Clinical Evaluation of Gender Differences in the Relationships of Erythropoietin with Haemoglobin, Iron and Ferritin in Presence and Absence of Anaemia in Healthy Young Adults

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ABSTRACT

Introduction: Erythropoietin increases erythropoiesis to overcome hypoxia. Synthesis of erythropoietin by kidneys and absorption of iron by intestine are regulated by hypoxia and iron levels. Haemoglobin is increased by androgens in males; haemoglobin, iron and ferritin are decreased by monthly blood loss in females leading to gender differences in erythropoietin and its correlation with these variables in presence and absence of anaemia. Present study aimed at investigating the influences of haemoglobin, iron and ferritin loss on EPO levels in the clinical setting.

Material and Methods: Participants of this observational cross sectional study, aged 18 to 25 years, from rural Central Kerala were evaluated clinically and from quantitative biochemical variables with cut off fixed to include anaemia, but exclude other secondary clinical influences (n = 159). Comparison of concentrations and correlations of erythropoietin were done with parametric and nonparametric methods.

Results: As there were gender differences in erythropoietin, haemoglobin, iron and ferritin, sample was partitioned into males and females. In the female sample, erythropoietin showed strong negative correlation with haemoglobin, iron and ferritin before and after exclusion of anaemia/hypoxia. But in the male sample, erythropoietin showed a moderate correlation only with iron before and after exclusion of anaemia. After excluding haemoglobin <125g/l, there was no gender difference in erythropoietin levels (P = 0.11) but there were differences when WHO criteria for anaemia was used as cut off.

Conclusion: Strong gender differences were observed in the negative correlations of erythropoietin with haemoglobin, iron and ferritin in females, with weak correlations in males. The residual correlation of erythropoietin with iron in males, after exclusion of hypoxia/anaemia indicated that in addition to hypoxia, iron deficiency also increased erythropoietin.

Keywords: Erythropoietin, Haemoglobin, Ferritin, Iron, Hypoxia, Anaemia, Gender Differences

INTRODUCTION

Major contribution of erythropoietin (EPO) levels in circulation is by the kidneys,[1] and may be in minor amounts by liver and brain.[2] EPO production by the peritubular fibroblasts in renal cortex, is stimulated by general hypoxia which is sensed by the kidneys through HIF-2.[1,2] In chronic kidney diseases, EPO from other organs is not able to substitute for the fall in circulating level of EPO from kidneys. The major site of action of EPO for erythropoiesis is at the colony forming units - erythroid, through EPO receptors and regulates at level of transcription.[3,4] EPO modulates the erythroid progenitor maturation and prolifereation,[4,5] microenvironment of bone marrow for erythropoiesis[6] and regulates components of heme synthesis pathway.[7,8] It takes 3 to 4 days for the reticulocyte counts to increase following the rise of plasma EPO.[9]

The higher hemoglobin and RBC counts in men when compared to women are from the augmentation of erythropoiesis by androgens and inhibition by oestrogens,[10] consequently, will the gender differences in haemoglobin levels inversely affect EPO levels, leading to higher EPO in women? 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.

There are major differences in the oxygen sensing mechanism and EPO expression in the hepatic and renal systems: Fetal hepatocytes are considered as a strong contributor to circulating EPO but after birth renal system that takes over. The renal fibroblasts responds with an all or none fashion to hypoxia[11] while the hepatic system may respond in a more graded way;[12] the location of hypoxia-response elements, transcription factors and transcription mechanisms differ in these two systems.

The EPO enhancer is activated by hypoxia-inducible transcriptional factors (HIFs). The α-subunit of HIF is sensitive to O_2 dependant and iron dependant degradation. Between HIF-1 and HIF-2, the transcription factor HIF-2 may be considered more important for EPO expression after birth.[13,14] O_2 - dependant degradation of HIF-α subunit is dependent on Fe^{2+} containing prolyl-4-hydroxylases (PHD-1, PHD-2 and PHD-3). These enzymes transfer one O-atom of O_2 to the proline and the other to 2-oxoglutarate yielding CO_2 and succinate.[15] The proline hydroxylated HIF-α combines with von Hippei-Lindau tumor suppressor protein (VHL) and undergoes proteosomal degradation.[16] PHD-2 and PHD-3 are themselves HIF-target genes, their expression increases

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How to cite this article: Nesheera KK, Sindu PC, Jose Jacob. Clinical evaluation of gender differences in the relationships of erythropoietin with haemoglobin, iron and ferritin in presence and absence of anaemia in healthy young adults. International Journal of Contemporary Medical Research 2017;4(8):1788-1795.

and HIF-α levels decline during long term hypoxic periods.[17] This feedback regulation may explain the declining EPO production during chronic anaemia or prolonged stay at high altitude.

The sensitivity of HIF to O_2 and PHD to iron in the short term may explain the inverse relationship of haemoglobin, iron and ferritin to EPO production. In healthy young adults, systemic hypoxia may be related to anaemia, especially in women. Is there still an inverse correlation between EPO and the three related variables even after exclusion of anaemia? The second part of the research problem in this study is the verification of this issue in the clinical setting.

The HIF system shows tissue specificity through variable expression of HIF alpha subunit isoforms (HIF-1α, HIF-2α, HIF-3α). The PHD isoforms are PHD1, PHD2 and PHD3. The HIF responsive target proteins are transferrin receptor 1, ceruloplasmin, heme oxygenase1and hepcidin.[18] HIF-2α with PHDs is emerging as potential iron sensors in the intestinal mucosa. DMT1 and Dcytb are highly upregulated by both iron deficiency and hypoxia in duodenum, mediated by HIF 2α.[19-22] HIF 2α mRNA was induced in the duodenum by iron deficiency. This induction was associated with intestinal – specific VHL degradation. Involvement of VHL indicates the role of PHD, iron levels and hypoxia in expressing Dcytb and DMT1.[19–26]

Systemic anaemia/hypoxia and low iron levels are sensed by intestinal mucosa and kidneys leading to increased iron absorption and EPO secretion, respectively. Therefore, higher EPO levels indicate anaemia and iron deficiency leading to increased negative correlations of EPO with haemoglobin, iron and ferritin. Present study was done at investigating the influences of haemoglobin, iron and ferritin loss on EPO levels in the clinical setting.

MATERIAL AND METHODS

Case control and sample collection

Healthy cross section of participants (n = 159) between 18 and 25 years, took part in this observational cross sectional study. Other age groups were excluded to decrease the influence of age, growth phase and environment. Participants were from the near sea level plains of rural Central Kerala, South India, avoiding mountainous regions of Western Ghats. Samples were collected from relatives and friends of students, research fellows and laboratory staff of this institution, who fulfilled the criteria for inclusion and exclusion.

Study was approved by the Institutional Research and Ethics Committees (AIMSIEC/07/2014 dated 31/01/2014). Informed written consent was obtained from each participant. Volunteers underwent a clinical evaluation for exclusion of individuals with disease conditions, injury, infection, inflammation, allergic reactions, alcoholism, or history of such conditions in the past 2 weeks. 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. Clinical Biochemistry laboratory evaluation was done for further exclusion of unhealthy individuals at the subclinical level and to permit inclusion of individuals with anaemia and iron deficiency. Exclusion criteria for these were: BMI >30 kg/m^2, serum triglyceride >200 mg/dl (2.26 mmol/l), 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), BP ≥140/90 mmHg, serum alanine aminotransferase above 60 U/l, high sensitivity C reactive protein (hsCRP) >5 mg/l, serum creatinine >1.3 mg/dl (114.9 µmol/l) in males and >1.2 mg/dl (106.1 µmol/l) in females. Some of these abnormal cut off levels were designed for this study. But individuals with increased EPO, anaemia and iron deficiency were included in the study. The lower cut off levels considered for exclusion of samples in this study for EPO was <3.5 IU/l; for anaemia according to WHO criteria it was <130 g/l in males and <120 g/l in females.[23] The cut off value of haemoglobin considered for exclusion of hypoxia in both males and females was 125 g/l.[9] The lower cut off considered for deficiency of iron were: iron <6.6 µmol/l (36.9 µg/dl) and ferritin <20 ng/ml. But iron and ferritin deficient samples were not excluded in this study.

Reference intervals used for the upper healthy limits in this study were EPO: 4 – 27 U/l[28]; Hemoglobin: male 133 – 162 g/l, female 120 – 158 g/l[28]; Iron: 7 – 25 µmol/l (41 – 141 µg/dl)[28]; Ferritin: Male 29 – 250 ng/ml, Female 10 – 150 ng/ml.[28,29]

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. Samples 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. All assays were done immediately after preparation of serum.

Inter conversion of Units of variables

Inter conversion between SI units (given in different Tables) 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; Cholesterol: (mg/dl X 0.0259) = mmol/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.

Analytical control and assays

Two auto analysers, an immunochemistry analyser Access 2 (Beckman Coulter, USA) and a chemistry analyser Vitros 5,1 FS (Ortho Clinical Diagnostics, USA) were used for assays. EPO and ferritin assays were done with Access 2 machine and their reagents, using immunometric assay with magnetic bead coated anti EPO or anti ferritin antibody.[25,26] The chemistry autoanalyser 5,1 FS, was used for assay of glucose, triglycerides, total cholesterol, HDL cholesterol, serum creatinine, iron and hsCRP. Haemoglobin estimation was done manually by Drabkin's method using colorimeter.

Both immunochemistry and chemistry autoanalysers fulfilled the criteria of coefficient of variation (CV) and bias below 4% each within the range of data collected for the immunochemistry and chemistry assays.

Limit of detection of EPO was taken as the lowest EPO concentration distinguishable from zero (calibrator as 0 U/l EPO) with 95% confidence, and was < 0.6 U/l. It was also far below the lowest linear six point EPO calibrator value (eg. 4.90 U/l). An example of actual linear six point calibration values for EPO in U/l from a particular lot of calibrators were 0, 4.90, 26, 128, 387, 797. Reference interval of EPO (manufacturer's) was 2.59–18.50 U/l.[25]

Daily continuous internal quality control data were analysed according to Westgard rules for acceptance or rejection of analyte data.[27] If there is a rejection, appropriate measures were taken to set right errors in machine functioning, reagents or calibration levels.

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 of analysis were used. Otherwise, non parametric methods were used. The Comparison of gender differences in the levels of EPO, hemoglobin, iron and ferritin in the sample were analysed by 95% confidence interval of mean (95% CI), two-tailed Student's t test and Mann Whitney U test. Correlations of EPO with the related parameters were done with parametric Pearson's correlation and nonparametric Spearman's rho. Scatter diagrams were used to visually verify the correlations.[24]

RESULTS

Influence of age and gender increased biological variations and heterogeneity in a healthy sample. The influence of age was decreased by selecting young healthy adults aged 18 to 25 years. General characteristics of EPO and parameters related to EPO in the sample population were evaluated (Table 1). Mean and 95% CI of mean were within the reference range. But there were low values outside the reference range or below cut off for haemoglobin, iron and ferritin in both males and females. There were EPO values above the reference range in females. These values were permitted to include subclinical conditions related to iron metabolism.

Gender differences were compared by 95% CI of mean and Student t test (or non parametric Mann Whitney U test). When the data distribution was not Gaussian, attempts were made to convert the distribution to Gaussian by log_{10} transformation. There were gender differences, as seen by Student t test, in EPO (P = 0.006), hemoglobin ($P < 0.001$), iron ($P < 0.001$), ferritin ($P < 0.001$), alanine aminotransferase (P = 0.001), triglycerides (P = 0.03), HDL cholesterol (P = 0.001) and creatinine ($P < 0.001$) (Table 1). These gender differences were also confirmed by 95% CI of mean. The gender differences were contributed by androgens which increased haemoglobin in males[10,23] and monthly blood loss which decreased haemoglobin, iron and ferritin in females. As expected, HDL cholesterol was higher in females, and creatinine, triglycerides and alanine aminotransferase were higher in males.

In the total sample, the distribution of EPO, hemoglobin and ferritin did not have Gaussian distribution and could not be transformed to Gaussian distribution. This may be due to heterogeneity in the composition of the sample due to gender and was removed by partitioning into male and female groups. Although after partitioning and log_{10} transformation, the distribution improved to Gaussian distribution, the male EPO sample, and the female EPO and haemoglobin samples did not have Gaussian distribution and could not be transformed to Gaussian distribution (Table 2A).

In the male sample, EPO had negative correlation with only iron (P = 0.04). But in the female sample EPO negatively correlated with ferritin, iron ($P < 0.001$) and haemoglobin (P = 0.001). In general, correlations were stronger in the female sample due to iron and ferritin deficiencies and anaemia (Table 2A).

X-Y scatter diagrams with EPO showed negative correlations which were visually observed to be least in males (Figure 1A to C) and better in females (Fig. 1D to F). In the female sample, maximum negative correlation was seen with ferritin and least with haemoglobin. These visual analyses of X-Y scatter diagrams (Fig. 1) confirmed the estimation of the correlations (Table 2A).

Exclusion of anaemic/hypoxic samples was done by removing haemoglobin <125g/l in both males and females, assuming that HIF-2 was equally sensitive to hypoxia in males and females.[9] There was decreased concentration of haemoglobin, iron and ferritin in the female sample ($P < 0.001$) (Table 3). These decreased levels in females did not cause a difference of EPO in the male and female samples (P = 0.11). These results indicate that a confounding factor is increasing EPO levels in males despite higher haemoglobin (Table 3A). This confounding factor may be increased muscular activity and muscle mass in young adult males leading increased hypoxia.

But when the exclusion of anaemia was according to the cut off of haemoglobin proposed by WHO for males (130g/l) and females (120g/l)[23], there was a difference in the level of EPO (P = 0.04). This difference is due to inclusion 18 samples in females by a lower cut off at 120g/l. When the cut off for haemoglobin was increased from 125 g/l to 130g/l in males, there was only one sample excluded and there was a difference in the EPO levels (Table 3B).

To study the contribution of anaemia/hypoxia on the negative correlations of EPO, haemoglobin <125 g/l were excluded. There was no correlation of EPO with haemoglobin and ferritin in the male sample but correlated with haemoglobin (P = 0.03) and ferritin ($P<0.001$) in the female sample. EPO

Variables in SI units	Total sample (n = 159) Mean±SD Minimum -Maximum 95% CI of mean	Comparison of male and female in total sample					
		Male (n = 49) Mean±SD Minimum -Maximum 95% CI of mean	Female (n = 110) Mean±SD Minimum -Maximum 95% CI of mean	after log_{10} transformation			
				Shapiro-Wilk Test, P		Levene's test, P	Student t test (or Mann-Whitney U test), P
				Male	Female		
Age (years)	21.91±1.86 18 – 25 21.61 – 22.20	21.22±1.97 18 – 25 20.66 – 21.79	22.21±1.74 18 – 25 21.88 – 22.54	---	---	---	---
BMI (kg/m^2)	21.24±3.21 15.32 – 28.40 20.74 – 21.75	21.58±3.24 15.32 – 28.40 20.65 – 22.51	21.09±3.20 16.22 – 28.21 20.49 – 21.70	0.285	0.001	0.958	(0.295)
Waist circumference (cm)	76.57±8.30 57.50 – 99.00 75.27 – 77.97	77.65±9.45 57.50 – 99.00 74.93 – 80.36	76.09±7.73 59.00 – 99.00 74.63 – 77.55	0.638	0.907	0.189	0.332
Fasting Glucose (mmol/l)	4.95±0.499 4.01 – 6.70 4.87 – 5.03	4.96±0.44 4.01 – 6.04 4.84 – 5.09	4.94±0.525 4.05 – 6.70 4.84 – 5.04	0.346	0.004	0.095	(0.475)
2 h. OGTT Glucose (mmol/l)	5.39±1.11 2.49 – 9.45 5.21 – 5.56	5.40±1.12 2.49 – 8.11 5.08 – 5.72	5.38±1.11 2.77 – 9.45 5.17 – 5.59	0.086	0.055	0.299	0.967
S. EPO (U/l)	8.95±5.09 3.67 – 36.10 8.15 – 9.75	7.40±2.80 4.03 – 14.52 6.60 – 8.20	9.64±5.70 3.67 – 36.10 8.56 -10.72	0.019	0.001	0.086	(0.006)
Blood Haemoglobin (g/l)	134.22±14.03 97.00 – 168.70 132.02 – 136.42	145.97±12.15 119.9 – 168.70 142.48– 149.46	128.93±11.29 97.0 – 154.00 126.80– 131.06	0.177	0.001	0.966	(<0.001)
S. Iron (µmol/l)	17.65±7.65 1.79 – 39.20 16.45 – 18.85	22.67±6.50 8.23 – 39.20 20.81 – 24.54	15.41±7.07 1.79 – 34.55 14.08 – 16.75	0.001	<0.001	0.003	(<0.001)
S. Ferritin (ng/ml)	30.89±26.95 2.50 – 139.70 26.66 – 35.11	52.04±30.42 10.90 – 139.70 43.30 – 60.78	21.47±18.84 2.50 – 121.90 17.91 – 25.03	0.149	0.126	0.054	<0.001
Alanine amino-transferase (U/l)	25.45±9.58 10.00 – 60.00 23.95 – 26.95	29.27±11.00 16.00 – 60.00 26.11 – 32.42	23.75±8.39 10.00 – 60.00 22.16 – 25.33	0.122	0.005	0.154	(0.001)
Total Cholesterol (mmol/l)	4.61±0.718 3.39 -6.89 4.50 – 4.73	4.53±0.782 3.39 – 6.89 4.31 – 4.76	4.65±0.689 3.42 – 6.37 4.52 – 4.78	0.381	0.126	0.378	0.267
Triglycerides (mmol/l)	0.89±0.329 0.37 – 2.15 0.838 – 0.941	0.952±0.306 0.37 – 1.83 0.864 – 1.04	0.862±0.336 0.42 – 2.15 0.799 – 0.926	0.345	0.019	0.944	(0.031)
LDL Cholesterol (mmol/l)	2.84±0.632 1.40 – 4.74 2.75 – 2.94	2.86±0.697 1.67 – 4.74 2.66 – 3.06	2.84±0.605 1.40 – 4.48 2.72 – 2.95	0.691	0.151	0.584	0.938
HDL Cholesterol (mmol/l)	1.36±0.322 0.80 – 2.25 1.31 – 1.41	1.24±0.257 0.85 – 2.25 1.17 – 1.31	1.41±0.334 0.80 – 2.20 1.35 – 1.48	0.037	0.123	0.027	(0.001)
S. Creatinine (µmol/l)	69.12±14.98 44.20 -106.08 66.76 – 71.45	86.24±12.04 53.04 – 106.08 82.78 – 89.69	61.48±8.42 44.20 – 88.40 59.89 – 63.07	0.001	<0.001	0.102	(<0.001)
High sensitive CRP (mg/l)	0.872±1.04 0.06 – 5.00 0.709 – 1.04	0.959±1.18 0.06 – 5.00 0.622 – 1.30	0.834±0.984 0.10 – 5.00 0.648 – 1.02	0.067	<0.001	0.711	(0.459)

Table-1: General characteristics of erythropoietin and the related parameters in the total (n = 159), male (n = 49) and female (n = 110) sample population. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed. The gender differences were compared by 95% CI of mean and Student t test (or Mann Whitney U test). The criteria for parametric and non parametric methods for comparison of gender differences are given under "statistical methods".

Variables in SI units	(A) Total Sample (n = 159)			(B) after excluding haemoglobin <125g/l, (n = 137)		(C) after excluding haemoglobin male <130g/l and female <120g/l, (n = 120)	
	Shapiro Wilk test after log_{10} transformation, P	Correlation coefficient, r (Spearman's rho ρ)	Significance of correlation P	Correlation coefficient, r (Spearman's rho ρ)	Significance of correlation P	Correlation coefficient, r (Spearman's rho ρ)	Significance of correlation P
S. EPO (U/l)	<0.001	-	-	-		-	
Blood Haemoglobin (g/l)	0.014	(-0.290)	<0.001				
S. Iron (µmol/l)	0.288 (before log_{10} transformation)	-0.455	<0.001				
S. Ferritin (ng/ml)	0.026	(-0.414)	<0.001				
Male (n = 49)				Male (n = 45)		Male (n = 46)	
S. EPO (U/l)	0.019	-	-	-	-	-	-
Blood Haemoglobin (g/l)	0.177	-0.071	0.629	(0.028)	0.855	(0.040)	0.796
S. Iron (µmol/l)	0.375 (before log_{10} transformation)	-0.295	0.040	-0.333	0.024	-0.353	0.017
S. Ferritin (ng/ml)	0.149	-0.215	0.138	-0.220	0.141	-0.221	0.145
Female (n = 110)				Female (n = 92)		Female (n = 74)	
S. EPO (U/l)	0.001	-	-	-	-	-	-
Blood Haemoglobin (g/l)	0.001	(-0.324)	0.001	(-0.256)	0.028	(-0.283)	0.006
S. Iron (µmol/l)	0.151 (before log_{10} transformation)	-0.454	<0.001	-0.443	<0.001	-0.417	<0.001
S. Ferritin (ng/ml)	0.126	-0.557	<0.001	-0.384	<0.001	-0.469	<0.001

Table-2: Correlation of EPO with haemoglobin, iron and ferritin in the total sample or after partitioning into males and females, before (A) and after (B) excluding haemoglobin <125g/L, or after excluding haemoglobin in males at <130g/L and in females at <120g/L (C) by parametric (Pearson's, r) and non parametric (Spearman's, ρ) methods. Iron was not log10 transformed as it had Gaussian distribution before transformation.

correlated with iron in the male (P = 0.02) and female samples (*P* <0.001), indicating greater iron deficiency in females (Table 2B). When the exclusion of haemoglobin was according to WHO criteria, there was no noticeable variation in the correlation with EPO (Table 2C).

DISCUSSION

EPO shows a diurnal variation with a nadir in the morning.[9] There are other variables used in this study that have postprandial variations. Almost all variables showed gender and/or age dependent differences. To reduce such influences, fasting blood sample obtained between 8 and 9 in the morning from participants aged 18 to 25 years was used for this study. Interferences of gender differences in the estimations were removed by partitioning the sample into male and female groups.

Correlations of EPO

Correlation between two variables arise when one increases or decreases in relation to the other. As an example, haemoglobin, iron or ferritin deficiency increased EPO and they gave rise to negative correlations with EPO (Table 2). Correlations may be decreased or lost when there was no deficiency of haemoglobin, iron or ferritin.

Female sample had lower haemoglobin, iron and ferritin than the male sample (Table 1). Lower the haemoglobin, iron and ferritin, better will be the correlations with EPO. Negative correlations of EPO with these variables may be influenced by heterogeneity in the sample resulting from gender differences. Therefore, the sample was partitioned into male and female groups.

Gender differences in the correlations of EPO

The correlations of EPO with haemoglobin, iron and ferritin were stronger in females (Table 2) due to deficiency of haemoglobin, iron and ferritin. X-Y scatter diagram of the variables may be used to visually confirm the negative correlations and discriminate it from artefacts arising from heterogeneity in the sample (Figure 1).

Comparison between males (n = 46) and females (n = 74) after excluding haemoglobin <125g/l						
Variables in SI units	**Male Mean±SD Minimum - Maximum 95% CI of mean**	**Female Mean±SD Minimum -Maximum 95%CI of mean**	**after log_{10} transformation**			
			Shapiro-Wilk test P		**Levene's test, P**	**Student t test (or Mann-Whitney U test), P**
			Male	**Female**		
S. EPO (U/l)	7.43±2.84 4.03 – 14.52 6.58 – 8.27	8.63±4.58 3.67 – 32.27 7.57 – 9.69	0.025	0.023	0.411	(0.105)
Blood Haemoglobin (g/l)	147.50±10.86 127.80 – 168.70 144.28 – 150.72	134.91±7.31 125.00 –160.60 133.22 –136.60	0.038	0.001	0.004	(<0.001)
S. Iron (µmol/l)	22.77±6.63 8.23 – 39.20 20.80 – 24.74	17.12±6.99 1.79 – 34.55 15.50 – 18.74	0.436	0.479	0.705 (before log_{10} transformation)	<0.001
S. Ferritin (ng/ml)	49.64±28.08 10.90 – 105.00 41.30 – 57.98	25.02±21.00 3.60 – 121.90 20.15 – 29.88	0.062	0.665	0.245	<0.001
Comparison between male and female after excluding haemoglobin Male <130g/l (n = 45) and female <120g/l (n = 92)						
S. EPO (U/l)	7.44±2.87 4.03 – 14.52 6.57 – 8.30	9.09±5.06 3.67 – 32.27 8.05 – 10.14	0.025	0.003	0.268	(0.035)
Blood Haemoglobin (g/l)	147.94±10.56 133.00 – 168.70 144.76 – 151.11	132.48±8.12 120.00 –160.60 130.81 –134.15	0.009	0.003	0.061	(<0.001)
S. Iron (µmol/l)	23.03±6.46 8.23 – 39.20 21.09 – 24.97	16.49±6.78 1.79 – 34.55 15.10 – 17.89	0.386	0.215	0.694 (before log_{10} transformation)	<0.001
S. Ferritin (ng/ml)	49.94±28.32 10.90 – 105.00 41.43– 58.45	22.96±19.72 2.50 – 121.90 18.90 – 27.02	0.062	0.880	0.175	<0.001

Table-3: Comparison of EPO, haemoglobin, iron and ferritin in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding anaemia/hypoxia. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed. The cut off levels for exclusion of haemoglobin were <125g/L in male and females (A), <130g/L in males and <120g/L in females (B).

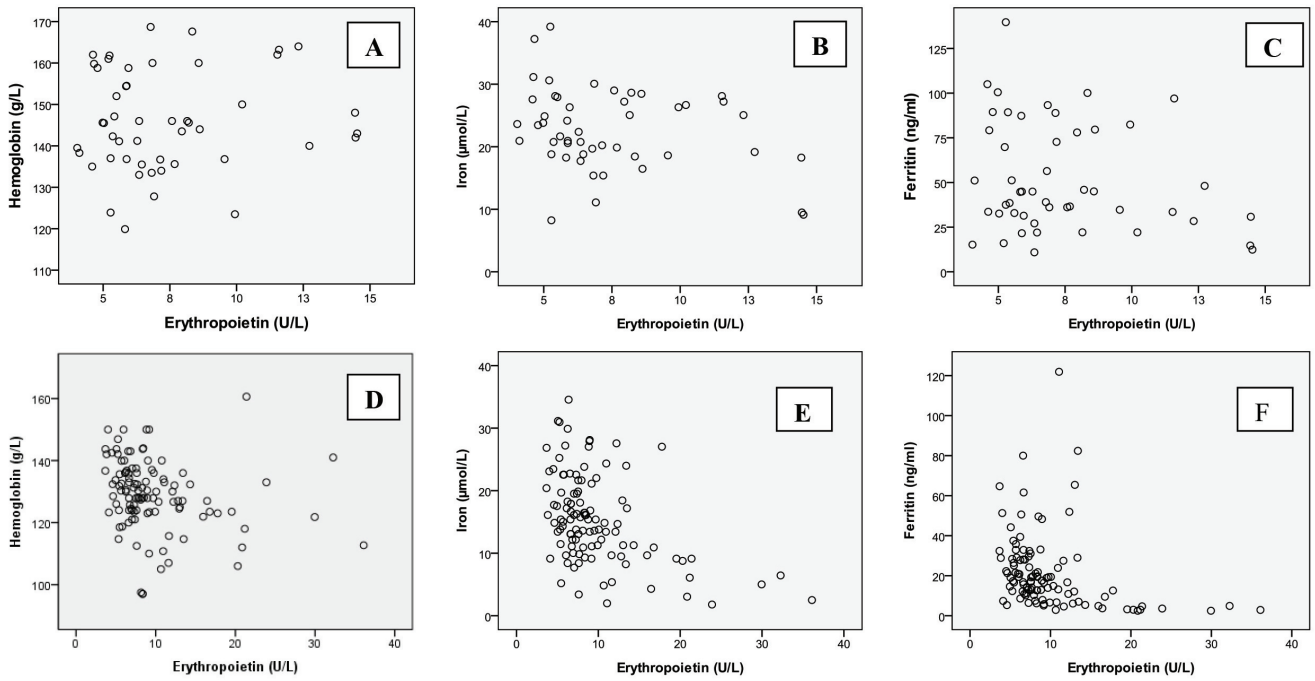

Figure-1: X-Y scatter diagram, in males (A – C; n = 49) and females (D – F; n = 110), of EPO with haemoglobin (A, D), iron (B, E) and ferritin (C, F).

Gender differences in the correlations with EPO were due to anaemia in females and androgens increasing haemoglobin in males[10] resulting in higher haemoglobin cut off for anaemia in males (130g/l) as compared to females (120g/l).[23] EPO levels increase exponentially when haemoglobin levels decreased below 125 g/l.[9,17]

Residual gender differences and correlations of EPO after exclusion of anaemia/hypoxia

When the samples with haemoglobin below 125 g/l were excluded to remove effects of hypoxia/anaemia on EPO, there were no gender differences in EPO despite decreased haemoglobin, iron and ferritin in the female sample (Table 3A). This may be due to increase of EPO in males contributed by increased muscular activity and hypoxia. The hypoxic sensitivity of HIF-2 may be same in males and females. But when the WHO criteria for cut off level of haemoglobin in anaemia were used in males and females[23] to exclude anaemia, there were gender differences in EPO, haemoglobin, iron and ferritin. Haemoglobin level was increased in males and this is further supported by the higher maximum of haemoglobin in males (Table 3B).

The consequent residual negative correlations of EPO with these variables were more in the female sample (Table 2B and C) due to lower haemoglobin, iron and ferritin (Table 3). In the male sample, EPO correlated only with iron. In the female sample, the correlations of EPO with iron and ferritin were stronger than that with haemoglobin. These two observations indicate that iron and ferritin may have independent influence on EPO, outside the influence of hypoxia and this may be due to the level of iron itself. O_2 - dependant degradation of HIF-α subunit is also dependent on Fe^{2+}- dependent prolyl-4-hydroxylases. The sensitivity of HIF to O_2 and PHD to iron[14] in the short term may explain the inverse relationship of haemoglobin, iron and ferritin to EPO production.

Strength, limitation and controversies

The clinical demonstration of the influence of iron on EPO independent of hypoxia/anaemia the gender differences in the correlations with EPO are the strong aspects. But the lack of gender differences in EPO when haemoglobin cut off is <125g/l, was due to increased EPO from increased muscle mass and exercise in young adult males leading to hypoxia, was not experimentally substantiated; it is only an argument and is a limitation. Assuming that there is no gender differences in the sensitivity of HIF-2 to hypoxia, setting a lower cut of 120g/l in females included large number subclinical anaemic samples (18 in number) which raised EPO levels and thus questions the lower cut off for anaemia in the female sample.[23]

CONCLUSION

There were gender differences in the correlations of EPO with haemoglobin, iron and ferritin with strong negative correlations in females. The residual correlation of EPO with iron in males, after exclusion of subclinical hypoxia/anaemia, indicated that in addition to hypoxia, iron deficiency also increased EPO. If subclinical anaemic and iron deficient samples which increased EPO levels are excluded with cut off for haemoglobin <125g/l, iron <36.9 µg/dl and ferritin <20 ng/ml, then a more healthy reference interval for EPO can be determined.

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 (Other centres)) at the Calicut University. Nesheera KK is a recipient of Junior Research Fellowship of Department of Science and technology, Government of India (No. DST/INSPIRE Fellowship/2013/15, Inspire Code IF130015). Authors acknowledge the contributions of Renjith G.

ABBREVIATIONS

EPO: erythropoietin; HIFs: hypoxia-inducible transcriptional factors; hsCRP: high sensitivity C reactive protein; OGTT: oral glucose tolerance test; PHD: prolyl-4-hydroxylases; VHL: von Hippei-Lindau tumor suppressor protein; DMT1: divalent metal ion transporter; Dcytb: putative brush border ferrireductase

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Source of Support: Nil; **Conflict of Interest:** None

Submitted: 06-08-2017; **Accepted:** 30-08-2017; **Published:** 14-09-2017