ORIGINAL RESEARCH PAPER

Volume-7 | Issue-9 | September-2018 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

EXCLUSION OF DEFICIENCIES IN VITAMIN D, HEMOGLOBIN AND IRON IN OTHERWISE HEALTHY YOUNG ADULTS BRINGS OUT THE GENDER DIFFERENCES IN THE RELATIONSHIPS OF PARATHYROID HORMONE

Biochemistry

Nesheera KK. Department of Biochemistry, Amala Institute of Medical Sciences, Department of Biochemistry, Amala Cancer Research Centre, Amala Nagar, Thrissur 680555, Kerala, India.

Sindu PC Department of Biochemistry, Amala Institute of Medical Sciences,

Jose Jacob* Department of Biochemistry, Amala Institute of Medical Sciences, Department of Biochemistry, Amala Cancer Research Centre, *Corresponding Author

ABSTRACT

BACKGROUND: Deficiencies influence clinical interpretations of parathyroid hormone. This study analysed gender differences in parathyroid hormone and related analytes after excluding deficiencies in healthy young adults.

METHODS: Healthy participants aged 18 to 25 years from near sea level Kerala, who were evaluated clinically and by quantitative biochemical analytes to exclude common diseases, but include deficiencies of analytes related to parathyroid hormone, took part in this observational cross sectional study.

RESULTS: Before exclusion of deficiencies, gender differences were seen in analytes related to parathyroid and correlations of parathyroid hormone were difficult to interpret. After exclusion of deficiencies, gender differences were lost in all analytes except hemoglobin. Correlations of parathyroid hormone were negative with hemoglobin, but positive with erythropoietin in females and negative with iron in males. Androgens increase hemoglobin causing loss of correlation of parathyroid hormone with erythropoietin.

CONCLUSION: Exclusion of deficiencies in analytes related to parathyroid hormone brought out differences in regulation of hemoglobin in healthy males and females.

KEYWORDS

Parathyroid hormone; Erythropoietin, Haemoglobin, Gender differences.

INTRODUCTION

Parathyroid hormone (PTH) and $1,25(OH)_2D_3$ increase plasma calcium by promoting bone resorption, calcium uptake by kidneys, phosphaturia and intestinal absorption of calcium[1]. Subclinical vitamin D deficiency, a common life style disorder, increased PTH, resulting from decreased $1,25(OH)_2D_3$[1,2]. We had reported that there were gender differences in the negative correlation of EPO with hemoglobin, iron and ferritin and the gender differences persisted even after excluding anaemia[3].

Hormone resistance may occur in diseases but may also be involved in apparently healthy individuals who have subclinical deficiencies. In dialysis patients with hyperparathyroidism is associated with low EPO, decrease in the formation of erythroid progenitors and shorter survival of red cells[4,5] resulting in anaemia[6,7]. Deficiency of EPO secretion from kidney damage in hemodialysis patients is the most important cause of anaemia and is corrected by recombinant human EPO[8,9]. But a significant number of these patients require large doses of EPO. There are many factors known to reduce the response of erythropoiesis by EPO[10]. Of these, the most important is iron deficiency[11]. Vitamin D deficiency may also decrease the effectiveness of PTH. In some of these patients, parathyroidectomy and control of secondary hyperparathyroidism increased haemoglobin, reduced the requirement of EPO stimulating agents and reduced fibrosis of bone marrow[6,7,12]. A major cause of EPO resistance is bone marrow fibrosis and there was significant reduction in osteoclastic activity[12].

EPO activated osteoclasts to induce bone resorption. Hematopoiesis required expansion of marrow cavity with bone loss. This activity of EPO may be directly on osteoclast or by inhibiting osteoblast and bone formation through an unknown intermediary[13,14]. A relationship between PTH and EPO may be required for altering the bone microenvironment during increased erythropoiesis that is seen in anaemia. Hematopoiesis also requires bone formation and EPO has been reported to be involved[15]. Increased osteoblastic activity with bone formation is required for recovery of bone compartment. Therefore, we hypothesised that a direct correlation of serum EPO with intact PTH may be clinically detected if deficiencies of related parameters are excluded.

It was also hypothesised in this study that there may be differential regulation of erythropoiesis in males and females, and may be detected clinically. The higher hemoglobin and RBC counts in men when compared to women are from the augmentation of erythropoiesis by androgens and inhibition by oestrogens[16]. Will the higher haemoglobin levels in males lead to gender differences in the relationship of EPO and PTH? The gender differences of haemoglobin levels are further increased by the monthly loss of blood in women, leading to decrease in haemoglobin, iron and ferritin. This study is an extension of our earlier report which showed gender differences in the negative correlation of EPO with hemoglobin, iron and ferritin after excluding anaemia[3]. In this study, we are further evaluating the cause of the residual gender differences in the absence of anaemia, after excluding deficiencies of vitamin D, hemoglobin, iron and ferritin that increased PTH and EPO levels, resulting in resistance to these hormones.

MATERIALS AND METHODS

STUDY SETTINGS AND 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 (AIMSIEC/07/2014 dated 31/01/2014). Clinical evaluation and partitioning of volunteers in this study was done in six stages (Table 1; Stages I to VI) for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample according to gender and age. 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, hypertension

Table 1. Number of participants (n) at various stages (I to VI) and steps (1 to 8) of implementation of exclusion criteria for selection of the PTH sample population (n = 142; at Stage IV), partitioning of samples according to age (18 – 25 years) and gender (males, M & females, F), and followed by exclusion of PTH related deficiencies (Stage V & VI).

Stages	Steps	Exclusion at various steps for selection of reference PTH sample	Sample number at various steps of clinical exclusions		
			(M+F) n, (% remaining after exclusion)	Male, n (% excluded at each step)	Female, n (% excluded at each step)
I (1 to 2)	1.	Participants before exclusion by clinical history and examination	>600	~300	~300

	2.	Participants after exclusion at step 1. Fasting blood and urine samples were taken for assays (all age groups)	385 (100%)	180 (120/300 = 40%)	205 (95/300 = 31.67%)
II (2 to 3)	**3.**	PTH samples selected after exclusion of subclinical disease states by Clinical Biochemistry evaluation, BMI and waist circumference.	274 (71.17%)	103 (77/180 = 42.78%)	171 (34/205 = 16.59%)
III (3 to 4)	**4.**	Participants after exclusion of growth phase <18 years and influence of age >25 years. Age 18 to 25 years.	175 (45.45%)	57 (46/103 = 44.66%)	118 (53/171 = 30.99%)
IV (4 to 5)	**5.**	Participants after excluding samples with high ferritin, osteocalcin, ostase, NTx and low EPO. Age 18 - 25 years.	142 (36.88%)	40 (17/57 = 29.82%)	102 (16/118 = 13.56%)
V (5 to 6, 5 to 7)	**6.**	PTH sample after excluding vitamin D deficiency.	68 (17.66%)	26 (14/40 = 35%)	42 (60/102 = 58.82%)
	7.	PTH sample after excluding anaemia and deficiency of iron.	97 (25.19%)	37 (3/40 = 7.5%)	60 (42/102 = 41.18%)
VI (5 to 8)	**8.**	PTH sample after excluding anaemia and deficiencies of iron, ferritin and vitamin D.	40 (10.39%)	22 (18/40 = 45%)	18 (84/102 = 82.35%)

and alcoholics at the time of examination or in the past one month. 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).

Exclusion of subclinical disease states and deficiencies by Clinical Biochemistry assays for sample selection and sample partitioning

Informed written consent was obtained from each participant at Stage II from whom fasting and post prandial blood and urine samples were obtained. Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of unhealthy individuals at the subclinical level. 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) (Table 1; Stage II).

PTH samples were selected after excluding growth phase at <18 years and influence of age at >25 years (18 to 25 years) (Table 1, Stage III), and after excluding certain intact PTH-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), EPO <3.5 IU/l (Table 1; Stage IV). These stringent exclusion criteria reduced the sample number from over 600 to 142. Even after selection of PTH samples, they were again subjected to exclusion of deficiencies of serum vitamin D <50 nmol/l, hemoglobin <125 g/l, iron <9.85 µmol/l (55 µg/dl) and ferritin <20 ng/ml (Stage V and VI), further reducing the sample size (n = 22 males and 18 females). The selected PTH sample (n = 142) was studied before (Table 2) and after (Table 3 and 4) exclusion of these four deficiencies.

The above cut off levels were designed for this study to include individuals with increased concentrations of EPO, intact PTH, and deficiencies of vitamin D, hemoglobin, iron and ferritin. Abnormal cut off levels, such as those for BMI, waist circumference, postprandial glucose, triglycerides and others, were also designed to include individuals with restricted variations but to rule out individuals with highly abnormal values such as obesity, postprandial glycosuria, high triglycerides and others.

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 or lipemic samples were excluded. 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. Blood samples were also collected 2 hours post prandial or after glucose challenge in oral glucose tolerance test.

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; EPO: (mIU/ml x 1.0) = U/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.

Analytical control and assays

Immunochemistry autoanalyser Access 2 (Beckman Coulter, USA) and their reagents were used for intact PTH, EPO and ferritin assays using immunometric method with magnetic bead coated anti PTH, anti EPO or anti ferritin antibodies[17]. 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[18]. Vitamin D assay was done by Diasorin Liaison (Italy)[19]. Haemoglobin estimation 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[20]. If there was a rejection, appropriate measures were taken to set right errors in machine functioning, reagents, storage or analyte calibration levels. Discontinuous 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 or 0 U/l EPO) with 95% confidence was 1 ng/l (0.1 pmol/l) for intact PTH and <0.6 U/l for EPO. It was also far below the lowest linear six point intact PTH calibrator value (eg. PTH: 10.7 ng/l; EPO: 4.90U/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; for EPO in U/l from a particular lot of calibrators were 0, 4.90, 26, 128, 387, 797[17].

Statistical Analysis

Normality of distribution was estimated by Shapiro-Wilk test. Equality of variances of groups compared was done by Levene's test. Statistical analysis and calculations were done with SPSS, version 23.0 software. When variables had Gaussian distribution (before or after $\log_{10}$ transformation) and when there was equality of variance in the groups compared, parametric methods (Student t test) of analysis were used. Otherwise, non parametric methods (Mann Whitney U test) were used. Comparisons of mean differences in two groups were also done by 95% confidence interval of mean (95% CI). Correlations were estimated with parametric Pearson's correlation when at least one of the correlating variable had Gaussian distribution before or after $\log_{10}$ transformation, otherwise nonparametric Spearman's rho was done[21,22].

RESULTS

Selection of Participants

Selection of participants by clinical history and examination was followed by exclusion of diseases at the subclinical level using cut off levels of quantitative biochemical analytes. The strict exclusion criteria reduced the sample size to 142 (Table 1; Stage IV) from the starting number >600 (Stage I) and further reduced during exclusion of deficiencies to 40 (Stage VI). After selection of participants at Stage II, the minimum and maximum of BMI, waist circumference, serum creatinine, fasting glucose, alanine aminotransferase, triglycerides and hsCRP were within the specified cut off levels (data not shown). Exclusions among the male participants from subclinical disease states were more at Stage I, II and IV, and that in female participants from deficiencies were more at Stage V and VI (Table 1).

Characteristics and gender differences in the quantitative analytes used for sample selection and those related to intact PTH

PTH sample at Stage IV in Table 1, partitioned into males (n = 40) and females (n = 102) were used for this study. Among the biochemical analytes used for sample selection at stage II, there were**Table 2.** General characteristics and gender differences of analytes used in the exclusion criteria for sample selection (A) and those related to intact PTH (B) in the selected PTH sample population. Shapiro-Wilk test for estimation of normality and Levene's test for equality of variances in male (M) and female (F) samples were done. Gender differences were compared by 95% CI of mean, Student t test (parametric method) or Mann Whitney U test (non parametric method).

Variables in SI units	Mean±SD, 95% CI of mean (M+F, n = 142)	Comparison of gender differences of analytes in the total sample					
		Mean±SD, 95% CI of mean (Males, n = 40)	Mean±SD, 95% CI of mean (Females, n = 102)	after log10 transformation			
				Shapiro – Wilk test, P		Levene's test (M & F), P	Student t test (Mann-Whitney U test), P
				M	F		
A. Quantitative analytes used in the exclusion criteria for sample selection at Stage II (Sample from Stage IV)							
Age (18-25 years)	22.2±1.75	22.05±1.54	22.28±1.85	---	---	---	---
BMI (kg/m^2)	21.31±3.07 20.8 – 21.8	21.38±3.21 20.35 – 22.41	21.28±3.06 20.69 – 21.88	0.305	<0.001	0.854	(0.713)
Waist circum- ference (cm)	77.07±7.81 75.8 – 78.4	78.59±8.36 75.92 – 81.27	76.47±7.55 74.99 – 77.95	0.627	0.340	0.473	0.149
S. Creatinine (µmol/l)	68.2±14.98 65.8 – 70.7	85.09±13.68 80.71 – 89.46	61.62±9.18 59.82 – 63.42	0.012	<0.001	0.048	(<0.001)
Fasting Glucose (mmol/l)	4.9±0.459 4.8 – 4.97	4.91±0.417 4.78 – 5.05	4.89±0.476 4.80 – 4.98	0.120	0.001	0.242	(0.471)
ALT (U/l)	25.7±9.1 24.2 – 27.2	30.00±10.71 26.57 – 33.43	24.00±7.81 22.47 – 25.53	0.322	0.063	0.101	<0.001
Triglycerides (mmol/l)	0.873±0.329 0.82 – 0.93	0.991±0.353 0.878 – 1.10	0.826±0.309 0.766 – 0.887	0.451	0.018	0.611	(0.002)
High sensitive CRP (mg/l)	0.89±0.95 0.73 – 1.04	0.830±0.888 0.546 – 1.11	0.907±0.979 0.715 – 1.10	0.304	<0.001	0.231	(0.973)
B. Quantitative analytes related to intact PTH sample at Stage IV; Table 1							
Intact PTH (ng/l)	32.69±15.55 30.11 – 35.27	26.72±11.61 23.01 – 30.44	35.04±16.31 31.83 – 38.24	0.534	0.196	0.535	0.003
S. EPO (U/l)	8.64±4.96 7.82 – 9.47	7.18±2.02 6.54 – 7.82	9.22±5.62 8.11 - 10.32	0.664	<0.001	0.017	(0.052)
Vitamin D (nmol/l)	58.20±34.50 52.47 – 63.92	76.48±46.80 61.52 – 91.45	51.03±25.12 46.09 – 55.96	0.099	0.179	0.078	0.001
Total Calcium (mmol/l)	2.44±0.132 2.42 – 2.46	2.51±0.122 2.47 – 2.54	2.42±0.128 2.39 – 2.44	0.253	0.242	0.384	<0.001
Hemoglobin (g/l)	132.80±13.18 130.62 – 134.99	142.53±11.69 138.80 – 146.27	128.99±11.73 126.68 – 131.29	0.302	<0.001	0.891	(<0.001)
S. Iron (µmol/l)	17.00±7.31 15.79 – 18.21	21.47±6.64 19.34 – 23.59	15.25±6.82 13.91 – 16.59	0.061	<0.001	0.032	(<0.001)
S. Ferritin (ng/ml)	31.96±30.08 26.97 – 36.95	60.26±37.21 48.36 – 72.16	20.86±16.95 17.53 – 24.19	0.597	0.190	0.063	<0.001

higher levels of triglycerides (P = 0.002), alanine aminotransferase (P <0001) and serum creatinine (P <0.001) in the male sample (Table 2A). These were the expected gender differences and were due to higher insulin resistance and muscle mass in young males.

All PTH-related analytes showed gender differences (Table 2B). The female sample had higher concentrations of intact PTH (P = 0.003) and EPO (P = 0.052) resulting from lower levels of vitamin D (P = 0.001), total calcium (P <0.001), hemoglobin (P <0.001), iron (P <0.001) and ferritin (P <0.001), as seen by both Student t test and 95% CI of mean. Higher concentrations of EPO and PTH in females, and consequently erythropoiesis may be due to anaemia and iron deficiency resulting from monthly blood loss[3] and due to vitamin D deficiency.

Gender difference in the concentration of EPO was lost and that of hemoglobin persisted after exclusion of deficiencies

The gender difference in EPO was lost during each step of exclusions (Table 3A, 3B & 3C). When all deficiencies were excluded, EPO was decreased in females but not in males, contributing to the loss of its gender difference. Surprisingly, even after all exclusions, gender difference in hemoglobin persisted with higher concentrations in males (Table 3C). This may be attributed to the effect of androgen to increase hemoglobin in males[3,16]. The gender difference in total calcium was too small (males = 2.5±0.115; females = 2.4±0.101).

Deficiencies influenced the correlations of PTH and showed complex gender differences

In males, there was negative correlation of PTH with calcium in the presence of vitamin D deficiency (Table 4A and 4C) but not after exclusion of vitamin D deficiency (Table 4B and 4D), indicating that vitamin D deficiency decreased calcium which led to increase in PTH. We expected similar negative correlation in the female sample, as vitamin D deficiency was more in females. The

Table 3. Influence of exclusion of samples deficient in vitamin D, hemoglobin, iron and ferritin (A to C) on the concentrations of intact PTH, EPO and their related parameters. Concentrations were compared before (Table 2B) and after exclusion of deficiencies (I) in

males (M & M) and in females (F & F). Gender differences were compared after exclusion (II). Comparisons were done by 95% CI of mean and Student t test (or Mann Whitney U test). Increase (↑) and decrease (↓) of mean is shown.

PTH and its related variables	I. Comparisons before and after sample exclusions		II. Gender difference after exclusions		
A. After exclusion of Vitamin D deficient samples. M (n = 26), F (n = 42) [Samples before exclusions for comparisons were from Table 2B. M (n = 40), F (n = 102)]					
	Student t test (Mann-Whitney U test), P		95% CI of mean		Student t test (Mann-Whitney U test), P
	M & M	F & F	Male	Female	M & F
Intact PTH (ng/l)	0.508	0.267	20.44 – 29.34	27.15 – 36.13	0.039
S. EPO (U/l)	0.611	(0.755)	6.12 – 7.74	7.22 – 9.43	0.112
Vitamin D (nmol/l)	0.018↑	(<0.001) ↑	80.97 – 116.04	68.05 – 81.18	(0.024)
Total Calcium (mmol/l)	0.762	0.363	2.47 – 2.56	2.36 – 2.43	<0.001
Hemoglobin (g/l)	0.435	(0.568)	136.02 – 144.37	125.78 – 134.22	(0.001)
S. Iron (µmol/l)	0.901	(0.345)	18.42 – 23.98	14.17 – 18.14	(0.005)
S. Ferritin (ng/ml)	0.839	0.040↑	43.18 – 75.10	20.91 – 32.83	<0.001
B. After exclusion of Hemoglobin and Iron deficient samples. M (n = 37), F (n = 60)					
Intact PTH (ng/l)	0.836	0.918	22.38 – 29.94	30.88 – 39.37	0.008
S. EPO (U/l)	0.874	(0.160)	6.44 – 7.77	6.90 – 8.15	0.515
Vitamin D (nmol/l)	0.980	0.610	60.88 – 92.81	46.76 – 60.22	0.020
Total Calcium (mmol/l)	0.713	0.470	2.48 – 2.55	2.40 – 2.47	0.002
Hemoglobin (g/l)	0.525	(0.001) ↑	140.49 – 147.66	132.80 – 137.02	(<0.001)
S. Iron (µmol/l)	0.761	(0.001) ↑	19.65 – 23.93	17.29 – 20.20	0.020
S. Ferritin (ng/ml)	0.641	0.015↑	44.40 – 67.49	21.57 – 31.33	<0.001
C. After exclusion of vitamin D, hemoglobin, iron, ferritin deficiencies. M (n = 22), F (n = 18)					
	M & M Mean±SD, P	F & F Mean±SD, P	Male 95% CI	Female 95% CI	M & F P
Intact PTH (ng/l)	24.6±11.9 0.394	32.6±16.9 0.430	19.3 – 29.8	24.2 – 41.1	0.106
S. EPO (U/l)	6.8±1.7 0.540	6.9±2.8 (0.041) ↓	6.05 – 7.58	5.52 – 8.32	(0.638)
Vitamin D (nmol/l)	101.4±45.3 (0.016) ↑	78.5±20.5 (<0.001) ↑	81.3 – 121.5	68.3 – 88.7	(0.180)
Total calcium (mmol/l)	2.5±0.115 0.713	2.4±0.101 0.746	2.47– 2.6	2.38 – 2.48	0.020
Hemoglobin (g/l)	142.1±9.8 0.978	135.5±6.5 (0.007) ↑	137.7 – 146.4	132.3 – 138.7	0.019
S. Iron (µmol/l)	21.3±7.2 0.905	17.8±4.9 (0.071) ↑	18.1 – 24.5	15.3 – 20.2	0.113
S. Ferritin (ng/ml)	58.6±36.4 0.916	42.3±19.1 (<0.001) ↑	42.5 – 74.7	32.8 – 51.8	0.060

absence of significant correlation may be due to confounding of PTH through increased EPO by hemoglobin and iron deficiencies.

In the female sample PTH positively correlated with EPO in presence or absence of deficiencies. But the correlation statistic increased after exclusion of hemoglobin and iron deficiencies (Table 4C, r = 0.367) and further increased when vitamin D, hemoglobin, iron and ferritin deficiencies were excluded (Table 4D, r = 0.577). Results indicated that deficiencies in the female sample influenced the direct correlation of PTH and EPO, decreasing hemoglobin levels. Exclusion of these deficiencies increased the correlation statistic and hemoglobin concentrations. In none of these circumstances PTH correlated with EPO in the male sample, indicating strong gender differences in the regulation of erythropoiesis.

After exclusion of all deficiencies, the negative correlation of PTH with hemoglobin in males and females could be explained

When all deficient states of PTH-related analytes were excluded, a clear gender difference in correlations of PTH emerged. PTH correlated negatively with hemoglobin in males ((r = -0.426; P = 0.048) and females (r = -0.494; P = 0.037) (Table 3D). In males, the negative correlation of PTH with hemoglobin may be related to the negative correlation of PTH with iron (r = -0.443; P = 0.039) (Table 3D). When hemoglobin is deficient, increased PTH may increase erythropoiesis by using and decreasing iron. The higher concentration of hemoglobin in males, when compared to females, is due to androgens. If higher levels of iron stores promote higher hemoglobin and lower PTH, then the inverse should also be true, thus suggesting the negative correlations of PTH with iron.

In the female sample, the negative correlation of PTH with hemoglobin may be related to the positive correlation of PTH with EPO (r = 0.557; P = 0.012). PTH did not correlate with iron in the female sample. In the absence of iron deficiency, increased hypoxia or anaemia in the female sample may lead to increase in EPO which in turn increased PTH levels to increase erythropoiesis

Table 4. Correlations of Intact PTH with selected PTH-related analytes in males (M) and females (F), before (A) and after (B, C and D) exclusion of samples deficient in vitamin D, hemoglobin, iron and ferritin. Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after $\log_{10}$ transformation.

Variables related to PTH	Correlation coefficient, r	P	Correlation coefficient, r	P
	Males		Females	
A. Correlations of PTH before exclusion of deficiencies (M, n = 40; F, n = 102)				
S. EPO (U/l)	-0.019	0.909	0.302	0.002
Vitamin D (nmol/l)	-0.311	0.051	0.166	0.095
Total Calcium (mmol/l)	-0.476	0.002	-0.157	0.114
Blood Hemoglobin (g/l)	-0.248	0.122	-0.086	0.388
S. Iron (µmol/l)	-0.365	0.020	-0.040	0.687
S. Ferritin (ng/ml)	0.055	0.735	-0.040	0.692
B. Correlations of PTH after exclusion of vitamin D deficiency (M, n = 26; F, n = 42)				
S. EPO (U/l)	0.056	0.784	0.301	0.052
Vitamin D (nmol/l)	-0.129	0.531	0.126	0.428
Total Calcium (mmol/l)	-0.236	0.246	-0.005	0.977
Blood Hemoglobin (g/l)	-0.411	0.037	-0.059	0.709
S. Iron (µmol/l)	-0.415	0.035	0.107	0.614
S. Ferritin (ng/ml)	-0.093	0.652	0.107	0.500

C. Correlations of PTH after exclusion of hemoglobin and iron deficiencies (M, n = 37; F, n = 60)				
S. EPO (U/l)	-0.093	0.583	0.367	0.004
Vitamin D (nmol/l)	-0.304	0.067	-0.136	0.299
Total Calcium (mmol/l)	-0.448	0.005	-0.206	0.114
Blood Hemoglobin (g/l)	-0.185	0.274	-0.185	0.158
S. Iron (µmol/l)	-0.298	0.073	0.167	0.202
S. Ferritin (ng/ml)	-0.007	0.967	0.078	0.552
D. Correlations of PTH after exclusion of vitamin D, hemoglobin, iron and ferritin deficiencies (M, n = 22; F, n = 18)				
S. EPO (U/l)	0.014	0.950	0.577	0.012
Vitamin D (nmol/l)	-0.094	0.677	0.065	0.799
Total Calcium (mmol/l)	-0.282	0.204	-0.141	0.578
Blood Hemoglobin (g/l)	-0.426	0.048	-0.494	0.037
S. Iron (µmol/l)	-0.443	0.039	-0.144	0.570
S. Ferritin (ng/ml)	-0.018	0.936	0.316	0.202

and to create bone marrow space for it. In the female sample, the strong relationships of PTH and EPO is emphasised by their positive correlations before and after exclusions of anaemia and iron deficiency or vitamin D deficiency or both. Oestrogens decrease the concentration of hemoglobin in females.

DISCUSSION

In an earlier report[3] we had shown gender difference in EPO concentrations and correlations after exclusion of samples with anaemia. Later we observed that clinical correlates of PTH, in addition to EPO, might be involved in erythropoiesis, and deficiencies of PTH-related analytes influenced these correlates. These observations on the gender difference in the concentrations and correlates of intact PTH are reported here.

Exclusion criteria and partitioning for selection of the PTH sample

Strict inclusion and exclusion criteria decreased the primary and secondary influences on PTH concentration. Such a procedure decreased the statistical outliers of distribution and β errors, resulting in increase in power of the sample population. This procedure facilitated better interpretation of the levels of intact PTH and its relationships in health and in disease. But sample number was severely reduced (Table 1). The decrease in sample size in males was more than that in the females at stages I, II and IV; The decrease in females was more than that in males at stages V and VI. Sample selection resulted in lower number of males in the PTH sample at stage IV but at stage VI there were almost equal number of males and females (Table 1). In males, the major causes for decrease in sample size were insulin resistance and life style disorders. In females, it was hypothyroidism and the deficiencies.

Subclinical deficiencies influenced the gender differences in PTH and related analytes

Gender differences (Table 2B) were due to higher PTH and EPO in females resulting from the deficiencies in vitamin D, hemoglobin, iron and ferritin. Vitamin D and calcium deficiencies are known to increase PTH[1,3]. Hemoglobin, iron and ferritin deficiencies increased EPO. EPO directly correlated with PTH in the female sample (Tables 4A to D) irrespective of presence or absence of deficiencies, indicating strength of the relationship. Deficiencies influenced the concentrations (Tables 2B, 3A, 3B) and correlations of PTH (Table 3A, 3B, 3C) which lead to difficulties in interpretation of the results.

Gender differences after exclusion of deficiencies

The higher hemoglobin concentration in males may be attributed to the effect of androgen to increase hemoglobin in males[3,16] (Table 3C). Gender differences in correlations of PTH might be due to the greater probabilities of hypoxia in females from lower concentrations of hemoglobin due to inhibition by oestrogens[3,16] (Table 4C) resulting in PTH directly correlating with EPO and inversely with hemoglobin. In males, PTH was inversely related to hemoglobin, but the correlation with EPO may be statistically confounded by the influence of androgens.

CONCLUSION

In females, higher concentrations of PTH and EPO might be due to deficiencies in hemoglobin, iron, ferritin and vitamin D resulting from monthly blood loss and decreased exposure to sunlight. In males and females, there was good negative correlation of PTH with hemoglobin only after exclusion of the deficiencies. The deficiencies caused PTH and EPO resistance and influenced correlations. After exclusion of deficiencies, hemoglobin was still higher in men and may be due to androgens; In males, PTH did not correlate with EPO but negatively correlated with hemoglobin, indicating that higher hemoglobin in males rarely caused general hypoxia and that the relationship of PTH with EPO may be confounded by the increased hemoglobin due to androgens.

ACKNOWLEDGEMENTS

Jose Jacob was granted Ph.D. guideship (No. CDC B1 2284/2003 RAG 10.01.2005) and Nesheera KK was registered for Ph.D. (Reg. No. DoR/B3/2234/Ph.D/2012) at the Calicut University. Nesheera KK is a recipient of Junior Research Fellowship of Department of Science & technology, Government of India (No. DST/INSPIRE Fellowship/2013/15, Inspire Code IF130015).

REFERENCES.

1. Risteli J, Winter WE, Kleerekoper M, Risteli L. Bone and Mineral Metabolism. In: Burtis CA, Ashwood ER, Bruns DE (eds) Tietz Textbook of Clinical Chemistry and Molecular diagnostics. 5th Ed. Saunders-Elsevier, New Delhi, 2012:1733 - 1801.
2. Brown EM. Calcium receptor and regulation of parathyroid hormone secretion. Rev Endocr Metab Disord. 2000; 1:307-15.
3. Nesheera KK, Sindu PC, Jacob J. Clinical evaluation of gender differences in the relationships of erythropoietin with hemoglobin, iron and ferritin in presence and absence of anaemia in healthy young adults. IJCMR. 2017; 4(8):1788 – 1795.
4. Meytes D, Bogin E, Ma A, Dukes PP, Massry SG. Effect of parathyroid hormone on erythropoiesis. J Clin Invest. 1981; 67:1263–1269.
5. Bogin E, Massry SG, Levi J, Djaldeti M, Bristol G, Smith J. Effect of parathyroid hormone on osmotic fragility of human erythrocytes. J Clin Invest. 1982; 69:1017–1025.
6. Goicoechea M, Gomez-Campdera F, Polo JR, Tejedor A, Ruiz MA, Vazquez I, Verde E, Valderrabano F. Secondary hyperparathyroidism as cause of resistance to treatment with erythropoietin: effect of parathyroidectomy. Clin Nephrol. 1996; 45:420–421.
7. Barbour GL. Effect of parathyroidectomy on anemia in chronic renal failure. Arch Intern Med. 1979; 139:889–891.
8. Erslev AJ. Erythropoietin. N Engl J Med. 1991; 324:1339-44.
9. Nissenson AR, Nimer S, Wolcott D. Recombinant human erythropoietin and renal anemia: molecular biology, clinical efficacy, and nervous system effects. Ann intern Med 1991; 114 (5):402 – 16.
10. Stivelman JC. Resistance to recombinant human erythropoietin therapy: a real clinical entity? Semin Nephrol. 1989; 9: Suppl 2: 8-11.
11. Van Wyck DB, Stivelman JC, Ruiz J, Kirlin LF, Katrz MA, Ogden DA. Iron status in patients receiving erythropoietin for dialysis – associated anemia. Kidney Int. 1989; 35: 712-6.
12. Sudhaker Rao D, Shih MS, Mohini R. Effect of serum parathyroid hormone and bone marrow fibrosis on the response to erythropoietin in uremia. N Engl. J Med. 1993; 328:171–175.
13. McGee SJ, Havens AM, Shiozawa Y, Jung Y, Taichman RS. Effects of erythropoietin on the bone microenvironment. Growth Factors 2012; 30(1): 22–28.
14. Bab SH, Liron T, Unger ND, Mittelman M, Gassmann M, Rauner M , et al. Erythropoietin directly stimulates osteoclast precursors and induces bone loss. The FASEB journal. 2015: 29:1890 – 1900.
15. Shiozawa Y, Jung Y, Ziegler AM, Pedersen EA, Wang J, Wang Z et al. Erythropoietin Couples Hematopoiesis with Bone Formation. PLoS ONE. 2010; 5(5): 1-14.
16. Murphy WG. The sex difference in haemoglobin levels in adults — Mechanisms, causes, and consequences. Blood Reviews. 2014; 28(2): 41–7.
17. Intact PTH, EPO and Ferritin In: Procedure manual. Access II Immunoassay systems, Beckman Coulter, USA. 2005.
18. Chemistry analytes. In: Procedure manual. Chemistry autoanalyser Vitros 5,1 FS (Ortho Clinical Diagnostics, USA. 2004.
19. Vitamin D. In: Procedure manual. Laison immunochemistry autoanalyser, Diasorin, Italy.
20. Klee GG, Westgard JO. Quality management. In: Burtis CA, Ashwood ER, Bruns DE (eds) Tietz Textbook of Clinical Chemistry and Molecular diagnostics. 5th Ed. Saunders-Elsevier, New Delhi 2012:163-203.
21. Altman DG. Principles of statistical analysis. Comparing groups. In: Practical statistics for medical research. Ist Ed. Chapman & Hall/CRC Press; Florida, USA, 1991:152-271.
22. Armitage P, Berry G, Mathews JNS. Comparison of several groups. In: Statistical methods in medical research. 4th edition. Blackwell Science Limited, Oxford, UK. 2002:208-35.