Association of human plasma erythropoietin levels with other analytes in health and in disease states

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(Faculty of Science)

By

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Under the Guidance of

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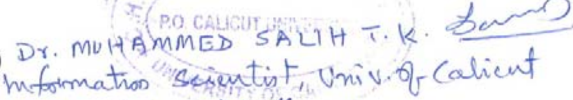

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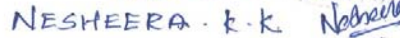

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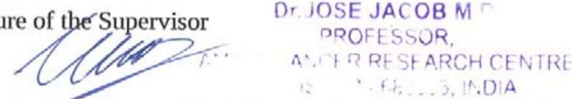

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ABBREVIATIONS

$1,25(OH)_2D$	1, 25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
3′-UTR	3′-untranslated region
ACD	Anemia of Chronic Disease
ACS	Acute Coronary Syndrome
AKT	AKT8 virus oncogene cellular homolog / protein kinase B
ALA	Aminolevulic acid
ALD	Alcoholic Liver Disease
ALT	Alanine Aminotransferase
AML	Acute Myeloid Leukaemia
AP2	Adaptor-related 2 protein complex
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASH	Alcoholic Steatohepatitis
BCE	Bone Collagen Equivalents
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma extra large
BFU	Burst Forming Unit
BMP	Bone Morphogenic Protein
BMI	Body Mass Index
C peptide	Connecting peptide
cAMP	cyclic Adinosine Monophosphate
CaSR	Calcium-Sensing Receptor
CaT1	Ca^{2+} transporter
CBP	CREB-Binding Protein
cDNA	complementary Deoxyribo Nucleic acid
CFU	Colony Forming Unit
CHF	Chronic Heart Failure
CI	Confidence Interval
c-IAP2	cellular Inhibitor of Apoptosis Protein 1

CIS	Cytokine induced SH2-containing protein
CKD	Chronic Kidney Disease
CPH	Carboxypeptidase H
CRP	C-reactive protein
DBP	Vitamin D–Binding protein
DcytB	Duodenal cytochrome b
DMT	Divalent Metal Transporter
DNA	Deoxyribonucleic Acid
ECs	Endothelial Cells
EKLF	Erythroid Kruppel- Like Factor
EPCs	Endothelial Progenitor Cells
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
ERGO	Ergocalciferol
ERK	Extracellular signal-regulated kinase
ESA	Erythrocyte-stimulating agents
FIH	Factor Inhibiting HIF
FOG -1	Friend of GATA-1
FPN	Ferroportin
Gab	GRB associated binder
GATA-1	GATA-binding factor 1
GLUT4	Glucose Transporter type 4
GM-CSF	granulocyte-macrophage colony stimulating factor
GRB	Growth factor receptor-bound protein
GSK-3β	Glycogen synthase kinase -3β
HAMP	Hepcidin Antimicrobial Peptide
HCP	hem-carrier protein
HDL	High Density Lipoprotein
HB	Hemoglobin
HFE	Human Factors Engineering
HIF	Hypoxia-Inducible transcriptional Factor

HJV	Hemojuvelin
HNF	Hepatocyte Nuclear Factors
HOMA	Homeostasis model assessment
hsCRP	high sensitivity C Reactive Protein
HSP	Heat Shock Proteins
IDA	Iron deficiency Anemia
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IL	Interleukin
INFγ	Interferon-gamma
IRE	Iron Responsive Element
IRIDA	Genetic Iron-Resistant Iron Deficient Anemia
IRP	Iron Regulatory Protein
IRS	Insulin/IGF Receptor Substrate
JAK	Janus Kinase
JNK	Jun N-terminal kinase
Klf1	Kruppel- Like Factor
K-S	Kolmogorov-Smirnova
LDL	Low-Density Lipoprotein
LT	Log_{10} transformed
MAPK	Mitogen-Activated Protein Kinase
MDS	Myelodysplastic Syndrome
MHC	Major Histocompatibility Complex
MI	Myocardial Injury
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic Acid
MT-2	Matriptase-2
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-alcoholic steatohepatitis
NF-kB	Nuclear Factor kappa B
NR	Nuclear Receptor

NTx	N-terminal telopeptide
O-DDD	O_2-Dependent Degradation Domains
OGTT	Oral Glucose Tolerance Test
PC	Prohormone Convertases
PCFT	Proton Coupled Folate Transporter
PHD	Prolyl Hydroxylases
PI	Phosphoinositide
PLC-γ	Phospholipase C-gamma
PO_2	O_2 partial pressure
PTH	intact Parathyroid Hormone
rhEPO	recombinant human Erythropoietin
SCF	Stem Cell Factor
SHC	SH 2-containing Collagen-related protein
SHP	SH2-containing Phosphatase
SOCS	Suppressor Of Cytokine Signalling
SOS	Son of sevenless guanine nucleotide exchange factor
STAT	Signal Transducer and Activator of Transcription
SW	Shapiro-Wilk
TAL-1/SCL	T-cell acute lymphocytic leukemia protein 1/ Stem cell leukemia
TBI	Transferrin-Bound Iron
TfR	Transferrin Receptor
TMPRSS6	Transmembrane Protease Serine 6
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
vHL	von Hippel–Lindau protein
VHL	von Hippel-Lindau tumour suppressor protein
WHO	World Health Organisation
XIAP	X-Linked Inhibitor of Apoptosis

INTRODUCTION

Concentrations of Erythropoietin in Circulation and Its Functions

Erythropoietin (EPO) is a cytokine that is known to regulate erythropoiesis (Lacombe and Mayeux, 1998; Krantz, 1991). The primary organs of EPO production and secretion are the kidney, liver, brain and uterus (Fliser and Haller, 2007; Maiese et al, 2008). EPO is mainly synthesised by the peritubular interstitial cells, peritubular endothelial cells, hepatocytes and kupffer cells (Lacombe et al, 1991; Mujais et al, 1999; Maiese et al, 2008). Major stimulus for secretion of EPO in blood circulation is hypoxia through hypoxia inducible factor (HIF) (Haase, 2010). Hypoxia results from conditions such as anemia, high altitude, heart failure, lung diseases and certain deficiencies. As EPO is predominantly produced in the kidney, decrease in EPO levels has been seen during early stages of kidney disease, and rhEPO (recombinant human EPO) has been widely used in patients with anemia resulting from renal disease (Eschbach et al, 1987). EPO, which regulates erythropoiesis, is also involved in improvement of type 2 diabetes mellitus, maintenance of cell functions, iron metabolism, among others. Therefore, the circulating blood concentration of EPO is related to and is influenced by multiple factors. The major contributor of circulating EPO is the kidney. EPO is assayed for investigation of the cause of anemia in patients with kidney diseases and for the treatment of the anemia using recombinant (rhEPO).

Research Problem

Identifying the factors that influence or are influenced by EPO will help in understanding the multiple roles and influences of EPO. These influences can be understood from concentrations and correlations of EPO and its related analytes in health and in disease or deficiency states. Such related analytes are intact parathyroid hormone (PTH), hemoglobin, iron, ferritin, troponin I, serum fasting glucose, fasting insulin, fasting C peptide, serum triglyceride, alanine aminotransferase (fatty liver), BMI and vitamin D. Also, if possible, changes in those concentrations and correlations after treatment. Preliminary studies have shown that there is a correlation between erythropoietin and various other blood analytes. These studies are also important in understanding the reference intervals of EPO and its variations in clinical and subclinical disease processes.

Objectives of the Dissertation

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment.

Organisation of the Thesis

The thesis comprises of eleven chapters. Chapters one and two are Review of Literature and Methodology, respectively. Fulfilment of the Objectives as results is described in Chapters 3 to 11.

Chapter 3. Gender Differences in the Correlations of Erythropoietin with Related Analytes, and their Concentrations in Healthy Young Adults

Introduction: Regulation of the synthesis of EPO by kidneys and absorption of iron by intestine are caused by hypoxia and iron levels. In males, hemoglobin is increased by androgens; in females, hemoglobin, iron and ferritin are decreased by monthly blood loss leading to increase in EPO. EPO increased erythropoiesis to overcome hypoxia. Concentration of hemoglobin, iron and ferritin influence EPO.

Methods: Participants of this observational cross sectional study, aged 18 to 25 years, from the near sea level plains of rural Central Kerala were evaluated clinically and from quantitative biochemical variables with cut off fixed to include anemia, but exclude other primary and secondary clinical conditions, to obtain the EPO study sample (From initial >600 participants, the EPO sample number was reduced to 159). Comparison of the

concentrations and correlations of EPO were done with parametric and nonparametric methods in various groups.

Results: As there were gender differences in the concentrations of EPO, hemoglobin, iron and ferritin, EPO sample was partitioned into males and females. After excluding deficiencies there was decreased or no gender difference in EPO levels (P = 0.105). In the female sample, EPO showed strong negative correlation with hemoglobin, iron and ferritin. But after exclusion of deficiencies in hemoglobin, iron and ferritin, there were major changes in the correlations. In the male sample, EPO showed a negative correlation only with iron. But exclusion of deficiencies influenced the correlations. Strong gender differences were observed in the negative correlations of EPO with hemoglobin, iron and ferritin in females, with weak correlations in males.

Conclusion and Significance: If subclinical anemia and iron deficient samples, which increased EPO levels are excluded, then a more healthy reference level for EPO can be determined (Ref. Chapter 7).

Chapter 4. The Gender Differences in Parathyroid Hormone and the Related Analytes in Healthy Young Adults

Introduction: EPO had shown positive correlations with intact PTH in several studies done for this dissertation. PTH resistance is known in diseases and during deficiencies. In this study, gender differences in PTH, EPO and analytes related to them were evaluated after excluding the deficiencies.

Methods: As above for EPO, but here for PTH sample.

Results: Before the exclusion of deficiencies, gender differences in concentrations were seen in all selected analytes related to PTH. In females, concentrations of PTH and EPO were higher when compared with males; while vitamin D, calcium, hemoglobin, iron and ferritin were lower in females. Exclusion of deficiencies of vitamin D, hemoglobin, iron and ferritin resulted in loss of gender differences in all analytes except hemoglobin, and markedly decreased sample size, especially in females. The strong gender differences in correlations of PTH with related analytes were markedly altered. But after the exclusions, there was a negative correlation of PTH with hemoglobin in males and females, and

positive correlation of PTH with EPO in females and its negative correlation with iron due its consumption for higher hemoglobin in males.

Conclusions and Significance: Higher hemoglobin in men and gender differences in the correlations may be due to androgens in males; in females the lower hemoglobin may be due to inhibition by oestrogens and monthly blood loss resulting in higher levels of EPO and parathyroid hormone. Exclusion of deficiencies in analytes related to parathyroid hormone brought out the gender differences that may indicate differences in regulation of hemoglobin concentrations in males and females.

Chapter 5. Gender Difference in the Relationship of Erythropoietin with C Peptide

Introduction: In the presence of increased insulin resistance in males, insulin secretion is higher. C peptide is closely related to insulin resistance. EPO has been shown to have a beneficial role in type 2 diabetes mellitus and was proposed to be due to decrease in insulin resistance.

Results: The concentrations of hemoglobin, iron and ferritin were lower in females resulting in higher EPO in females. But the concentration of C peptide was higher in male sample due to increased insulin resistance. The relationships of EPO with C peptide were analysed in the male and female sample after exclusion of samples with deficiencies. The higher hemoglobin in males will not increase EPO and, therefore, EPO was not related to C peptide. In the female sample, insulin resistance is lower. Therefore, EPO directly correlated with PTH and with C peptide.

Conclusion and Significance: The increased EPO resulted in increased insulin secretion in the female sample. The direct correlation of EPO with C peptide in females with decreased in insulin resistance indicated increase in insulin secretion. As insulin resistance is lower in females, EPO increased insulin secretion.

Chapter 6. Gender Differences in Concentrations and Correlations of Fasting Insulin, before and after Exclusion of Deficiencies in Hemoglobin, Iron and Ferritin.

Introduction: One of the causes of increased fasting insulin or hyperinsulinaemia is insulin resistance. Insulin resistance increased insulin secretion as a compensatory mechanism. In males insulin resistance is higher than in females resulting in increased fasting insulin in males. EPO has been shown to be beneficial for type 2 diabetes, probably due to decrease in insulin resistance.

Results: In this study, we have shown that there was no gender difference in the fasting insulin concentration, and fasting insulin correlated with EPO in the female sample and not in the male sample. This gender difference may be due to EPO decreasing insulin resistance in males, resulting in decrease of insulin secretion. But increase in insulin secretion directly by EPO may be confounding the correlation between insulin, C peptide and EPO in the male sample. This confounding was less in the female sample where EPO correlated with insulin before exclusion of deficiencies. But after exclusion of deficiencies, EPO decreased in the female sample resulting in loss of gender differences in the correlation of EPO with fasting insulin.

Conclusion and Significance: There is gender difference in the concentration and correlations of insulin with EPO and the related analytes before and after exclusion of deficiencies.

Chapter 7. Baseline Reference Interval of Erythropoietin Sample Population, and Partitioned Male and Female Groups

Introduction: The reference interval reported for EPO was about 4 – 27 U/L. In calculating the reference intervals, it was assumed that α error was 2.5.to 5% of area on either side of the distribution plot by parametric methods or 2.5 to 5% of the sample number in the non parametric percentile method was abnormal and were excluded. But we observed that the number of α and β errors were much more than 5% each.

Methods: Clinical exclusion, clinical biochemistry exclusion and partitioning of samples were used to exclude samples with disease states and deficiencies to obtain the baseline

reference sample. The three reference levels used were 95% non parametric when sample number was ≥120, 95% parametric when the sample number was 30 – 120 and the minimum-maximum levels were used as reference intervals, as errors were removed to a maximum after all three exclusions. When the sample number was below 30, the only method used was the non parametric minimum-maximum levels as the reference interval.

Results: Reference interval chosen for fasting erythropoietin**. (1)** Reference Interval of fasting sample population (n = 411): selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.84 – 26.7 U/L. This is similar to the reported reference interval of EPO. **(2)** Reference Interval of fasting EPO male sample: selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.61 – 19.91 U/L. **(3)** Reference Interval of fasting EPO female sample: selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.84 – 32.28 U/L. The upper limits of the reference intervals showed gender differences. (**4)** Baseline Reference Interval of fasting EPO in male sample aged 18 – 25 years, after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin: selected method non parametric range (minimum-maximum levels); calculated reference interval: 4.11 – 12.73 U/L. **(5)** Baseline Reference Interval of fasting EPO in female sample aged 18 – 25 years, after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin: selected method non parametric range (minimum-maximum levels); calculated reference interval: 3.67 – 13.41 U/L. There was marked decrease in the upper limits of the reference intervals.

Chapter 8. Deficiencies of Hemoglobin, Iron and Ferritin, and their Influence on Erythropoietin and the Related Analytes

Deficiencies in hemoglobin (anemia), iron and ferritin were found to influence concentrations of related analytes such as EPO and their correlations. The relative prevalence and biochemical functions of analytes made us group the samples according to absence of deficiencies, presence of deficiencies in hemoglobin, iron and ferritin in isolation or in combination. Deficiencies were more in the female sample. In the female sample, ferritin deficiency was the most abundant, followed by deficiency of hemoglobin in isolation and deficiency of hemoglobin in combination. Differences in concentrations

of analytes and in their correlations with EPO were observed with and without deficiencies in the major groups. Differences in the correlations influenced the regulation of analytes and caused alterations in the biochemical functions. In samples with anemia and iron deficiency, there were major differences in the correlations of EPO with C peptide. In the female sample, correlation of EPO with hemoglobin was better in the absence of deficiency. In the male sample, EPO did not correlate with hemoglobin in presence or absence of deficiencies. Subclinical deficiencies, therefore, can influence the interpretation of clinical data.

Chapter 9. Influence of Vitamin D Deficiency in Combination with Other Deficiencies, on Erythropoietin and the Related Analytes

Deficiencies in vitamin D in isolation or in combination with deficiencies in hemoglobin, iron and ferritin were found to influence concentrations of related analytes resulting in changes in their correlations with EPO. In the vitamin D sample (n = 148), the most abundant deficiency group was vitamin D deficiency in isolation (n = 33) followed by vitamin D deficiency in combination with ferritin (n = 24) and hemoglobin (n = 24). In the groups formed by the presence and absence of vitamin D deficiency, there were no major differences in concentrations of related analytes and in their correlations with EPO, both in the male and in the female samples. But in the samples with deficiency of vitamin D, there were significant differences both in the concentrations of analytes and in their correlations with EPO and PTH. Though interpretations of the influences of few of the deficiencies could be made, in most cases the interpretations were complex. After correction of vitamin D in the participants, there were no major changes in the concentrations of analytes. But there were major change in the correlations of PTH with calcium in the male sample. In the female sample, there were major changes in the correlations of EPO and PTH in whom the deficiencies were more number of samples.

Chapter 10. Influence of Fasting Hyperglycemia on the Erythropoietin and the Related Analytes in the Presence and Absence of Deficiencies

In the male and female sample glucose, insulin and C peptide increased sequentially from control sample to IFG to diabetes mellitus. This indicated increased insulin resistance

from control to diabetes mellitus resulting in increase in all the parameters related to insulin resistance, such as fasting triglyceride, waist circumference, ALT and hsCRP. The deficiencies in hemoglobin, iron, ferritin and vitamin D had no influence on the relative concentration of fasting glucose, fasting insulin, fasting C peptide and EPO. The gender difference in PTH, which was earlier demonstrated in this study, was also present. As before, there were influences of the deficiencies on the correlation of EPO. These influences on the correlations were complex and could not be explained.

Chapter 11. Influence of Increased Troponin I in Patients Clinically Suspected of Myocardial Injury on Erythropoietin and Its Related Analytes

Atheromatous lesions leading to myocardial injury can influence EPO concentration resulting from the influence of cytokines and myocardial failure. The influence of cytokines from the atheromatous plaque leads to anemia and iron deficiency, with retention of ferritin at the stores. These influences of anemia and iron deficiency can further complicate the outcome of myocardial injury. Myocardial injury may or may not lead to hypoxia. Hypoxia resulting from myocardial injury further increases EPO. It was observed that positive correlation of EPO with troponin I as a result of hypoxia was influenced by anemia and iron deficiency.

Chapter1.

Review of Literature

1.1. Erythropoietin

Erythropoietin (EPO), now classified as cytokine, is a glycoprotein mainly produced by kidney and is the major humoral regulator of red cell production or erythropoiesis.

1.1.1. Historical Aspects of Erythropoietin

The major function of EPO, **erythropoiesis**, was first described by Francois-Gilbert Viault in 1890, who observed a rise in his red blood cell count from 5.0 million to 7.1 million/mm^3 after traveling from sea level to a **high altitude** mountain area at Morococha, Peru at 4500 m above sea level. This observation provided the first demonstration of erythropoiesis, that the specific stimulus for EPO expression is a fall in **tissue O_2 partial pressure (PO_2)** (Jelkmann, 2011; Bunn, 2013).

Anemia as cause for increased erythropoiesis was shown by Carnot and Deflandrein 1906, who demonstrated an increase in red blood cell counts in normal rabbits after injection of serum from anemic rabbits and postulated that erythropoiesis, is regulated by a **humoral factor** in the plasma called hemopoietin. Krumdieck in 1943 and Erslev in 1953 confirmed the experimental design of Carnot and Deflandre by taking the accurate measurements of **reticulocytes** and showed that it takes **3–6 days for the induction of new red cell** production in rabbits following injection of anemic serum. Hence they confirmed the existence of humoral factor that control erythropoiesis, and **named it as erythropoietin** by Bonsdorff and Jalavisto in 1948 (Kasper, 2003; Bunn, 2013).

Hypoxia as a factor for stimulation of erythropoiesis was demonstrated by experiments of Reissmann in 1950 (Kasper, 2003). In the following years, it was observed that **kidney was the major site EPO production** by organ ablation studies in rats (Jacobson et al, 1957) and man (Nathan et al, 1964). First attempt to **isolate and purify EPO** from urine of severely anaemic patients were by Goldwasser and Kung in 1977 (Miyake et al, 1977). Identification of **amino-terminal amino acid sequence** enabled the synthesis of EPO DNA probes and used for **isolation and molecular cloning of the EPO gene** (Jacobs et al, 1985; Lin et al, 1985). This opened up the functions and

molecular biology of EPO and resulted in the development of recombinant human EPO, which is used as a therapeutic agent for the treatment of patients with various types of anemia.

1.1.2. Structure of Erythropoietin

EPO is a small acidic glycoprotein with a **molecular mass of 30.4 kDa**. Therefore, it can cross the glomerular basement membrane into the filtrate and then pass into urine. The EPO gene is located on chromosome 7, contains as a single copy in a 5.4 kb region, and encodes a polypeptide chain containing 193 amino acids (Jacobs et al, 1985). It has a leader sequence of 27 amino acids at the NH_2 terminus and an arginyl residue from the carboxyl terminus, are cleaved intracellularly and a **165-residue polypeptide is released** (Lai et al, 1986; Rency et al, 1987).This amino acid sequence residues forms four antiparallel α-helices, two β-sheets and two intra-chain disulfide bridges (Cys7-Cys161, Cys29-Cys33) (Bazan, 1990a; Boissel et al, 1993). It is heavily glycosylated with approximately **40% of carbohydrate moieties**. The carbohydrate portion comprises of three N- linked (Asp 24, 38 and 83) and one O-linked (Ser 126) oligosaccharide groups which are required for the correct processing and export of the hormone, molecular stability and for its in vivo activity (Delorme et al, 1992; Higuchi et al, 1992). The carbohydrate moiety contains fructose, mannose, N-acetylglucosamine, galactose and N-acetyl neuraminic acid (Dorald et al, 1985). Glycosylation slightly impedes the biological activity of EPO but it is essential for ensuring prolonged circulation in the plasma (Goldwasser et al, 1974). The primary structure of **recombinant human erythropoietin (rhEPO)** was shown to be similar to that of the endogenous hormone except for the in vivo post translational cleavage of arginyl residue from the carboxyl terminus. The molecular cloning of the EPO gene led to high production of rhEPO in cell culture with sufficient purity and quantity for both definitive scientific pursuits and for the development of therapy (Bunn, 2013).

1.1.3. Site of Erythropoietin Production

The **peritubular fibroblasts of the renal cortex** are the primary site of EPO production (Wintour et al, 1996). The renal synthesis of EPO was first demonstrated by Jacobson et al, in 1957. He showed that in rats nephrectomy completely abolished the

increased EPO production in response to **anemia** or the **administration of cobalt**. In patients with **end-stage renal failure and severe anemia** serum EPO levels is found to be extremely low, but after successful renal transplantation the levels will revert to normal (Denny et al, 1966). Histochemistry studies in mouse by **in situ hybridization experiments** showed that EPO mRNA was produced by interstitial cells of the kidney cortex (Koury et al, 1988; Lacombe et al, 1988). EPO mRNAwas detected in interstitial cells of peritubular capillary bed with fibroblast like characteristics in various species within cyst walls of polycystic kidneys (Eckard et al, 1989; Bachmann et al, 1993; Fisher et al. 1961).

Intracellular storage site for EPO was not detected. **Anemia causes an exponential increase in the number of interstitial cells with expotential increase in EPO mRNA and serum EPO** (Koury et al, 1989). Tumor cells of patients with renal adenocarcinoma and polycythemia produce erythropoietin (Da Silva et al, 1990).

In situ hybridization showed that **hepatocytes surrounding central veins** were responsible for the EPO production in the liver (Koury et al, 1991). The second major site of EPO production in adult human is **liver accounting for about 20% of circulating plasma levels**. Hepatic production does not compensate the loss of kidney production of EPO from chronic renal failure. Liver is the major site of EPO synthesis in **fetal stage** with a shift from liver to kidney during gestation (Zanjani et al, 1981).

A small amount of EPO mRNA has also been found in **lung, testes and spleen** (Fandrey and Bunn, 1993; Tan et al, 1991). EPO is also found in the **brain astrocytes** and its receptors are detected in mouse brain (Masuda et al, 1994; Digicaylioglu, 1995) and in cell lines with neuronal properties (Masuda, 1993).

1.1.4. Regulation of Erythropoietin Production

EPO production is markedly **up-regulated by hypoxia** that leads to an increase **in gene transcription** (Schuster, 1989). There are no intracellular storage sites for EPO. Complex interactions between DNA and nuclear proteins controlled the EPO gene expression. Transgenic mice experiments by Semenza et al developed several constructs of human EPO gene containing various lengths of cis regulatory regions (Semenza et al, 1989). Sequences necessary for kidney-specific expression, **kidney inducible elements**

have been localized in a region 9.5 to 14 kb from the 5' end of the human EPO gene (Semenza et al, 1991a). A **negative regulatory element** is present between 0.4 and 6 kb from the 5'end of the human EPO transcription start site (Semenza et al, 1990). Sequences responsible for liver expression, **Liver inducible element** are located within 0.5 kb of 5'-flanking sequences and 0.7 kb of 3'-flanking sequences.

A **50 bp enhancer** has been identified approximately 120 bp upstream from the 3' end of the polyadenylation site, and is responsible for **hypoxia-inducible EPO gene expression** (Semenza et al, 1991b; Beck et al, 1991; Blanchard et al, 1992). This enhancer contains three different segments (Semenza et al, 1992). A conserved sequence is located near the 5'end of the enhancer, where the transcription factor **hypoxia-inducible factor1 (HIF-1) binds** (Beck et al, 1993; Wang and Semenza, 1993). The C-terminal portion of HIF-1 specifically binds to **p300, a transcriptional coactivator** (Arany et al, 1996). A functional domain conveying oxygen regulates response has been identified in the middle segment (Pugh, 1994). The third part corresponds to 3' end is a binding site for hepatocyte nuclear factor 4 (HNF-4) (Galson et al, 1995). **DNA-binding proteins** interact with the transcriptional coactivator **p300/CBP** and enhance hypoxic induction (Arany et al, 1996). Thus, hypoxia induces the formation of a complex of proteins, which directly or indirectly binds to the enhancer and transduces a signal to the promoter, thereby permitting EPO gene transcription (Huang et al, 1997).

1.1.4.1. Hypoxia-inducible factor1 or HIF

Wang and Semenza in 1995 purified HIF by affinity chromatography. HIF-1 is a heterodimer transcription factor containing **two basic helix – loop - loop- helix proteins**, **an O_2-labile α subunit** (120 kDa; isoforms 1α, 2α or 3α) and a constitutive **β-subunit** (90–95 kDa) (Wang and Semenza, 1995). HIF belongs to the PAS [PER/arylhydrocarbon receptor nuclear translocator (ARNT)/singleminded (SIM)] family of transcription factors (Wang et al, 1995). HIF- β has been previously cloned and identified as aryl hydrocarbon receptor nuclear translocator (ARNT) (Hoffman et al, 1991).

In man and other mammals, **three isoforms of HIF-α gene** have been identified: HIF-1α, HIF-2α (Tian et al, 1997), and HIF-3α (Gu et al, 1998). HIF-1α is more abundant and present in almost all organs and tissues. Whereas originally thought that HIF-2α has

more restricted expression and is limited to the vascular endothelium (Tian et al, 1997). Later studies showed that **HIF-2** (also called EPAS1 for endothelial PAS domain protein 1) **is the primary transcription factor inducing EPO expression** (Warnecke et al, 2004; Haase, 2010). Production and up-regulation of EPO by hypoxia require HIF-2α (Gruber et al, 2007) in kidney (Scortegagna et al, 2005; Kapitsinou et al, 2010; Paliege et al, 2010) and in fetal and adult liver (Rankin et al, 2007). HIF-1α is involved in facilitating oxygen delivery and cellular adaptation to hypoxia by stimulating several biological processes, such as **erythropoiesis, angiogenesis, and anaerobic glucose metabolism** (Semenza, 2001). On using Cre-lox P recombination to ablate renal HIF-2α by Kapitsinou et al. shown that hepatic HIF-2 takes over as the main regulator of the serum EPO level. Hepatocyte-**derived HIF-2 is also involved in the regulation of genes in iron metabolism**, supporting the role of HIF-2 in the coordination of EPO synthesis with iron homeostasis (Kapitsinou et al, 2010).

1.1.4.2. Oxygen sensing of HIF

To understand the mechanism of activation of HIF-1, it is necessary to determine how hypoxic induction impacts on the expression of its two subunits. **In hypoxic conditions, the levels of the mRNAs encoding HIF-α or HIF-β does not get altered**. This steady-state in level of mRNA suggests that the activity of the HIF-α-ARNT complex is **regulated by a post-transcriptional mechanism** (Kallio et al, 1997).

At the protein level, irrespective of the oxygenation of the cell the ARNT subunit remains abundant and HIF-α subunit cannot be detected in oxygenated cells (Wang et al, 1995; Huang et al, 1996). **HIF-α protein can only be detected in deoxygenated cells or in those exposed to iron chelators** or certain transition metal ions like cobalt, all of which induce HIF-dependent gene transcription (Semenza and Wang, 1992). These observations suggest that the activation of HIF-1 depends on an increase in the amount of HIF-α protein in deoxygenated cells.

In normoxia, HIF-α is remarkably unstable (Huang et al, 1996) and involves the formation of a complex with **von Hippel–Lindau protein (vHL)** (Maxwell et al, 1999), enabling it to under go ubiquitination and subsequent degradation in proteasomes.

O_2 - dependant degradation of HIF-α subunit is depends on a large (200-residue) interior segment at the C-terminus called O_2-dependent degradation domains (O-DDD) (Huang et al, 1998). These domains are prolyl hydroxylated (at Pro402 and Pro564 in HIF-1α, and Pro405 and Pro531 in HIF-2α) in the presence of O_2 (Epstein et al, 2001; Bruick & McKnight, 2001; Jaakkola et al, 2001; Ivan et al, 2001; Yu et al, 2001). The reaction is catalysed by **prolyl-4-hydroxylases** (PHD-1, PHD-2 and PHD-3) and **requires molecular oxygen, ferrous iron, and ascorbate** (Schofield and Ratcliffe, 2004). These enzymes transfer one O-atom of O_2 to the proline and the other to 2-oxoglutarate yielding CO_2 and succinate (Bruegge et al, 2007). The **proline hydroxylated HIF-α combines with von Hippel-Lindau tumour suppressor protein** (VHL)/E3 ligase and and undergoes proteosomal degradation (Pugh et al, 1997; Huang et al, 1998; Maxwell et al, 1999).

Furthermore, the transcriptional activity of the HIFs is suppressed by **oxygen-dependent asparagine hydroxylation** (at Asn803 in HIF-1α and Asn847 in HIF-2α) via **factor inhibiting HIF (FIH)**, which blocks the binding of the transcriptional co-activators CREB-binding protein (CBP) and p300. FIH-1 is another Fe^{2+}-containing and 2-oxoglutarate-requiring dioxygenase. FIH inactivation facilitates CBP/p300 recruitment to the HIF transcriptional complex and causes an increase in the expression of HIF target gene under hypoxia or in VHL-deficient cell lines (Mahon et al, 2001; McNeill et al, 2002; Stolze et al, 2004). According to in vitro assays, Km values of the three PHDs for O_2 are above the arterial PO_2 (~170 mmHg), whereas for **FIH-1 it is lower PO_2 (~60 mmHg)** (Koivunen et al, 2004). Thus, the HIF-α PHDs are the primary O_2 sensors in control of EPO production.

In hypoxia, HIF-α subunit is not degraded and can enter the nucleus and where it forms a stable heterodimer with HIF-β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-2α/β heterodimers bind to the **HIF consensus binding site 5'-RCGTG-3' and increase EPO transcription** in the presence of coactivators CBP and p300 (Salceda and Caro, 1997).

Degradation of HIF-α subunit from oxidative modification is **inhibited by iron chelators and cobaltous ion**, which presumably displaces iron from the active site and

thought to be enhancing EPO expression. The discovery that Fe^{2+} is required for HIF-α degradation may provide an explanation for the increased level of plasma EPO in patients treated with the iron chelator like deferoxamine (Kling et al, 1996). **2-Oxoglutarate competitors** inhibit the hydroxylation of HIF-α and shown to stimulate EPO production in vitro and in mice (Hsieh et al, 2007). **Nitric oxide, reactive oxygen species, and Krebs cycle metabolites like succinate and fumarate** also inhibit HIF PHDs and cause an increased EPO transcription (Haase, 2010).

Prolyl hydroxylases enzymes are the proximate oxygen sensor for regulating HIF. Among the three prolyl hydroxylases, **PHD-2** plays the predominant role in the renal production of EPO (Minamishima et al, 2008; Takeda et al, 2008). PHD-2 and PHD-3 are themselves HIF-target genes and increases transcription of PHD2 and PHD3. So their expression increases and HIF-α levels decline during long-term hypoxic periods (Aprelikova et al, 2004; Stiehl et al, 2006).

This **feedback regulation** may explain the declining EPO production during **chronic anemia or prolonged stay at high altitude.** Furthermore, protein turnover of PHD1 and PHD3 is hypoxically regulated by Siah proteins, which themselves are hypoxia inducible (Nakayama et al, 2004).

1.2. Action of Erythropoietin

1.2.1. Structure of the EPO Receptor

Radiolabeled EPO were used to demonstrate specific binding to cells derived from the **erythroid lineage**. Cells used for these experiments were **normal erythroid progenitors, virally transformed spleen cells, murine and human erythro leukemia cells, and cells from human fetal liver**. Then **cloning of the EPO-R cDNA** was done. The results showed that there is 80% protein homology between the murine and human EPO polypeptide (D'Andrea and Zon, 1990). Erythropoietin receptor (EPO-R) from a cDNA library of murine erythroleukemic cells has also been cloned. This cloning experiment provided a new insights into the understanding of EPO-induced signal transduction (D'Andrea, 1989).

The human EPO receptor gene is located on chromosome 19 (Winkelman et al, 1990) and contains eight exons and seven introns, encoding a 507 aminoacid peptide with a **molecular weight of 66 kDa**. In human genes, exons 1–5 encode the 251 aminoacid extracellular domain, exon 6, a 20 aminoacid transmembrane α-helical region, and exons 7–8 the 236 amino acid cytoplasmic domain (Maouche et al, 1991; Noguchi et al, 1991). In addition to this cloned chain, cross-linking of EPO to the cell surface of erythroid cells detects **two accessory molecules of 85 kDa and 100 kDa that are associated with p66** but not recognized by anti-p66 antibodies (Mayeux et al, 1991; D'Andrea and Zon, 1990).

EPO-R belongs to the **cytokine class I receptor family** with a single membrane-spanning domain. This family shares specific structural motifs with other members of the receptor family. The most striking similarity is the presence of four cysteines and a WSXWS motif (i.e. tryptophan-serine-any amino acid-tryptophan-serine) positioned in the extra-cellular ligand-binding region (Cosman et al, 1990; Bazan, 1990b). EPO-R extracellular region comprises two immunoglobulin-like domains, each formed by a β-sandwich-like structure containing 7 β-strands. The membrane-distal (D1) domain contains the four conserved cysteine residues, which form two intra molecular disulfide bridges that stabilize D1 (Livnah et al, 1996; Syed et al, 1998). The membrane-proximal (D2) domain is linked by a short hinge and contains the conserved WSXWS motif. This motif appears to be important for the EPO-R folding and stabilizes its tertiary structure (Livnah et al, 1999; Syed et al, 1998). The cytoplasmic region of EPO-R **is rich in amino acids proline, glutamine and aspartase** contains a box 1 domain that is specific to JAK2 (Zhuang, 1994) and contains eight phosphotyrosine sites. The interaction between EPO and its receptor revealed that **EPO has two discrete binding sites for the EPO-R** (Syed et al, 1998). The first EPO-R molecule binds EPO with a high affinity with a dissociation constant of approximately 1 nM and is thought to contribute the majority of the ligand binding energy (Syed et al, 1998), whereas the second EPO-R molecule binds to the complex with affinity approximately 1000-fold lower (dissociation constant of ~1μM) (Philo et al, 1996). The EPO-R is **expressed in hematopoietic cells** as well as in nonerythroid tissues including **brain** (Digicaylioglu et al, 1995), **heart** (Depping et al, 2004), **uterus** (Yasuda et al, 1998), kidney (Westenfelder et al, 1999), pancreatic islets (Fenjeves et al, 2003).

1.2.2. Erythropoietin Signaling Pathways

Erythropoietin receptor does not have endogenous tyrosine kinase domain within its cytoplasmic region. This indicates that protein tyrosine kinase function is carried out by a distinct factor, **JAK2 protein tyrosine kinase** was identified as the principal kinase involved in mediating EPO-responsive signal transduction (Witthuhn et al, 1993; Yoshimura and Lodish, 1992). EPO-R signals through multiple pathways include the signal transduction and activator of transcription (STAT) 5 pathway, the phosphatidylinositol 3-kinase/ protein kinase B (PI-3K/AKT) and MAPK/ERK pathways (Jelkmann et al, 2008).

JAK2 molecule specifically binds to box 1 domain of the EPO-R, and dimerization with conformational changes brings both JAK2 molecules into close proximity leading to **transphosphorylation and activation of JAK2**. The activation of JAK2 causes phosphorylation of tyrosine residues of the intracellular EPO-R domainand recruits binding of various intracellular **transcription factors that contain src homology 2 domains**. These include **STAT5A and STAT5B, the p85 subunit of phosphoinositol 3'-kinase (PI3K), nuclear factor kappa B (NF-kB) and p42/44 mitogen-activated protein kinase (MAPK)** (Chin et al, 1996; Damen et al, 1995a; Damen et al, 1993; Klingmuller et al, 1995; Matsumoto et al, 1997; Kwon et al, 2014). Deletion of the JAK2 gene in mice causes embryonic death by day 12–13 from severe anemia (Parganas et al, 1998). Thus JAK2 is essential for EPO-dependent definitive erythropoiesis. But **all of these signaling pathways** are known not only for promoting **red blood cell proliferation,** but also for **vasodilation** (Serizawa et al, 2015**), insulin-sensitization** (Alliouachene et al, 2015), and for having **antithrombotic, anti-inflammatory and anti-apoptotic actions** (Bouvard et al, 2015)

1.2.2.1. STAT (the signal transducer and activator of transcription) pathway

In cytokine induced signaling the **STAT (the signal transducer and activator of transcription) pathways** plays a major role. The STAT5A and STAT5B proteins are predominant signal transducers for EPO-R. JAK2 mediated STAT5 phosphorylation leads to the formation of stable STAT5 homodimers. These proteins accumulate in the nucleus and bind to the specific regulatory sequences and mediate EPO-responsive

transcription. It induces **transcription of genes** involved in **erythroid survival, proliferation and differentiation signals** (Damen et al, 1995b; Klingmuller et al, 1996 Watowich, 2011). STAT5 bind to cis-acting elements and **increases Bcl-X_L and Bcl-2 expression** and thereby **protecting proerythroblasts from apoptosis** (Silva et al, 1996). STAT pathway appears to be the **most important signaling pathway for EPO action**.

Adult mice deficient in both STAT5 isoforms (STAT5A and STAT5B) have a normal hematocrit indicated that activity was non-essential for adult erythropoiesis in homeostatic conditions (Teglund et al, 1998). However, STAT5 deficient embryonic mice cause a severe anemia shows a role in fetal erythropoiesis (Socolovsky et al, 1999). Erythropoietic rate could not efficiently increase in STAT5 deficient mice in response to stress or had chronic anemia indicating a critical role for STAT5 in demand-driven erythropoiesis (Socolovsky et al, 2001).

1.2.2.2. Phosphatidylinositol 3-kinase/ protein kinase B (PI-3K/AKT)

Other signaling cascades such as the PI3K/Akt and Ras/MAPK pathways are evoked upon EPO-R activation. PI3K/Akt signaling has been identified as the major transduction pathway for EPO-mediated **cell protection in various mammalian non-hematopoietic tissues** (Chateauvieux et al, 2011; Zhang et al, 2015). This pathway is reported to play an important **role in cell growth, survival and metabolism**. This pathway also mediates anti-apoptotic, proliferative and differentiation responses of erythroid cells (Haseyama et al, 1999; Uddin et al, 2000; Bouscary et al, 2003). The Phospholipase C-gamma (PLC-γ) pathway is also activated via the EPO-R and appears to generate signaling phospholipids and Ca^{2+} mobilization (Ren et al, 1994; Halupa et al, 2005).

1.2.2.3. MAPK/ERK pathway

The mitogen-activated proteins kinases (MAPKs) play crucial roles in cellular signaling pathways and gene expression and consisting of three major members: **Extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminalkinase (JNK)** (Honda et al, 2016). These molecules represent three different intracellular-signaling cascades. Initiation of the ERK/MAPK cascade involves activation of Ras, Raf, and MAPK/ERK kinase. The ras/raf/MAPK pathway is initiated by the association of

SHC/GRB2/SOS complex to the phosphorylated EPO-R eventually leads to the translocation of active MAPK to the nucleus to phosphorylate transcription factors such as ELK-1 that promote **inhibition of apoptosis, cell proliferation and differentiation** (Strack, 2002: Carroll et al, 1991). In contrast with erythroid cell types, the EPO-mediated signaling pathways are less well characterized for non-erythroid cells.

The p38 MAP kinase and JNK pathways are also activated by EPO in erythroid cells (Nagata et al, 1997a; Nagata et al, 1997b) and appear to be important for the proliferation, differentiation and apoptosis (Nagata and Todokoro, 1999; Shan et al, 1999).

Recent data have shown that other signaling factors may also be involved in erythropoietin signaling. The **Src family tyrosine kinase Lyn** phosphorylates the receptor and activates STAT5 and showed dramatic effect on erythroid cell maturation (Tilbrook et al, 1997; Chinet al, 1998). SHP-1 and SHP-2 are the two tyrosine phosphatases involved in erythropoietin induced signaling. Dephosphorylation of the EPO receptor is achieved by SHP-1. It acts as a negative regulator, which binds through its SH2 domain to phosphorylated tyrosines of the receptor. SHP-1 also mediates the inactivation of JAK2 through dephosphorylation (Klingmuller et al, 1995).While SHP-2 play a positive role in stimulating cell proliferation signals (Li et al, 2011).

Another mechanism for down regulating the EPO receptor involves the **SOCS family of proteins**; include CIS, SOCS-3, and SOCS- 1. These are induced by activation of the EPO-R and their translated proteins can directly bind the receptor and JAK2 (Sasak et al, 2000; Jegalian and Wu, 2002). This leads to competitive blocking of STAT to the receptor and initiate ubiquitin-mediated proteasomal degradation of JAK2 (Endo et al, 1997; Ungureanu et al, 2002). Inactivation of the Src-family kinase Lyn occurs via the transmembrane phosphatase CD45 (Harashima et al, 2002).

1.3. Erythropoiesis

The entire process by which red cells are produced in the bone marrow is termed as erythropoiesis. The various stages of erythropoiesis includes the 1) **commitment of pluripotent stem cell progeny into erythroid differentiation**, 2) **the EPO-independent or early phase of erythropoiesis** and the 3) **EPO-dependent or late phase of erythropoiesis**. Erythropoiesis involves a great variety and number of cells at different stages of maturation like commitment, differentiation and proliferation of progenitor cells. These cells progressively acquire distinct functional and morphological characteristics.

Erythropoiesis in humans and other mammals proceeds normally at a low basal rate by replacing senescent red blood cells with young reticulocytes. In humans, erythropoiesis can be enhanced eightfold times than the baseline rate in various clinical conditions like hemorrhage, hemolysis, and other types of stress that impair the oxygenation of arterial blood or the delivery of oxygen to the tissues. **EPO** is the sole mediator of **hypoxic induction of erythropoiesis**. EPO is essential for erythropoiesis. EPO is mainly produced by hepatocytes during the fetal state, following birth, peritubular fibroblasts in the renal cortex become the main production site. Erythropoiesis is a slow-acting process. It takes **3 - 4 days** before reticulocytosis becomes apparent following a rise in plasma EPO. EPO concentrations are usually expressed in international units (IU) (Jelkmann, 2007). The rate of erythropoietin synthesis is inversely related to hemoglobin concentration. EPO concentration may increase from approximately 10mIU/ml at normal hemoglobin concentrations to 10,000 mIU/ml in severe anemia.

1.3.1. Commitment of Stem Cell Progeny to Erythroid Differentiation

The cells that give rise to colonies were termed as **colony forming unit-spleen (CFU-S)** and these cells are identical or closely related to multipotent hematopoietic stemcells. Both commitment and differentiations are irreversible events. Under normal conditions, once commitment occurs, differentiation proceeds fully and mature cells are formed.

1.3.1.1. Erythroid progenitor

Erythroblasts in the bone marrow are generated from proliferation and differentiation of erythroid progenitors. These progenitor cells cannot identify morphologically, but detected functionally by their ability to form colonies of erythroblasts.The two erythroid progenitors in the bone marrow are **burst forming unit-erythroid (BFU-E)** and **colony forming unit-erythroid (CFU-E)** (Dessytris and Sawyer, 2009).

1.3.1.1.1. Burst forming unit-erythroid (BFU-E)

The first committed erythroid progenitor is the burst forming unit-erythroid (BFU-E). In the presence of EPO, and under the influence of other factors acting on early hematopoietic cells such as IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), thrombopoietin and stem cell factor give rise to clusture of many erythroid colonies, which takes approximately **10 - 14 days** to form large, often broken, hemoglobin-containing colony of erythroblasts. BFU-E **expresses EPO receptors** (Dessytris and Sawyer, 2009).

1.3.1.1.2. Colony forming unit-erythroid (CFU-E)

BFU- E then matures in to colony forming unit-erythroid (CFU-E), which is the more mature erythroid progenitor. These cells mature within **3 to 5 days** and form small, tightly packedcolonies of more differentiated erythroid progenitor. Both BFU-E and CFU-E cannot be identified by microscopy as they have no distinct morphological features, and appear as typical primitive hemopoietic blast cells. However EPO-R density and EPO dependency increase at the level of CFU-E (Krantz, 1991; Prchal, 2010). Studies on knockout mice lacking EPO or the EPO-R have shown that **EPO is crucial for the proliferation and survival of CFU-E** and their irreversible terminal differentiation. But it is not required for generation of BFU-E and their differentiation to CFU-E (Kieran et, 1996; Lin et al, 1996). EPO mainly act as a survival factor, by maintaining cell proliferation and the induction of expression of erythroid specific proteins. Thus, EPO does not appear to be involved in the commitment of the erythroid lineage (Wu et al, 1995; Dessytris and Sawyer, 2009).

1.3.2. Differentiation of Erythroid Precursors

Cells that constitute the latter stages of erythropoiesis can be identified by light microscopy. The first morphologically recognizable erythroid precursor cells are **proerythroblasts**, which are derived from CFU-E. Proerythroblasts also contains EPO-R. These cells develop sequentially into **basophilic erythroblasts, polychromatic erythroblasts and eventually into orthochromatic erythroblasts,** at this state nuclei are extruded and form reticulocytes. Morphological characteristics of each stage are seen with ordinary microscope. The cytoplasm of erythroid precursor contains ribosome, which remains free in the cytoplasm rather than being part of endoplasmic reticulum. Mitochondria in erythroid cells are round or oval and the cristae are less distinct.

1.3.3. Proliferation and Maturation

Approximately **50 erythroblast and 124 recticulocytes** are formed from each proerythroblast. Each erythroblast undergoes five mitotic divisions over 5 days before the orthochromatic erythroblast loses its nucleus and enters a 2 to 3 day period of maturation before its release from the marrow. These enucleated cells released into the circulation as mature disc-shaped biconcave erythrocytes (Prchal, 2010).

The stages of morphological maturation are characterized by a gradual reduction in cell size, nuclear condensation, cytoplasmic acidification, and with increased hemoglobin concentration (Alter, 1994). After erythrocytes have been in the circulation for an extended period, the outer lipid layer of erythrocytes begins to express phosphatidylserine and is recognized by reticuloendothelial macrophages. This results in the degradation of the aging erythrocytes and recycles most of the red cell components (Schroit et al, 1985).

Normal development of erythroid precursors occurs in **erythroblastic islands**, which is the anatomical unit of erythropoiesis. It consisit of a centrally located macrophage, surrounded with differentiating and maturing erythroid cells (Bernard, 1991; Sadahira and Mori, 1999). These islands are observed in adult bone marrow and in splenic red pulp, the secondary site of erythropoiesis. When the erythroblast is mature sufficiently for nuclear expulsion, it makes contact with endothelial cells and passes

through a pore in cytoplasm of the endothelial cell via an unclear mechanism. These cells then enter the circulation as recticulocytes. Within the erythroblastic islands, resident macrophages are responsible for phagocytosing the extruded nuclei of reticulocytes, as well as engulfing other eliminated organelles. Macrophages also produce stimulatory role in erythropoiesis as depletion of macrophages suppresses erythropoiesis.

1.3.4. Regulation of Erythropoiesis

Normal erythropoiesis requires precise and well coordinated expression of transcription factors, including **GATA-1** (Pevny et al, 1991), **FOG-1** (Tsang et al, 1997), **TAL-1/SCL** (Shivdasani et al, 1995), and **EKLF/Klf1** (Perkins etal, 1995). These transcriptional factors bind to the DNA of promoter or enhancer of many genes and achieve commitment, proliferation, and differentiation of erythroid cells. Of these transcription factors, **GATA-1** is the most extensively studied which promote the establishment of an erythroid phenotype and suppresses alternative lineage or multipotent progenitor capacity of the cell (Perry and Soreq, 2002). The GATA-1 consensus DNA binding site (T/AGATAA/G) is present in the regulatory elements of both erythropoietin and erythropoietin receptor gene (Chiba et al, 1991). Mice deficient for GATA-1 die in utero due to apoptosis of erythroblasts (Pevny et al, 1991), demonstrating a crucial, non-redundant role for GATA-1 in erythroid commitment.

GATA-1 interacts with FOG-1 and helps to promote GATA-1 to establish an erythroid phenotype on progenitor cells (Tsang et al, 1997). TAL-1/SCL is also incorporates GATA-1 and is involved in promoting phenotype and terminal differentiation of erythroid cells (Wadman et al, 1997). EKLF/Klf1 is a transcription factor essential for regulating β-globin gene expression in the late stages of erythroid differentiation and elimination of this gene produces a severe anemia (Spadaccini et al, 1998).

The different stages of differentiation cross-regulate each other, via expression of Fas death receptors on immature erythroblasts, while in contrast, more mature erythroid cells produce Fas ligands. Thus, an excess of mature erythroid cells inhibits the differentiation of less developed erythroid precursors (De Maria et al, 1999). Studies showed that **INFγ** (interferon-γ) down regulates SCF (stem cell factor) and EPO-R at the

surface of the erythroid progenitors, thus leading to reduction of the survival and growth of these cells and eventually to apoptosis of the progenitors (Taniguchi et al, 1997). Further studies showed that interferon-γ induced the concomitant expression of Fas and Fas ligand at the surface of the erythroid progenitors, and leads to apoptotic cell death (Dai et al, 1998).

1.4. Heme and Hemoglobin

Hemoglobin is the main protein in mature red cells and contains more than 600 million hemoglobin molecules. Hemoglobin is a tetramer which is held by non-covalent interactions. Each subunit of hemoglobin is composed of **a heme and four globin chains** (2 alpha and 2 beta).

Hb A1 ($\alpha_2\beta_2$) is the major (98%) form of hemoglobin in adults.hemoglobin is the oxygen carrying component of RBCs and its main function is to carry oxygen from the lungs to the tissues and return carbon dioxide (CO2) from the tissue to the lungs.

1.4.1. Biosynthesis of Hemoglobin

Erythropoiesis is a biological process which consumes most of the body iron for hemoglobin synthesis. As hemoglobin accounts for approximately 90% of dry weight of erythrocyte, the biosynthesis of hemoglobin is closely related to erythropoiesis. The production of hemoglobin begins during the proerythroblast stage of erythropoiesis. Heme is not only the structural component of hemoglobin but also plays multiple regulatory roles in the differentiation of erythroid precursor cells. Maturation of erythrocyte precursors are related to hemoglobin production and content. More over the differentiation of CFU-E into erythrocyte precursors require the activation of gene involved in hemoglobin synthesis (Dessytris and Sawyer, 2009).

1.4.1.1. Heme synthesis.

Heme synthesis is a complex process involving two stages: **biosynthesis of porphobilinogen** and **conversion of porphobilinogen to heme**. The synthesis takes place in the mitochondria and in the cytosol by a series of biochemical reactions.

1.4.1.1.1. Biosynthesis of porphobilinogen.

The first step in heme synthesis is takes place in the mitochondrion, with the **condensation of glycine with succinyl CoA**. The reaction is catalyzed by **aminolevulinic acid Synthase**, with Pyridoxal phosphate as a cofactor to form **5-aminolevulic acid (ALA)**. This reaction is the rate limiting step and is the highly regulated reaction in heme biosynthesis. The enzyme ALA synthase is highly regulated by the amount of heme in the cell. ALA then leaves the mitochondria and form porphobilinogen by the condensation of two molecules of ALA. This reaction is catalyzed by ALA dehydrase. The porphobilinogen is unstable and chemically reactive (Chiabrando et al, 2014; Burnhann, 1968).

1.4.1.1.2. Conversion of porphobilinogen to heme

Four molecules of porphobilinogen undergo condensation reaction by uroporphyrinogen-I synthase and form **uroporphyrinogen-I**. The uroporphyrinogen-1 then converted to form **uroporphyrinogen-III** by uroporphyrinogen-III cosynthase. Decarboxylation of four acetic acid side chains of uroporphyrinogen-III results in the formation of **coproporphyrinogen-III** which is catalyzed by uroporphyrinogen decarboxylase. These reactions were occurred in the cytosol.

Coproporphyrinogen-III is then transported to mitochondria and then forms **protoporphyrinogen-IX** from coproporphyrinogen-III. This reaction is catalyzed by a mitochondrial enzyme coproporphyrinogen oxidase. Oxidation of protoporphyrinogen-IX by protoporphyrinogen oxidase forms **protoporphyrin-IX**. The insertion of ferrous ion (Fe^{2+}) into the middle of protoporphyrin-IX is catalyzed by ferrochelatase (heme synthase) to form **heme**. Heme is the prosthetic group for hemoglobin, myoglobin and cytochromes. Heme also present in enzymes like catalase and peroxidase. Heme exits in mitochondria, which then combines with the globin molecules synthesized in the ribosome (Chiabrando et al, 2014; Burnhann, 1968).

A **disruption in the production of heme** causes a variety of anemias. Iron deficiency impairs heme synthesis and thereby causing anemia. A number of drugs and toxins also interfere with enzymes involved in heme biosynthesis and inhibit heme

production. Lead poisoning commonly produces anemia as lead inhibits heme synthesis (Bottomley, 1968).

1.4.1.2. Globin synthesis

Human globins are encoded by α and β gene clusters, in which α gene cluster is located on the short arm of chromosome 16 where as β gene cluster is located on chromosome 11. The α-gene cluster contains genes for two adult α_1, α_2 and an embryonic ζ chain. The β-gene cluster comprises genes for an embryonic ε, duplicated fetal γ and adult β and δ globins (Diesseroth et al, 1977; Fritsch et al, 1980).

Two α-globin chains and two β-globin chains are arranged in pair forming the tetrameric molecule or globin portion of the hemoglobin. Each globin chain has a covalently bound heme moiety a reduced ferrous ion component, which helps in the reversible binding of oxygen, carbon dioxide and carbon monoxide. The globin portion of normal hemoglobin consists of two α chains and two β chains of these α chain contains 141 amino acids and β chain contains 146 amino acids. The δ chain contains a variation of 10 amino acid and γ chain by 39 amino acid compare to β chain (Dessytris and Sawyer, 2009; Higgins et al, 2012).

The location and rate of synthesis of globin chains vary from embryonic, to fetal and then to adult globin synthesis. Globin synthesis is controlled by the interaction between several transcription factors, the locus control regions upstream of the gene cluster and the promoter regions of the individual genes. The expression of α and β-genes is closely balanced to avoid the accumulation of free globin chains by an unknown mechanism. Balanced gene expression is required for normal red cell function and any disturbances in this balance will produce a disorder called **thalassemia**.

1.5. Iron Metabolism

Iron is essential for several vital biological processes like cellular processes, energy production, biosynthesis, replication and locomotion. The most common forms of iron in the human body are **ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron**. Iron when presented on its free form has toxic properties. It has the ability to mediate electron transfer, changing between the +2 and +3 oxidation states and is easily achieved in a way that regular

reducing agents perform the reduction of aqueous Fe^{3+} to Fe^{2+}, while dioxygen (O_2) promotes the reverse reaction (Beutler, 2010).

In normal physiological O_2 concentrations the most stable form of iron is Fe^{3+}. Superoxide radicals are formed by the reduction of O_2 by Fe^{2+} (Walling et al, 1975). This may elicit the production of reactive oxygen species responsible for cellular and tissue damage. These radicals are responsible for the attack to proteins, nucleic acids and carbohydrates, for triggering the propagation of lipid peroxidation and, ultimately, cell apoptosis. Therefore, the organism has developed mechanisms to prevent this toxic property of free iron. Iron is usually **found coupled with proteins** to avoid the negative effects of reactive oxygen species. In serum, it is mainly associated with **transferrin**, while within the cells stored as **ferritin** (Silva and Faustino, 2015). The mechanism by which iron homeostasis is regulated by dietary **iron absorption** by the duodenum, **transport** of iron in the circulation, cellular uptake and consumption, **recycling** by macrophages and **storage** in the liver (Silva and Faustino, 2015).

1.5.1. Absorption

Dietary iron is classified as **heme or non-heme iron**, in which heme iron is highly abundant in meat. It is a component of the hemoproteins, hemoglobin and myoglobin, normally comprises about one third dietary protein. Iron absorption is mainly occurs through the duodenum. Absorption of iron from diet is strictly regulated according to the body needs (Beutler, 2010).

The low pH of the stomach coupled to proteolytic enzymes is responsible for the release of heme from hemoproteins. The mechanism responsible for heme uptake is not well understood (Silva and Faustino, 2015). It is seem to be occurring by receptor-mediated endocytosis. The HCP1 (heme-carrier protein 1), has been identified as the receptor involved in this process. It is identical to the proton coupled folate transporter (PCFT) and has low-affinity to heme and is more involved in folate absorption (Laftah et al, 2009)**.**

Dietary **ferritin** is absorbed through an adaptor-related 2 protein complex (AP2)-dependent endocytosis mechanism by the enterocytes (San Martin et al, 2008). In

enterocyte, heme is broken by heme oxygenase 1 and iron is released as ferric iron. Ferric iron is (Fe^{3+}) is reduced to Fe^{2+} by **duodenal cytochrome b (DcytB) reductase**, and is transported into the intestinal vilius by the proton-coupled **divalent metal transporter 1 (DMT-1)**. The basolateral export of iron from the enterocyte to the circulation is mediated through **ferroportin** in association with **hephaestin** and is to be associated with the circulatory transferrin (Yeh et al, 2009).

In the cytoplasm of enterocyte iron is either driven to the basolateral membrane or stored as ferritin. Here, the 'mucosal block' mediated by ferritin plays a critical role in the effective absorption of iron (Beutler, 2010).

1.5.2. Transport, Cellular Uptake and Storage

Iron is distributed to body tissues by transferrin, which transport iron in the plasma. Iron that is absorbed from the diet by intestinal enterocytes must be delivered to cells for either general or specific functions. Iron must ultimately transit from the cytoplasm of the cells, across their cell membrane, to the plasma iron carrier protein, transferrin. Recent studies showed that the transferrin iron pool is not homogeneous but is composed of apo-transferrin (apo-Tf), when no iron is bound; monoferric transferrin (bounded to a single iron atom); and diferric transferring (bounded to two iron atoms) (Young and Bomford, 1984).

The cellular uptake of transferrin-bound iron (TBI) is mainly mediated by the **transferrin receptor 1 (TfR1)**, located at the cell membrane. Upon binding, this TBI–TfR1 complex undergoes a clathrin-dependent endocytosis. The endosome pH is decreased by the entry of H+ and Fe^{3+} is released from transferrin. The apotransferrin remains bound to TfR1 and this complex then returns to the cell membrane and is released to the plasma. In the erythroblast, free iron is reduced by **Steap 3** and transported to the cytoplasm by **DMT-1**, which induces the release of Fe^{2+} in to the cytosol, where it is taken up by mitochondria for heme synthesis (Beutler, 2010; Silva and Faustino, 2015).

The organism has a high requirement for iron, as majority of iron is used by the erythroblasts for hemoglobin synthesis. Circulating erythrocytes consist mainly of

hemoglobin which constitutes about 70% of iron in the body. Mitochondrion is the main cell organelle responsible for maintaining the iron homeostasis.

Iron is **stored as ferritin** and storage prevents the presence of free iron whenever body iron levels increase. Stored iron also ensures its immediate availability during iron deficiency. The release of iron from ferritin is driven by both lysosome- and proteasome-mediated degradation mechanisms (Mehlhase et al, 2005).

1.5.3. Recycling

During senescent, the erythrocytes are scavenged by splenic and hepatic macrophages and cause the release of iron from hemoglobin and rendering it available for another hemoglobin cycle. Recycling macrophages release approximately 20 percent of hemoglobin iron form the cell to the plasma, from these 80 percent of iron is reincorporated into hemoglobin. The remaining iron is enters the storage pool as ferritin or hemosederin. When there is an increased demand of iron for hemoglobin synthesis, stored iron is released more rapidly. Where as **in inflammatory condition** or **infection or malignancy** the usage of iron for hemoglobin synthesis is reduced and **stored iron remains high** (Beutler, 2010; Silva and Faustino, 2015).

1.5.4. Regulation of Iron Metabolism

Human beings have several mechanisms through which iron levels are kept in homeostasis. Iron homeostasis is regulated at the level of dietary iron absorption by the duodenum, transport of iron in the circulation, cellular uptake and consumption, recycling by macrophages and storage in the liver (Charlton and Bothwell, 1983).

Iron homeostasis is controlled by iron absorption, recycling and storage. There are no active ways for iron excretion. The **daily requirement** of a human is approximately **1 mg of dietary iron**. This iron overcomes the non-specific iron losses through bleeding, sweat and sloughing of epithelial cells. The organism increases its duodenal absorption whenever more iron is required, and release from macrophages and from storing cells. On the other hand, when there is a condition of iron overload the absorption is inhibited and storage form of iron is increased, in order to prevent the toxic effects of free iron excess (Finch, 1994).

Commonly, iron homeostasis is controlled by **hepcidin,** a small peptide of 25 amino acids, produced in the liver, secreted into the plasma, and excreted through the kidneys. It plays a central role in regulating iron absorption from intestine mucosal cells and its release from macrophages. The regulation of hepcidin production is entirely transcriptional. Hepcidin gene (HAMP) transcription is increased by high iron levels, infection and inflammatory stimuli, and is decreased by anemia, hypoxia, HIF-1 and erythropoiesis. Hepcidin acts by binding to the cell surface **ferroportin-1**, a transmembrane iron-transport protein expressed both on intestinal mucosal cells and macrophages. Binding of hepcidin with ferroportin induces its internalization and degradation. Depletion of membrane ferroportin prevents iron release from enterocytes, macrophages and hepatocytes and results in a fall in iron level (Ganz, 2005). Alterations in hepcidin production may also lead to changes in iron absorption and recycling. Multiple factors including iron, erythropoiesis, inflammation, and growth factors regulate hepcidin expression.

The two transferrin receptors **TfR1 and TfR2**, and **HFE**, a **MHC class I-like membrane protein**, may serve as holotransferrin sensors (Ganz and Nemeth, 2011). The holotransferrin/HFE/TfR2 complex stimulates hepcidin expression through an incompletely understood pathway, possibly by potentiating BMP (bone morphogenic protein-2) pathway signaling. HFE, alone may regulate hepcidin expression without complexing with TfR2 (Schmidt and Fleming, 2012). Mutations of HFE or TfR2 in humans or mice cause hepcidin deficiency and hemochromatosis (Ganz and Nemeth, 2011).

The **BMP pathway** had a central role in the regulation of hepcidin transcription. BMP pathway signaling to hepcidin is modulated by a coreceptor **hemojuvelin**. Loss of hemojuvelin or BMP6 in mice reduces hepcidin expression and impairs hepcidin response to acute or chronic iron loading (Ramos et al, 2011). Hemojuvelin mutations in humans result in severe hepcidin deficiency and cause juvenile hemochromatosis (Papanikolaou et al, 2004). Pathogenic human mutations in BMP6 had not been identified yet.

The membrane protein, a serine protease **matriptase-2** (MT-2, also called transmembrane protease serine 6, TMPRSS6) also influences hepcidin synthesis by modulating hemojuvelin concentration on the cell membrane. Mutations in MT-2 lead to increased hepcidin expression in both humans and in mice and results in the development of iron-restricted anemia (Silvestri et al, 2008).

Besides the systemic regulation of iron homeostasis provided by hepcidin, iron metabolism is regulated of by cellular regulatory processes. That is, the cytoplasmic mRNA level by interaction of **iron regulatory protein (IRP)/iron responsive element (IRE)**. It controls both mRNA stability and translation of transcripts coding for proteins apoferritin and transferin receptor. These proteins are involved in iron uptake, export, transport and storage. Whenever these mechanisms are altered, due to genetic or environmental factors, iron overload or iron deficiency pathologies may arise (Anderson et al, 2012).

1.5.5. Disorders

Low hepcidin levels are seen in iron overload, while elevated hepcidin levels in anemia. Alteration in systemic iron homeostasis results in two major classes of disease, anemia and hemochromatosis. These iron-related diseases are caused mainly by disturbances in hepcidin regulation (Zhang and Rovin, 2013).

1.5.5.1. Anemia

World Health Organization reported that anemia is an indicator of poor nutrition and low health status. Approximately 126 million peoples were suffering from anemia worldwide (McLean et al, 2009). The World Health Organization defines that anemia occurs if the hemoglobin level drops **below 120 g/l for women and below 130 g/l** for men at sea level (World Health Organization, 1968). Generally, in anemic patients the quality of life is impaired due to fatigue, dizziness, and impaired cognitive functions. The symptoms are due to iron deficiency in red blood cells which diminishes the oxygen supply for the body. Iron deficiency can occur either with or without anemia. The prevalence of different anemia subtypes is: about 50% of anemias arise from nutritional iron deficiency, 42% are caused by inflammation and infection and 8% of anemias

develop due to nutritional deficiencies (such as vitamin A, vitamin B12, folate, riboflavin, or copper), or are genetically caused (Denic and Agarwal, 2007).

1.5.5.1. 1. Iron deficiency anemia

Iron deficiency anemia (IDA) is the most frequent form of anemia caused by a relative or absolute deficiency of dietary iron. In IDA, iron is insufficient to satisfy the iron demand for erythropoiesis. In earlier stage, iron deficiency occurs without anemia, but later anemia develops as a result of depleted iron stores. In this case, treatment is by oral or intravenous iron supplementation (Clark, 2008).

Pathogenesis of iron deficiency anemia: Pathogenic factors in anemia of iron deficiency are impaired hemoglobin synthesis as a consequence of reduced iron supply, defect in cellular proliferation and reduced survival of erythroid precursors and erythrocytes, when the anemia is severe. Iron-containing proteins like cytochrome c, cytochrome oxidase, glycerophosphate oxidase, muscle myoglobin, succinic dehydrogenase, and aconitase are also reduced in iron deficiency, and may be responsible for clinical and pathologic manifestations.

1.5.5.1. 2. Anemia of chronic disease

Anemia of chronic disease (ACD) is usually normocytic, normochromic anemia which occurs in patients with chronic infection, inflammation, or neoplasia. Serum iron levels are usually decreased due to hepcidin activation by cytokines. Transferrin saturation is low in ACD, but ferritin levels are high due to inflammation. In ACD, oral supplementation of iron is not effective as elevated hepcidin levels impair the intestinal absorption of iron. But treatment with intravenous iron is effective. This marginally improves the anemic state as intravenous iron is trapped in the storage sites like macrophages and hepatocytes as a consequence of ferroportin degradation by hepcidin. ACD is categorized as anemia of heart failure, anemia of chronic kidney disease, anemia in inflammatory rheumatic diseases, anemia of the elderly (Weinstein et al, 2002).

Anemia in Chronic kidney disease (CKD) is inevitable as disease progress, so that it is mandatory to assess anemia in CKD patients. The main etiology of anemia in CKD is the **failure in EPO production by the kidney** which leads to decrease in the

level of hemoglobin. Several observational studies showed that anemia is associated with increased mortality rate and increased hospitalization in dialysis patients with CKD (Delaney et al, 2012).

In patients with CKD and low endogeneous serum erythropoietin level **rhEPO-epoitin alfa and beta** was used to correct anemia. 90-95% of patients treated with rhEPO are effective in correcting anemia of CKD. The most common side effect of rhEPO treatment is hypertension. Correcting anemia with rhEPO therapy have positive responses like improves exercise capacity, cognitive function and quality of life (Winearl et al, 1986; Eschbach et al, 1987).

1.5.5.1.3 Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is a heterogeneous group of disorders caused by genetic aberrations in genes that control hematopoiesis. Different degrees of anemia are observed in MDS. Clinically, in MDS the cellular blood count is abnormal. MDS is diagnosed by bone marrow biopsy and visual examination of morphological evidence of dysplasia of the bone marrow. MDS is characterized by the loss/reduction of the number of red cells and results in anemia. Blood transfusions are mainly used to treat severe anemia and as a consequence, iron overload is also observed. It was speculated that iron overload in MDS leads to acute myeloid leukaemia (AML), when ferritin levels rise above 2500 ng/ml. In this case, iron chelators are recommended as treatment. Curative treatment is yet unavailable, so that bone marrow transplantation is the only way to treat the disease (Garcia-Manero, 2012).

1.5.5.1. 4. Anemias caused by genetic defects

The prevalence of anemia caused by genetic defects is less than five cases per 10,000. Anemias caused by genetic defects include hemolytic anemias, such as **hemoglobinopathies**. These are caused by inherited defects in the hemoglobin structure (e.g., sickle cell disease) or in Hb synthesis (beta-thalassemia). This may also arises due to mutations in proteins responsible for the regular shape of erythrocytes or metabolic deficiencies (e.g., in glucose-6-phosphate hydrogenase) that increase oxidative stress in erythrocytes. Alterations in the shape or viability of erythrocytes cause hemolysis, which can be acute or chronic. These diseases are commonly under diagnosed and

inappropriately treated due to the lack of availability of diagnostic strategies (Gulbis et al, 2010).

Genetic iron-resistant iron deficient anemia (IRIDA) is a microcytic anemia, resistant to both oral and parenteral iron supplementation. It is caused by mutations in the TMPRSS6 gene encoding matriptase-2. This leads to elevated hepcidin levels and internalization and degradation of ferroportin. As a result, iron cannot be sufficiently absorbed from the intestine or released from iron stores. Anemia is not observed at the time of birth, but develops at one month of age, and usually more pronounced in children than in adults (Camaschella and Poggiali, 2011).

1.5.5.1.5. Etiology of iron deficiency

Iron deficiency is the end result of a long period of negative iron balance, as a result of major blood loss, or because of failure to meet the increased physiologic need for iron. As the total body iron level begins to fall, the iron stores in the hepatocytes and the macrophages of the liver, spleen, and bone marrow are depleted results in decreased plasma iron content. This causes inadequate supply of iron to marrow for normal hemoglobin production and the blood hemoglobin level decreases. Factors leading to iron deficiency anemia were **negative iron balance**, **increased requirements of iron**, or **inadequate iron for erythropoiesis**.

Malabsorption and **intestinal blood loss** were the main factors resulted in the development of iron deficiency. **Achlorhydri**a, **gastric infection** with Helicobacter pylori, celiac disease and gastric surgeries, impaired iron absorption and cause iron deficiency anemia. As iron absorption was mostly takes place in the duodenum, iron deficiency was common when the duodenum is surgically by passed. **Copper deficiency** was also associated with abnormalities in iron metabolism and cause iron deficiency anemia (Andrews NC, 2009).

Inadequate diet, **blood loss**, such as that associated with menstruation is a significant cause of iron deficiency anemia. **Gastrointestinal bleeding** due to duodenal or gastric ulcers or gastritis was resulted in iron deficiency. Certain drugs like **nonsteroidal anti-inflammatory drugs (NSAIDs)**, including aspirin, were also associated with gastrointestinal bleeding.

In **carcinoma** of the cecum, carcinomas of other parts of the colon, stomach and the ampulla of Vater, iron deficiency anemia may be the only initial symptom. Ulcerative colitis, infection with intestinal and genitourinary parasites, including whipworm, Trichuris trichiura, hookworm, Necator americanus and schisotosomes was associated with iron deficiency.

Increased Requirements of iron in infancy, pregnancy and lactation also cause iron deficiency. Frequent blood donation, hemoglobinuria, alveolar hemorrhage in the pulmonary alveoli may also contribute to iron deficiency. Iron deficiency was also seen in **chronic renal failure patients** treated with hemodialysis (Andrews NC, 2009).

1.5.5.2. Iron overload

Low hepcidin levels are seen in overload or hemochromatosis. Hemochromatosis is caused by increased iron export from enterocytes, hepatocytes and macrophages. This increased iron export is **due to low hepcidin levels and ferroportin overexpression**. Iron is accumulated in heart, liver, and pancreas and cause oxidative damages which leads to cirrhosis, cancer, diabetes, and cardiomyopathy. It is the most frequent genetic disorder develops due to mutations in genes that play an important role in iron homeostasis. These include activators of hepcidin transcription (HFE), transferrin receptor 2 (TFR2), hemojuvelin (HFE2, or HJV), FPN and hepcidin (HAMP). The most common form of hemochromatosis is caused by the mutation of HFE gene. Mutations of HFE gene lead to an adult onset of hemochromatosis, whereas mutations in TFR2, HJV, and HAMP cause a juvenile subtype. In hemochromatosis serum levels of iron, transferrin saturation and ferritin are increased. The symptoms of hemochromatosis are typically non-specific (e.g., fatigue, arthralgia, malaise, darker skin, or increase in transferrin saturation) (Pietrangelo, 2011).

Iron accumulation is also seen in acquired diseases like chronic liver diseases such as alcoholic (ALD) and non-alcoholic fatty liver disease (NAFLD), steatohepatitis (ASH and NASH), chronic hepatitis C infections, and end-stage liver disease (Pietrangelo, 2009). Increased iron stores may predispose to insulin resistance and later diabetic complications occurs (Martinelli et al, 2012). In addition, increased body iron stores correlate with the risk for atherosclerosis and cardiovascular diseases. An increase in the

brain iron content is seen in patients with Alzheimer's disease or Parkinson's disease, which leads to disturbances in brain iron homeostasis and may contribute to the pathogenesis of these disorders (Altamura and Muckenthaler, 2009).

1.6. Ferritin

Ferritin is the storage form of iron found in both prokaryotes and eukaryotes. Ferritin exists as a **24-subunit multimer of heavy (H) and light (L) polypeptide chains**. When synthesized, these chains self assemble, forming a spherical protein shell that is able to store up to 4500 iron atoms on its core. Ferritin is localized in cell cytosol, nucleus and mitochondria, but is also present in the serum. Ferritin is found in animal and plant cells as well as in fungi and bacteria. Iron is incorporated into ferritin as Fe^{2+}, but oxidized quickly to Fe^{3+} within its spherical shell by H-ferritin ferroxidase (Wang et al, 2010).

The major function of ferritin is to provide a storage site for iron which may be used for heme synthesis whenever required. Ferritin is stored in liver and spleen and is mostly contains L-subunits. Ferritin protects the cells from iron toxicity. Ferritin synthesis is mainly regulated by translation rather than transcription. Ferritin mRNA has IRE and an increase in intracellular free iron results in the translational increase of ferritin production (Klausner et al, 1993). Degradation pathway of ferritin is remains largely a mystery. The relationship between degradation of ferritin and formation of hemeosiderin is yet unclear.

1.6.1. Ferritin Levels

Serum ferritin concentrations are normally within the range 15–200 ng/ml and are lower in children than adults. Ferritin levels are lower in women before the menopause than in men. It is mainly due to blood losses during menstruation and childbirth. But after menopause the ferritin concentration increases but remains lower than in men. Low serum ferritin was observed in patients with iron deficiency anemia and is typically less than 12–15 ng/ml (Worwood, 1982). Reference interval for serum ferritin concentration in **adult man is 20 – 250 ng/ml** and for **adult women is 20 – 200 ng/ml** (Higgins et al, 2012).

A high concentration of serum ferritin is found during iron overload, but there are other causes as well. Serum ferritin concentration was also higher with increasing body mass index. High ferritin is seen in acute inflammatory conditions in which ferritin is mobilized without iron excess. Ferritin is also high in disorders, such as hemochromatosis or hemosiderosis. As ferritin is an acute-phase protein, it is often elevated in the course of disease. A high concentration of ferritin is also seen in patients with pancreatic carcinoma, lung cancer, hepatoma and neuroblastoma (Wang et al, 2010).

1.6.2. Hemeosiderin

Ferritin is a soluble protein, which degraded and forms an insoluble hemeosiderin and accumulates in lysosomes. Both ferritin and hemeosiderin are storage forms of iron which provide iron available for protein and heme synthesis. Normally much of the stored iron in the body is present as ferritin, but when there is iron overload the proportion of hemeosiderin increases. A small proportion of molecules in the form of dimers, trimers and other oligomers were present in the Purified preparations of ferritin (Williams and Harrison, 1968). These may be intermediates of hemeosiderin formation.

1.7. Erythropoietin beyond Erythropoiesis

In addition to erythropoiesis, erythropoietin plays a broader biological role (Brines and Cerami, 2006). Initial studies showed that the erythropoietin receptor was selectively expressed in cells of the erythroid lineage, restricting the effect of erythropoietin on red blood cell production. However, subsequent studies disclosed a more widespread expression of EPO receptor, suggesting the possibility of **extrahemeatopoietic effects** of erythropoietin. For example, In addition to kidneys and liver, a minimal amount of **EPO mRNA has been detected in brain, retina, heart, skeletal muscle, kidney and endothelial cells** (Lacombe and Mayeux, 1999; Juul et al, 1998; Digicaylioglu, 1995).The identification of the existence of EPO-R expression on different cell types results in a search for nonerythropoietic effects of EPO. As a result, now we know that EPO has diverse biological activities, like direct effects on immune cells (Broxmeyer, 2011), endothelial cells, bone marrow stromal cells, cells of the heart, reproductive system, gastrointestinal tract, muscle, kidney, pancreas, and nervous

systems (Brines and Cerami, 2006; Choi et al, 2010; Hand and Brines, 2011; McGee et al, 2012; Sytkowski, 2011).

1.7.1. EPO and Diabetes Mellitus

In both clinical and experimental studies, EPO has been closely associated with the modulation of cellular metabolism. Diabetes mellitus (DM) condition is associated with cellular dysfunction in relation to cellular metabolism (Maiese et al, 2007). At least 350 million individuals were affected by DM and its incidence is increasing in worldwide. Both Type 1 and type 2 diabetes mellitus are chronic disorders of insulin insufficiency leads to the dysregulation of glucose homeostasis, hyperglycemia, and vascular complications. A common feature among all forms of diabetes mellitus is the insufficiency of functional pancreatic β-cell mass to maintain the glucose level. Thus the main goal in the treatment of all types of diabetes is the preservation and the promotion of β-cell growth and survival (Bach, 1994; Mathis et al, 2001; Kahn, 2003).

Both type 1 and type 2 DM can cause long-term complications throughout the body and thus required important health concerns (Daneman, 2006; Maiese et al, 2007). DM can affect the entire body. It affect the immune system (Zhao et al, 2015), kidney (Nakazawa et al, 2010), liver (Lee et al, 2014), musculoskeletal function (Deblon et al, 2012), and cardiovascular system (Aragno et al, 2012). DM patients can develop severe neurological and vascular disease (Donahoe et al., 2007) which leads to an increased risk of cognitive loss especially from vascular disease (Chong et al., 2005). Disease of the nervous system can become the most debilitating complication for DM and affect sensitive cognitive regions of the brain, such as the hippocampus, which leads to ischemic disease of the brain, peripheral nerve disease, visual deterioration, loss of memory function impairment and dementia (Awad et al, 2004; Gerozissis, 2003).

EPO is under investigation for the treatment of variety of diseases, but provides exciting prospects for the treatment of diabetes mellitus (Zhang et al, 2014). In addition for the treatment of anemia, the use of EPO can improve cardiac function, decrease fatigue, and improve cognition in patients with DM. EPO also help in regulating cellular energy metabolism, obesity, apoptosis, autophagy, tissue repair and regeneration in experimental models of DM (Silverberg et al, 2006).

In experimental animal models of DM, EPO **reduce blood glucose levels and obesity** (Katz, 2010). EPO also protect against the detrimental effects of obesity (Zhang et al, 2014) and can treat diabetic peripheral neuropathy (Chattopadhyay et al, 2009) in animal models. EPO has been shown to reduce oxidative stress in renal tubular cells due to high glucose level (Dang et al, 2010). EPO can also control cellular mitochondrial function (Chong et al, 2003), and maintain energy metabolism (Wang et al, 2014). In expiremental DM models, EPO has shown to have cardio protective effect with the inhibition of glycogen synthase kinase-3β (GSK-3β) (Ghaboura et al, 2011) that can limit Wnt signaling pathways (Maiese et al, 2008). Several studies have shown that EPO can improve wound healing and wound closure in diabetic mice through decreased apoptotic cell death and increased angiogenesis (Hamed et al, 2011; Hamed et al, 2014). EPO can provide direct protection against endothelial cell apoptosis due to elevated glucose through activation of Wnt1 (Chong et al, 2007) and the inhibition of GSK-3β and FoxO3a (Chong et al, 2011).

Choi et al through their studies showed that recombinant human EPO treatment can protect against diabetes development in type 1 (streptozotocin model) and type 2 (db/db mouse model) diabetes. Protection in these models was mediated by JAK2 signaling pathways directly in pancreatic β cells, which exert antiapoptotic, proliferative, anti-inflammatory, and angiogenic effects (Choi et al, 2010). In nonerythroid cells EPO can signal via a heterodimeric receptor composed of an EPO-R monomer chain and CD131 (Brines and Cerami, 2006) and its activation requires much higher concentrations of EPO compared with that of the homodimeric EPO-R (Hand and Brines, 2011). EPO receptor and JAK2 knockout mice show defects in β-cell mass and function (Liu et al, 2004). These results showed that the effects of EPO result from **direct biological effects on β cells** and that JAK2 is an essential intracellular mediator and thus may berelevant to diabetes.

EPO deficiency and a higher incidence of anemia in diabetic patients suggests the potential beneficial effects of EPO in the setting of diabetes (McGill and Bell, 2006; Thomas, 2006). During diabetic pregnancies there is an increased EPO secretion, which may represent the body’s attempt at endogenous protection against the complications of DM (Teramo et al, 2004). EPO treatment in patients with chronic renal failure was

associated with an adverse effect of increase in the incidence of hypoglycemia which raises the intriguing possibility of a direct effect of EPO on pancreatic β cells (Drüeke et al, 2006).

EPO also have adverse effects that involve the vasculature system and unchecked cellular proliferation. In patients with DM and renal disease, EPO administration results in a two-fold increase in stroke, which may be due to increase in blood viscosity with a reduction in cerebral blood flow (Frietsch et al, 2007). In animal models systemic administration of EPO may block retinopathy (Shen et al, 2014). Elevated EPO concentrations in patients with DM also may lead to proliferative diabetic retinopathy (Semeraro et al, 2014) and was associated with excessive vascular growth and may lead to hypertension (Maiese et al, 2010). Sustained erythrocytosis with EPO may also cause the activation of inflammatory pathways and blood-brain barrier dysfunction (Ogunshola et al, 2013). As a proliferative agent, EPO can help in new tumor growth as well as foster the progression of existing tumors (Hedley et al, 2011).

1.7.2. EPO and Tissue Protection

EPO **prevents** programmed cell death that is **apoptosis** of erythrocyte precursor cells. EPO-R is expressed in the endothelial cell (Anagnostou et al, 1990), the central nervous system, and by astrocytes (Digicaylioglu, 1995). EPO has a role in tissue-protection, as EPO prevent apoptosis. Under relative hypoxic conditions reactive free radicals are produced as by-products of cellular metabolism, which mediates injury. Chronic renal hypoxia with subsequent tubulointerstitial injury leads to end-stage renal failure. Endogenous EPO provide considerable protection from injury under these circumstances (Brines and Cerami, 2006).

Capillary endothelial cells obtained from a variety of tissues respond to local ischemia by producing EPO and potentially provide EPO-mediated protection. Tissue protection following injury is attributable by EPO-mediated survival of the capillary endothelium itself from destruction by micro infarction. EPO also strongly mobilizes and regulates endothelial progenitor cells (Bahlman et al, 2004).

1.7.2.1. Nervous system protection

Understanding of the biology of EPO-mediated tissue protection is initially developed from the studies on the nervous system. Several studies showed that exogenous EPO administration before or after injury are highly effective for the prevention of permanent or transient ischemic injury in multiple organs and tissues. EPO administration is also found to be protecting from trauma, toxins, and infection (Brines and Cerami, 2005).

Intraventricular infusion of erythropoietin can protect neurons against ischemeic injury (Sakanaka et al, 1998). Several experimental studies have shown that erythropoietin can cross the blood–brain barrier and systemic injections of large doses of erythropoietin can decrease brain or spinal cord damage (Brines et al, 2000).

In experimental models EPO shows a protective effects on multiple sclerosis or status epilepticus (Agnello et al, 2002) and also can prevent or partially reverse experimental diabetic neuropathy (Bianchi et al, 2004) . These neuroprotective effects of EPO are associated with a strong inhibition of apoptosis and with neurotrophic activity of erythropoietin (Siren et al, 2001). Furthermore, EPO also antagonizes the activities of proinflammatory cytokines and promotes healing following injury by stimulating capillary growth. However, inflammatory cytokines suppress EPO production, so that maximum protective benefit is only achieved by administration of exogenous EPO (Villa et al, 2003; Brines and Cerami, 2006).

1.7.2.2. EPO and Cardiovascular protection

Several clinical studies showed that EPO has an important protective role in the cardiovascular system (Maiese et al, 2005). Experimental study results from knockout mice showed that the heart expresses functional erythropoietin receptors and injections of large doses of EPO protect from the consequences of transient or permanent coronary artery occlusion. EPO treated animals exhibit a reduction in myocardial damage and were associated with decreased apoptotic cell death (Parsa et al, 2003). Administration of EPO in patients with congestive heart failure or diabetes combined with congestive heart failure showed an improvement in the cardiac output and a decrease in medical resource utilization (Silverberg et al, 2006). Patients with anemia and congestive heart failure can

improve exercise tolerance, renal function, and left ventricular systolic function by the administration of EPO (Palazzuoli et al, 2006). Clinical studies showed that administration of EPO in patients with anemia or on chronic hemodialysis can increase left ventricular ejection fraction, stroke volume, and cardiac output, indicating improved cardiac function secondary to the correction of anemia (Maiese et al, 2005; Silverberg et al, 2006). Recently, several studies showed that patients with acute myocardial infarction have an increased plasma EPO levels, and were raised within seven days of the cardiac insult. These results suggest that the raise in EPO levels may be a possible protective response from the body (Ferrario et al, 2007). Serum EPO levels may use as a biomarker for cardiovascular injury (Fu and Van Eyk, 2006).

1.7.2.3. EPO and Renal protection

The non-hematopoietic role of EPO in the kidney was first implied by the identification of EPO-R protein expression on both proximal and distal tubular cells of kidney (Westenfelder et al, 1999). Work from experimental studies illustrates that EPO plays a protective role in the renal system (Sharples and Yaqoob, 2006). EPO administration suppresses the inflammatory response to ischemia–reperfusion in the kidney by reducing proinflammatory cytokine production, subsequent leukocyte recruitment, and amplification of damage and improves renal function. EPO also protects kidney from the toxic effects of cyclosporine and cisplatin (Chatterjee, 2005). A number of clinical trials have concluded that predialysis patients treated with early EPO administration slow down the progress of kidney disease. However, the chronic administration of EPO increases hemoglobin concentrations and cause complications (Gouva et al, 2004). Small proportion (35%) of patients with end-stage renal disease will exhibit a dose-dependent increase in blood pressure but others only had a moderate increase or no effect on blood pressure following EPO administration. Acute EPO administration also reduces cortical renal blood flow and urinary solute excretion (Coleman et al, 2007). However, potential effects on renal nerves have not been assessed completely.

1.7.2.4. Therapeutic approaches of tissue protection

Therapeutic utility of EPO mediated tissue protection though the EPO-R could be complicated by increases in red cell mass and development of a prothrombotic state. This problem is avoided by ensuring selective tissue protection without activating erythropoiesis. This can be accomplished by using desialated EPO which has a very short half-life. In several preclinical models, this analogue demonstrates potent tissue protection without any detectable stimulation of the bone marrow (Erbayraktar et al, 2003). Another approach to avoid this problem is that the selectively targeting tissue protection was predicted from the mismatch between the affinity of the non-hematopoietic, tissue EPO receptor and the much lower levels of circulating EPO. In non hematopoietic tissues, EPO receptor isoform is different from the homodimer mediating erythropoiesis. Non hematopoietic tissue EPO-R consists of a heteromeric complex containing EPO-R and the beta receptor subunit (CD131) (Brines et al, 2004). This was demonstrated by several experimental studies with knockout of the beta common receptor gene fully abolishes tissue protective properties of EPO in the nervous system and heart.

1.7.2.5. Cellular mediators of tissue protection

The cytoprotective effects of erythropoietin are mediated through the binding of EPO to the heterodimer receptor containing the erythropoietin receptor and the common beta receptor (Brines et al, 2004). Signaling pathways activated by EPO in mediating tissue protection is complicated, and often conflicting. Subsequent study has identified that unlike erythropoiesis, survival pathways do not appear to be redundant, as inhibition of any single pathway generally blocks tissue protection. The main signaling pathways involved in mediating tissue protection are: the **Jak2-STAT-Bcl-2 pathway**, which is the dominant EPO-dependent pathway mediating antiapoptotic mechanism for hematopoietic cells, also has been implicated in tissue protection. This pathway is regulated by induction of antiapoptotic molecules like Bcl-2 and Bcl-XL or by decrease in proapoptotic molecules like bax and bak (Brines and Cerami, 2005).

The neuroprotective effect of EPO involves the activation of Ras/mitogen activated protein kinase or the phosphatidylinositol 3-kinase pathway. Binding of EPO to its receptor can lead to the phosphorylation of PI3-kinase, which in turn phosphorylates

protein kinase B/Akt. Akt then phosphorylate and cause inactivation of proapoptotic molecules, such as caspase 9, Bad or glycogen synthase kinase-3b (Kashii et al, 2000).

Jak2- dependent activation of nuclear factor-kB also proposed to mediate neuroprotection of cortical neurons in vitro and inhibition of these messengers blocked protection (Brines and Cerami, 2005). The activation of transcription factor NF-kB in turn enhances the transcriptional activity of target genes encoding antiapoptotic molecules, such as XIAP and c-IAP2 (Digicaylioglu and Lipton, 2001).

In kidney, erythropoietin induces heat shock protein 70 (Hsp70) and the inhibition of Hsp70 expression eliminate the protective effects of erythropoietin. The antiapoptotic effects of Hsp70 include inhibition of apoptosis protease-activating factor-1 and of apoptosis-inducing factor (Yang et al, 2003).

1.7.3. EPO and Angiogenesis

In the vascular system, EPO promotes angiogenesis and stabilizes vascular integrity, increases the number of endothelial cells (ECs), protects these cells against ischemia and apoptosis (Chong et al, 2002). EPO-R is expressed in vascular endothelial cells and cancer cells (Hardee et al, 2006). The signaling of EPO in ECs is mediated via phosphorylation of JAK-2 and STAT-5 similar to that occurring in erythroid cells and cause cell proliferation, production of matrix metalloproteinase-2 (MMP-2), and vascular differentiation (Haller et al, 1996).

EPO-R is expressed in ECs derived from bone marrow of patients with monoclonal gammopathy and patients with multiple myeloma. EPO enhances chondrogenic and angiogenic responses during bone repair and could serve as a therapeutic agent to support skeletal regeneration (Holstein et al, 2011). EPO treatment also enhances the expression of angiogenic factors, microvascular density, and reduces inflammation and apoptosis of human fat tissue that has been injected into nude mice (Hamed et al, 2010).

In cultured human and bovine ECs, EPO stimulates the proliferation and migration of ECs (Anagnostou et al., 1990). EPO could stimulate neovascularization and improve cardiac function in ischemic hearts (Westenbrink et al, 2008). The mechanism is

probably controlled by local VEGF and EPO-R expression. EPO upregulate VEGF for the mobilization of endothelial progenitor cells (EPCs) in local ischemic tissue and improves microvascularization and cardiac function. The presence of ischemia and hypoxia together exponentially increases EPO-R expression and the vascular EPO/EPO-R system plays a crucial protective role against hypoxia/ischemia, providing a new therapeutic target in cardiovascular medicine (Satoh et al, 2011). The vascular EPO-R as well as EPO promotes post-ischemic angiogenesis via increase VEGF secretion from ischemic muscle, EPCs mobilization, and recruitment of bone marrow-derived proangiogenic cells to the ischemic tissue (Nakano et al, 2007). Furthermore, EPO treatment improves revascularization and the inhibition of cardiac fibrosis in diabetic rats (Lu et al, 2012).

Moreover, the uterine endometrium and ovaries are dependent upon EPO for inducing angiogenesis to compensate the vessels lost during the estrus cycle. EPO has been shown to be necessary to enhance blood vessel formation in the endometrium of ovariectomized mice and also required for the formation of a capillary network for the development of follicles and the corpora lutea (Yasuda et al., 1998). Intraperitoneal administration of rhEPO after focal ischemia reduces cell death of ECs, enhances angiogenesis, and clearly restores the local cerebral blood flow (Li et al, 2006). rhEPO also enhances angiogenesis, reduces white matter damage, and promotes cognitive recovery in anoxia rats (Yan et al, 2016). Furthermore, EPO mediates neurovascular remodeling and neurobehavioral recovery in rats after traumatic brain injury (Xiong et al, 2011). During experimental cardiac ischemia, EPO may promote the viability of transplanted marrow stromal cells and enhance capillary density (Zhang et al, 2007).

In retinopathy early EPO supplementation stimulates the transport of EPCs from bone marrow into the retina, their differentiation into ECs, and the revascularization of injured vasculature of the retina (Otani et al, 2002), but late EPO treatment does not have any protective effect on retinal vasculature.

EPO had a role in both growth and angiogenesis of tumors and some studies confirmed a direct effect of EPO on the proliferation of ECs and/or angiogenesis of tumors. EPO promotes angiogenesis in chemically induced murine hepatic tumors

(Nakamatsu et al, 2004). EPO secreted by glioma tumor cells affects glioma vascular ECs and promotes angiogenesis (Nico et al, 2011). EPO administration enhances the growth of pituitary adenomas by promoting angiogenesis via the EPO–JAK-2–STAT-3–VEGF signaling pathway (Yang et al, 2012).

1.8. Parathyroid Hormone (PTH)

Parathyroid glands located on the thyroid gland capsule, synthesise and secrete PTH. The chief cells of the gland synthesise, store and secrete PTH hormone.

1.8.1. Biochemistry and Functions of PTH

PTH increases the plasma free **calcium** and decrease the plasma **phosphate**. PTH is **metabolised and cleared** by the liver and kidneys. The **regulators of PTH secretion** are ionic calcium, 1, 25-dihydroxyvitamin D (calcitriol) and phosphate. **PTH acts** directly on bone and kidneys, but indirectly on intestine.

1.8.1.1. Synthesis and secretion of PTH

A. Synthesis of PTH: The pre-pro-PTH of **115 amino acid** is the **primary translation product** (Fitzpatrick and Bilezikian, 2006; Nissenson and Jüppner, 2008). The amino terminal hydrophobic **leader sequence** (pre-hormone) helps in transport of PTH across the endoplasmic reticulum membrane into the cisternae of Golgi. The 'pre' sequence and the 6 amino acid long N-terminal **"pro" sequence**s are enzymatically cleaved for intracellular processing and packaging in the Golgi apparatus. The PTH molecule contains a large number of **basic amino acids**. The **final intact PTH of 84 amino acids** with a molecular mass of 9425 Da is stored, secreted or degraded intracellularly

B. Secretion of PTH: Parathyroid cells have **few secretory granules** for storage of PTH. PTH should be **synthesized by translation** of mRNA when required for secretion. The **intracellular degradation is increased** when plasma **ionic calcium concentration is high** and **secretion of PTH is low**. PTH secretion is inversely related to intracellular degration and plasma ionic calcium. **Pro-PTH is not secreted** and does not circulate in detectable concenrations. But several **C-terminal fragments of PTH are**

secreted from the parathyroid along with intact PTH (Murray et al, 2005). Biological activity of PTH are in the **N-terminal third** of the molecule (Hodsman et al, 2005; Murray et al, 2005). **Synthetic PTH (1-34)** is nearly as potent as intact PTH (1-84) in interacting with the PTH/PTHrP receptor (PTH1R, type 1 PTH receptor) and for increasing ionic calcium, phosphaturia and functions in kidney and bone.

C. Regulation of PTH syntheis and secretion: Regulation of PTH protein synthesis by calcium and phosphate is mainly at **the post-transcriptional level**. Specific proteins bind in a sequence specific manner in the 3′-untranslated region **(3′-UTR)** of PTH mRNA. This binding alters the stability of the mRNA (Naveh-Many, 2010). As **the parathyroid cells have few secretory granules for storage of PTH**, the hormone must be synthesized when required for secretion.

(1) Free ionic calcium in plasma: The immediate **short term regulator** of PTH synthesis, metabolism and secretion is the free ionic calcium in plasma or extracellular fluid (Brown, 2000; Fitzpatrick and Bilezikian, 2006; Nissenson and Jüppner, 2008). Free calcium is sensed by a **G-protein–coupled calcium-sensing receptor** (CaSR) on the cell surface of parathyroid cells. The relationship is **inverse sigmoid between PTH secretion and free extracellular calcium** (Nissenson and Jüppner, 2008).

Maximal slope of this plot indicating secretion and suppression of secretion are attained **with mild hypocalcemia and mild hypercalcemia**, respectively. Midpoint of this relationship or the set point is the calcium concentration at which PTH secretion is half maximal (Favus and Goltzman, 2008; Fitzpatrick and Bilezikian, 2006; Nissenson and Jüppner, 2008).

(2) $1,25(OH)_2D$ interacts with vitamin D receptors in the parathyroid glands to chronically decrease PTH secretion by suppressing transcription of PTH gene and subsequent secretion. It is **the intermediate and long term regulator of PTH secretion**.

(3) Hyperphosphatemia and hypophosphatemia increase and decrease PTH synthesis and secretion (Almaden et al, 1998), respectively, and the hyperphosphatemia of chronic renal failure lead to **parathyroid hyperplasia** and hyperparathyroidism.

(4) Magnesium usually does not play an important role in PTH secretion except at the extremes of magnesium concentration (Favus and Goltzman, 2008). Chronic severe hypomagnesemia has been associated with impaired PTH secretion, whereas acute hypomagnesemia may stimulate secretion. Chronic hypomagnesemia can cause PTH resistance (Leicht and Biro, 1992) whereas hypermagnesemia suppresses PTH secretion through the calcium-sensing receptor, although not as effectively as does calcium (Favus and Goltzman, 2008).

1.8.1.2. Biological actions of PTH

PTH regulates calcium and phosphate homeostasis **directly through bone and kidney, and indirectly by the intestine**. These actions are mediated by 1,25$(OH)_2$D or caklcitriol (Fitzpatrick and Bilezikian, 2006; Nissenson and Jüppner, 2008). PTH acts by binding to **type 1 PTH receptors** (PTH/PTHrP receptors) located in the plasma membrane of target cells. This initiates intracellular activation of signalling pathways, such as synthesis of cAMP, activation of kinases, phosphorylation of proteins, rise in cytosolic calcium and calcium stimulated phospholipase C activation, followed by generation of diacylglycerol and phosphoinositide-activated enzyme and transport systems, and secretion of lysosomal enzymes (Fitzpatrick and Bilezikian, 2006; Partridge et al, 2006).

1.8.1.2.1. Functions of PTH in kidneys

1. Induction of 25-hydroxyvitamin D-1α-hydroxylase, resulting in the formation of 1,25$(OH)_2$D, which in turn stimulates intestinal absorption of both calcium and phosphate.

2. **Increases reabsorption of calcium** in the distal convoluted tubule of the nephron.

3. **Decreases phosphate reabsorption** by the proximal tubule.

4. **Inhibits Na^+-H^+ antiporter activity**, which favors **mild hyperchloremic metabolic acidosis** in hyperparathyroid states.

1.8.1.2.2. Functions of PTH in bone

The actions of PTH on bone are complex, as evidenced by its stimulation of bone resorption or bone formation. The activity depends on the concentration of PTH and the

duration of exposure (Fitzpatrick and Bilezikian, 2006; Hodsman et al, 2005). Chronic exposure to high concentrations of PTH results in increased bone resorption. Immediate bone resorption is important for the maintenance of calcium homeostasis. But the delayed skeletal effects of PTH are important for extreme systemic needs and skeletal homeostasis.

(a) Bone resorption by PTH: It was not been possible to find PTH receptors on osteoclast. **PTH receptors are found on osteoblasts and adjacent bone marrow stromal cells**. These cells transmit PTH action to osteoclasts by several **cytokines** (Murray et al, 2005). Actions of cytokines on osteoclasts are **enhanced differentiation of osteoclast precursors** and **stimulation of resorbing activity** in mature osteoclasts. Osteoblasts, through the action of macrophage-colony stimulating factor (M-CSF), **stimulate osteoclast precursor formation from macrophages**. Therefore, **osteoclasts** are ultimately **derived from the bone marrow and from hematopoietic stem cells** located in the bone marrow.

(b) Anabolic effect of PTH on skeleton: PTH increases the number of osteoblasts and enhances their differentiation from stromal cells. Different fragments and the intact PTH hormone may have different effects on osteoblast gene expression (Fitzpatrick and Bilezikian, 2006). Because of its anabolic effect on bone, **PTH (1-34) is used as a therapeutic agent** for osteoporosis under the name of teriparatide (Hodsman et al, 2005).

1.8.1.2.3. Integration of the effects of PTH on calcium and phosphate levels

Integration of the direct effects of PTH on bone and kidney, and the indirect effects on intestine through 1,25$(OH)_2$D, results in changes in calcium and phosphate concentrations in blood and urine. **In blood, total and free calcium concentrations are increased and phosphate is decreased**. But, **in urine, inorganic phosphate and cAMP concentrations are increased.** Urinary **calcium excretion usually is increased.** This is due to the larger filtered load of calcium, derived from bone resorption and intestinal calcium absorption, which overrides the increased tubular reabsorption of calcium. In the absence of disease, the increase in blood calcium decreases PTH secretion through a negative feedback loop, maintaining homeostasis.

1.8.1.3. Heterogeneity of circulating PTH

PTH circulates in our body partially as the **biologically active hormone** and partially as a series of **N-terminally truncated fragments** containing the midregion and C-terminal amino acids (Bringhurst, 2003; Lopez et al, 2010). The heterogeneity is due to the secretion of both intact hormone and C-terminal fragments by the parathyroids, peripheral metabolism of intact hormone by liver and kidney to C-terminal fragments, and the renal clearance of intact hormone and C-terminal fragments.

In the parathyroids, secretion of intact PTH is increased by hypocalcemia and is decresed or absent in hypercalcemia, however some secretion of C-terminal fragments persists in hypercalcemia

Biologically active intact PTH (amino acids 1-84) is rapidly **cleared from plasma (half-life <5 minutes)** by Kupffer cells of the liver (60 to 70%) and by glomerular filtration (20 to 30%) (Fitzpatrick and Bilezikian, 2006). Peripheral metabolism inactivate intact hormone without releasing measurable concentrations of biologically active N-terminal fragments (Bringhurst, 2003; Murray et al, 2005).

Plasma PTH measured by **most immunoassays include both inactive fragments and intact hormone.** Fragments consisting of the middle and carboxyl regions of the molecule (e.g., amino acids 34-84, 36-84) are devoid of the N-terminal region and classical PTH biological activity. These molecules were earlier considered to be inactive degradation products, but several reports have identified separate receptors for C-terminal PTH in bone cells and have suggested that such fragments may affect the maturation and biological activity of these cells. C-terminal fragments are cleared by glomerular filtration and have a half-life of less than 1 hour. In individuals with impaired renal function the half-life and circulating concentration of C-terminal fragments are significantly increased.

In individuals with normal renal function, 5 to 25% of the total circulating PTH is intact hormone and 75 to 95% consists of C-terminal fragments (Fitzpatrick and Bilezikian, 2006). There are specific assays for the biologically active intact PTH.

1.8.2. Clinical Significance

Determination of PTH is useful in the **differential diagnosis of both hypercalcemia and hypocalcemia**, for **monitoring parathyroid function in renal failure**, and for **evaluating parathyroid function in bone and mineral disorders**.

1.9. Vitamin D and Its Metabolites

Vitamin D is produced endogenously through the exposure of skin to sunlight and is absorbed mainly from foods containing or supplemented with vitamin D. The vitamin is metabolized to its **biologically active form, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D)** which regulates calcium and phosphate metabolism. Vitamin D deficiency can cause impaired formation of bone, rickets in children and osteomalacia in adults.

1.9.1. Biochemistry and Nutrition of Vitamin D

Vitamin D and their metabolites may be grouped as cholecalciferols or ergocalciferols. **Cholecalciferol (vitamin D_3)** is the parent compound produced in the skin from 7-dehydrocholesterol on exposure to the ultraviolet B portion of sunlight (Bikle et al, 2008; Lensmeyer et al, 2006). The quantity of production of vitamin D_3 by skin is influenced by latitude, season, aging, sunscreen use and skin pigmentation. **Ergocalciferol (Vitamin D_2)**, the parent compound of the other family, is manufactured by irradiation of ergosterol produced by yeasts. Vitamin D_2 and vitamin D_3 differ by the double bond between carbon 22 and carbon 23 and a methyl group on carbon 24.

Major sources of vitamin D: In our diet, the major sources are fish liver oils, fatty fish, egg yolks and liver, and contain hgher concentrations of vitamin D. Before supplemention with vitamin D_2 or vitamin D_3 from these sources, most vitamin D in the body was that produced by synthesis in the skin. Vitamin D is also acquired by ingestion of fortified foods such as cereals, bread products and milk or vitamin D supplements.

The common **Recommended daily allowance** (RDA) is 400 IU (10 µg). Some European countries have doubled the dose as recommendation for people older than 60 years (800 IU or 20 µg). In the United States, up to 2000 IU of vitamin D daily as given

for treatment or to prevent osteoporosis and to increase dietary calcium absorption (Heaney et al, 2009).

1.9.2. Metabolism, Regulation, Transport and Plasma Levels

They are metabolized to 25- hydroxyl vitamin D [25(OH)D] in the liver by the enzyme vitamin D 25- hydroxylase and cytochrome P450 (DeLuca, 2004; Lensmeye et al, 2006). The **concentration of 25(OH)D in serum** is 10 to 65 ng/ml or 25 to 162 nmol/l (conversion factor = 2.5) and its half-life is 2 to 3 weeks. Dietary calcium absorption is maximal when the concentration of 25(OH)D is near 30 ng/ml. Therefore, any **reference interval for 25(OH)D should not be confused with the "optimal" or "healthy" range** for 25(OH)D.

$25(OH)D_2$ and $25(OH)D_3$ are metabolized to the biologically active hormone 1, 25- dihydroxyvitamin D ($1,25(OH)_2D$) by 25(OH)D 1α-hydroxylase, a cytochrome P450 enzyme, in kidney and placenta. Normal **circulating concentrations of $1,25(OH)_2D$** are approximately 15 to 60 pg/ml or 36 to 144 pmol/l and its half-life 4 to 6 hours.

Regulation of circulating $1,25(OH)_2D$ concentrations: Circulating concentrations of $1,25(OH)_2D$ are regulated, primarily by PTH, phosphate, calcium, and $1,25(OH)_2D$ (Carmeliet et al, 2006; DeLuca, 2004). PTH and hypophosphatemia increase the synthesis of $1,25(OH)_2D$ by increasing the activity of 25(OH)D-1α- hydroxylase, whereas hypocalcemia acts indirectly by enhancing the secretion of PTH. Hypercalcemia, hyperphosphatemia, and $1,25(OH)_2D$ reduce 25(OH)D 1α-hydroxylase activity and $1,25(OH)_2D$. $1,25(OH)_2D$ also induces 25(OH)D 24-hydroxylase enzyme activity that produce 24,25- dihydroxyvitamin D [$24,25(OH)_2D$], the most prevalent dihydroxylated vitamin D form in serum.

Transport of vitamin D: In circulating plasma, vitamin D, 25(OH)D, and $1,25(OH)_2D$ are bound to **vitamin D–binding protein (DBP)**, a specific, high-affinity transport protein also known as group-specific component of serum or Gc-globulin (Bikle et al, 2008; Carmeliet et al, 2006; Haddad, 1995). DBP belongs to the albumin and α-fetoprotein gene family and contains 458 amino acid residues and has a molecular mass of 51,335 Da. DBP is constitutively synthesized by the liver and circulates in excess amout (at about 400 mg/l), with less than 5% of the vitamin D binding sites normally

occupied. DBP binds with vitamin D and its metabolites, particularly the 25-hydroxylated metabolites 25(OH)D, 24,25$(OH)_2$D, and 1,25$(OH)_2$D (Bikle et al, 2008; Haddad, 1995). Normally, only 0.03% of 25(OH)D and 0.4% of 1,25$(OH)_2$D are see free in plasma. The concentration of DBP is increased in pregnancy and with estrogen therapy and is reduced in nephrotic syndrome.

1.9.3. Functions of Vitamin D

1.9.3.1. Absorption of calcium and phosphate

Biological actions of 1,25-Dihydroxyvitamin D (1,25$(OH)_2$D) helps to maintain calcium and phosphate in blood through its actions on intestine, bone, kidney, and the parathyroids. In the small intestine, 1,25$(OH)_2$D stimulates calcium absorption from the duodenum, and phosphate absorption by the jejunum and ileum (Bikle et al, 2008; Carmeliet et al, 2006; DeLuca, 2004). The three main events which serve the absorption of calcium from the diet are: (1) calcium entry into the brush border cytoplasm, mediated by an epithelial Ca2+ transporter or channel (CaT1); (2) diffusion of calcium within the cell fostered by calbindin-D9k, which is a cytosolic calcium-binding protein and (3) exit of calcium from the cell across its basolateral membrane by the action of a CaATPase (e.g., a Na+/Ca2+ exchanger). Approximately 90% of CaT1 synthesis is vitamin D dependent, and calbindin D synthesis is completely dependent on vitamin D. High calcium diet downregulates CaT1 and calbindin D expression by downregulating the production of 1,25$(OH)_2$D.

1.9.3.2. Bone metabolism

At high concentrations, 1,25$(OH)_2$D increases bone resorption by inducing the differentiation of monocytic stem cells in bone marrow into osteoclasts and by stimulating osteoblasts to produce cytokines and other factors that influence osteoclast activity. By stimulating osteoblasts, 1,25$(OH)_2$D also increases alkaline phosphatase and osteocalcin concentration in circulation. In the kidneys, 1,25$(OH)_2$D exhibits an ultra-short negative feedback loop mechanism, by inhibiting its own synthesis and stimulates its own metabolism. 1,25$(OH)_2$D also acts directly on the parathyroid gland to inhibit the synthesis and secretion of PTH. 1,25$(OH)_2$D also increases the concentrations of the

calcium sensing receptor in the parathyroid gland, thus sensitizing the gland to calcium inhibition (Bikle et al, 2008).

1.9.3.3. Action on target tissues

$1,25(OH)_2D$ exerts its actions on target cells by associating with a specific nuclear vitamin D receptor (VDR). This receptor is analogous to the steroid receptors for androgens, estrogens, and corticosteroids and is expressed widely in tissues, and most cells respond to $1,25(OH)_2D$ (Bouillon et al, 2008). The vitamin D receptor can form heterodimers with members of the retinoid X receptor and is a member of the NR1I family. The other members in this family include the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR).

1.9.3.4. Other actions of vitamin D

In addition to the effect of vitamin D on calcium metabolism, increasing evidence suggests the role of $1,25(OH)_2D$ in regulation of the immune response and in epithelial differentiation. Several studies showed inverse relationships between vitamin D metabolite concentration in blood and the incidence of certain cancers (Davis, 2008) and various other disorders. These findings have led to increased demand for vitamin D testing and also increase the recommended daily intake of vitamin D. However, it is difficult to demonstrate the substantial reductions in the rate of development of various cancers through the use of vitamin D in clinical trials (Davis, 2008).

1.9.4. Clinical Significance

Determination of circulating 25(OH)D may be useful in the differential diagnosis of **hypocalcemia, hypercalcemia, or hypercalciuria** and for evaluating vitamin D status in health and in bone and mineral disorders. Only measurements of 25(OH)D and $1,25(OH)_2D$ have proven clinical value (Lee et al, 2007; Prentice et al, 2008).

The nutritional status of vitamin D is best determined through the **measurement of 25(OH)D**, rather than vitamin D, because (1) 25(OH)D is the **main circulating form of vitamin D**, (2) have **longer half-life** and is less affected by day to- day variation, exposure to sunlight, or food intake and (3) **measurement of 25(OH)D is** relatively **easy** compared to vitamin D which require more technically complicated methods (Hollis,

2007; Hollis, 2008; Lensmeyer et al, 2006). Nutritional vitamin D deficiency is seen higher in breast-fed infants, strict vegetarians who abstain from eggs and milk, individuals with darker skin pigmentation, and the elderly.

Circulating concentrations of 25(OH)D may be decreased by reduced availability of vitamin D, inadequate conversion of vitamin D to 25(OH)D, increased metabolism of 25(OH)D, and urinary loss of 25(OH)D with its transport protein DBP. Reduced availability of vitamin D is mainly due to the inadequate exposure to sunlight, dietary deficiency, malabsorption syndromes, and gastric or small bowel resection.

Severe **hepatocellular disease** has been associated with **vitamin D deficiency** and may be due to the inadequate conversion of vitamin D to 25(OH)D. Drugs such as phenytoin, phenobarbital, and rifampin stimulate drug metabolizing enzymes which accelerate the metabolism of vitamin D and its metabolites. In **nephrotic syndrome**, the serum 25(OH)D concentrations may be reduced, because of the urinary loss of DBP and 25(OH)D.

Vitamin D deficiency is associated with a greater risk of **anemia, lower hemoglobin** and higher usage of erythrocyte-stimulating agents (Sim et al, 2010). Vitamin D deficiency is also associated with a number of clinical consequences like **cardiovascular disease** (Zittermann, 2006; Bischoff et al, 2006), **decreased bone mineralization and early mortality** (Bouillon et al, 1993; DeLuca, 2004).

The main role of vitamin D is to regulate bone and mineral metabolism. In addition to this, it had many other biological actions including **muscle strengthening, cellular proliferation and differentiation, immune system modulation** (DeLuca, 2004), inhibition of rennin synthesis (Li, 2003), **insulin production** (Chiu et al, 2004) and also **have a role in erythropoiesis** (Sim et al, 2010).

Vitamin D supplementations in hemodialysis patients was associated with a reduction in erythrocyte-stimulating agents (ESA), increased reticulocytosis and also affect marrow function (Saab et al, 2007). Correction of 25-D deficiency appears to have a limited impact on renal osteodystrophy in patients with end stage renal disease; it may improve other clinical outcomes, such as anemia in renal disease. ERGO supplementation

in hemodialysis patients may decrease the need for EPO but probably has no impact on markers of mineral metabolism (Kumar et al, 2011).

Measurement of circulating 1,25$(OH)_2$D is diagnostic in vitamin D–dependent rickets types 1 and 2, and in disease states associated with overproduction of 1,25$(OH)_2$D such as sarcoidosis, tuberculosis, Hodgkin's disease, fungal infection, Wegener's granulomatosis, and lymphoma. 1,25$(OH)_2$D test result also gives confirmatory information in the evaluation of hypercalcemia, hypercalciuria, hypocalcemia, and bone and mineral disorders. Concentrations of 1,25$(OH)_2$D are increased in type 2 vitamin D–dependent rickets, primary hyperparathyroidism, although its diagnosis does not require measurement of 1,25$(OH)_2$D and in 1,25$(OH)_2$D intoxication. Reduced concentrations of 1,25$(OH)_2$D can be observed in patients with renal failure, hypercalcemia of malignancy, hyperphosphatemia, hypoparathyroidism, pseudohypoparathyroidism, type 1 vitamin D–dependent rickets, hypomagnesemia, nephrotic syndrome, and severe hepatocellular disease.

Diagnosis of vitamin D deficiency is not based on measurement of 1,25$(OH)_2$D, because the circulating concentration of this metabolite is often normal as a compensatory hyperparathyroidism (Lips, 2007). This assay is not useful in confirming intoxication with vitamin D or 25(OH)D, because in this situation, 1,25$(OH)_2$ concentrations may be low, normal, or increased.

1.9.5. Reference Intervals of Vitamin D

Reference intervals for vitamin D metabolite, 1,25$(OH)_2$D are method dependent and is 15 to 60 pg/ml or 36 to 144 pmol/l. Lower limits of the reference interval for 25(OH)D of 10 or 15 ng/ml (25 to 37 nmol/l) have been inappropriately low, as even above this limit the vitamin D status of the individual is insufficient (Lips et al, 2008). Vitamin D levels less than 20 to 30 ng/ml (50 to 75 nmol/l) can be associated with increased serum PTH concentrations and reduced calcium absorption. In late 2010, the United States Institute of Medicine published a recommendation that the lower limit for vitamin D sufficiency is lowered from 30 to 20 ng/ml (50 nmol/l) and that concentrations of vitamin D above 50 ng/ml can be considered as a cause for concern.

In 2002 and 2003, the National Health and Nutrition Examination Survey (NHANES) III study reported a high prevalence of vitamin D insufficiency was seen in adults and adolescents in North America (Calvo and Whiting, 2003; Looker et al, 2002). Circulating concentrations of 25(OH)D are increased by exposure to sunlight and also show a seasonal variation, with the highest concentrations in summer and the lowest concentrations in winter or spring (Sherman et al, 1990). The concentrations are influenced by latitude, sunscreen use, and skin pigmentation. Serum 25(OH)D concentrations of 100 ng/ml (250 nmol/) are common in people with extensive sun exposure, such as lifeguards.

Concentrations of vitamin D metabolites (1,25$(OH)_2$D) vary with age and are increased in pregnancy (Kovacs and Kronenberg, 1997) and in children than in adults, with the highest concentrations occurring during periods of growth (Misra et al, 2008). Both 25(OH)D and 1,25$(OH)_2$D concentrations have been decrease with age. This may be a consequence of poor nutrition, reduced exposure to sunlight, and declining health. In healthy and active subjects the concentrations of these metabolites were unchanged with age.

1.10. Insulin and C Peptide: Biosynthesis, Processing and Action

Insulin is a protein produced by the β-cells of the islets of Langerhans in the pancreas. Human insulin (molecular weight 5808 Da) consist of 51 amino acids in two chains A & B, joined by two disulfide bridges, with a third intra disulfide bridge within A chain.

It is an anabolic hormone that stimulates the uptake of glucose into fat and muscle, promotes the conversion of glucose to glycogen or fat for storage, inhibits glucose production from liver, stimulates protein synthesis and inhibits protein breakdown.

1.10.1. Biosynthesis of Insulin

The biosynthesis and processing of the insulin molecule along the secretory pathway of the β-cell is a highly regulated and dynamic process (Steiner et al, 1967). Preproinsulin is the protein precursor for proinsulin. It consists of about 100 amino acids and is formed by ribosomes in the rough endoplasmic reticulum of the pancreatic β-cells. Preproinsulin is not detectable in the circulation under normal conditions because it is enzymatically cleaved and converted to proinsulin.

Proinsulin is an 86 amino acid polypeptide, stored in secretory granules in the Golgi complex of the β-cells. Proteolytic cleavage of proinsulin is catalyzed by two Ca^{2+} regulated endopeptidases: prohormone convertases 1 and 2 (PC1 and PC2). PC1 (sometimes designated as PC3), cleave at the Arg31, Arg32 site of proinsulin to yield split32, 33-proinsulin and PC2 at Lys64 & Arg65 site to generate split-65, 66-proinsulin.

A third proteolytic enzyme is also involved in proinsulin processing is carboxypeptidase H (CPH). It is a Ni^{2+}-requiring exopeptidase that sequentially cleaved by PC3 at Arg31, Arg32 to generate des-31, 32 proinsulin, which is trimmed by CPH and then processed by PC2 at Lys64, Arg65 to obtain spilt-65,66-proinsulin and finally trimmed by CPH to produce insulin, C-peptide and free basic amino acids (Orci et al, 1986).

1.10.2. Insulin Secretion

The secretion of insulin is mainly by two pathways, constitutive and regulated pathways. The constitutive pathway occured by the rapid transport of proteins from trans golgi to plasma membrane in small vesicles. These vesicles fused with the plasma membrane and secreted the protein to extracellular environment. The regulated pathway involves the selective packaging of secretory proteins into secretory granules and secretion (exocytosis) is followed in response to a stimulus. Before the release of insulin via exocytosis, an insulin secretory granule must be transported from an intracellular storage pool to the β-cell surface. These are mediated via an interaction with the β-cell cytoskeleton framework of microtubules and microfilaments.

For the secretion of insulin specific signal is needed otherwise it is in the resting state. Exocytosis of insulin is triggered by certain intracellular secondary signals, including an increase in cytosolic [Ca^{2+}]; and also by several secretory granules, cytosolic and plasma membrane proteins. Glucose, amino acids, pancreatic and gastrointestinal hormones like glucagon, gastrin, secretin, gastrointestinal polypeptide and some medications (e.g., sulfonylureas, β-adrenergic agonists) stimulate insulin secretion. Hypoglycemia, somatostatin and various drugs suchas α-adrenergic agonists, β-adrenergic blockers, diazoxide, phenytoin, phenothiazines, nicotinic acid inhibit insulin secretion (Pfeifer et al, 1981).

1.10.2.1. Regulation of insulin biosynthesis in the β-Cell

In healthy individuals, secretion of insulin is in a pulsatile fashion, with glucose and insulin the main signals in the feedback loop. Glucose elicits the release of insulin from the pancreas in two phases; the first phase begins 1 to 2 minutes after intravenous injection of glucose and ends within 10 minutes and the second phase begins at the point where the first phase ends. This phase depends on continuing insulin synthesis and release and lasts until normoglycemia has been restored. It takes usually within 60 to 120 minutes. The threshold concentration of glucose required to stimulate insulin secretion is 4-6 mM and a maximum biosynthesis is reached at a glucose concentration of 10-12 mM. With progressive failure of β-cell function, the firstphase of insulin response to glucose is lost, but other stimuli like glucagon or amino acids can able to elicit this response. Although the second-phase of insulin response is preserved in most type 2 diabetes mellitus patients, both the first-phase response and normal pulsatile insulin secretion are lost. However patients with type 1 diabetes mellitus exhibit a minimal or no insulin response (Campbell et al, 1982).

1.10.3. Degradation of Insulin

Approximately 50% of insulin is extracted by the liver in portal circulation and degraded. As the extracted amount of insulin accurately reflect the rate not may the plasma insulin concentrations is vary, of insulin secretion. Additional insulin is degraded in the kidneys, where insulin is filtered through the glomeruli, reabsorbed, and degraded

in the proximal tubule. In the circulation the half-life of insulin is between 4 and 5 minutes.

1.10.4. Insulin Action

The action of insulin is initiated by binding of insulin to its specific receptors in the plasma membrane. The insulin receptor is a **heterotetramer,** consisting of **two α-subunits** and **two β-subunits**. It belongs to a subfamily of receptor tyrosine kinases that also includes the insulin-like growth factor-1 (IGF-1) receptor (Zhang & Roth, 1992). The α-subunit is located on the outer surface of the plasma membrane and contains the insulin binding site. The β-subunit extends intracellularly through the plasma membrane and contains an intrinsic tyrosine kinase domain. The insulin receptor is widely distributed throughout the body.

Insulin binding to the α-subunit induces a conformational change in the receptor, resulting in activation of the kinase activity in the β-subunit, which catalyzes the phosphorylation of tyrosine residues on several proteins. One of the major substrates for this tyrosine kinase activity is the receptor itself. In addition to phosphorylating itself, the insulin receptor catalyzes the phosphorylation of tyrosine residues of various specific intracellular proteins. These include four insulin-receptor substrate (IRS) proteins (IRS-1, IRS-2, IRS-3, and IRS-4), Shc, and Gab-1. The phosphorylated tyrosine residues on these target proteins serve as ‘docking sites’ for intracellular molecules that contain SH2 (Src-homology 2) domains (Taniguchi et al, 2006).

The SH2 proteins that bind to phosphorylated IRS proteins fall into two major categories, include those labeled phosphatidylinositol 3-kinase (PI3K) and growth factor receptor–bound protein 2 (Grb2), both of which mediate downstream signal transduction events. These molecules associates with SOS to activate the mitogen-activated protein (MAP) kinase cascade via Ras (King & Johnson, 1985). Phosphatidylinositol 3′-kinase also activates Akt /protein kinase C (PKC) cascade. The latter enzymes regulate glucose transport by modulating translocation of GLUT4 to the plasma membrane. Akt also phosphorylates and inactivates glycogen synthase kinase-3 and thereby enhance glycogen synthesis.

The main effect of insulin on glucose homeostasis is its ability to stimulate glucose transport in fat and muscle cells. This occurs through the translocation of GLUT4 glucose transporters from intracellular site to the plasma membrane. Insulin regulates the glycogen/ lipid/protein synthesis, cell growth and differentiations and also some gene expression. Other functions of insulin are regulate hormone production, secretory function and signal sensing in neural or endocrine cells, regulation of estrogen/androgen balance in ovarian granulose cells, promote vasodilatation in endothelial cells.

1.10.5. Insulin Resistance

Insulin resistance is where a normal or elevated concentration of insulin produces an attenuated biological response ie, impaired sensitivity to insulin mediated glucose disposal. Compensatory hyperinsulinaemia occurs when pancreatic β cell secretion increases to maintain normal blood glucose levels in the setting of peripheral insulin resistance in muscle and adipose tissue (Wilcox, 2005).

Insulin resistance has been divided into two: decreased insulin sensitivity (normal response but increased hormonal level) and decreased responsiveness (normal level of hormone but less response). Insulin resistance has been seen in certain disorders such as type 2 diabetes, obesity, hypertension, polycystic ovarian and a variety of genetic syndromes, and in physiologic conditions such as puberty and pregnancy (Kahn, 1978). Insulin resistance is also present in stress conditions, in association with infection and secondary to treatment with a variety of drugs, particularly glucocorticoids. Insulin resistance plays an important role in type 2 diabetes and metabolic syndrome.

The pathophysiologic defects of insulin resistance have not been identified, but it is usually attributed to a defect in insulin action. Measurement of insulin resistance in a routine clinical setting is difficult, and therefore, measures fasting insulin concentration or the euglycemic insulin clamp, which provide an indirect assessment of insulin function (American Diabetes Association, 1998).

Insulin resistance syndrome refers to the cluster of abnormalities and related physical outcomes that occur mainly in insulin resistant individuals. Metabolic syndrome is associated with insulin resistance, hyperinsulinemia, obesity, dyslipidemia (high triglyceride and low high-density lipoprotein (HDL) cholesterol), and hypertension

(Reaven, 2004). Individuals with this syndrome are at increased risk for cardiovascular disease.

1.10.6. C Peptide

Proinsulin is cleaved to a 31 amino acid connecting peptide (C peptide) having molecular weight of 3600 Da and insulin. C peptide is secreted by pancreatic β-cells along with insulin into the portal circulation. Although insulin and C peptide are secreted in equimolar amounts, fasting C peptide concentrations are five to 10 fold times higher than those of insulin owing to the longer half-life of C peptide, which is approximately 35 minutes (Orci et al, 1988).

Unlike insulin, C peptide is not significantly cleared by the liver and is removed from the circulation by the kidneys and degraded, with a fraction excreted unchanged in the urine. Therefore, it has been suggested that peripheral C peptide levels are more closely reflect pancreatic insulin secretion than peripheral insulin levels.

It was believed for many years that C peptide was important only for the correct folding of insulin and otherwise it was inactive. But now, several studies showed that C peptide exerts direct effect on renal function, to augment glucose utilization and to improve autonomic nervous function in insulin dependent diabetes mellitus, as well as insulin secretion. All these effects are mediated by direct impact of C peptide on Na+K+-ATPase activity in various tissues (Wahren et al, 2000).

Measurement of C peptide concentrations provides estimates of a patient's insulin secretory capacity and rate. Measurement of C peptide concentration has a number of advantages over insulin measurement. Because hepatic metabolism of C peptide is negligible, its concentrations are better indicators of β-cell function than peripheral insulin concentration (Polonsky et al, 1986).

Measuring C peptide is an indication for the evaluation of fasting hypoglycemia. Some patients with β-cell tumors particularly if hyperinsulinism is intermittent, may exhibit increased C peptide concentrations with normal insulin concentrations. When hypoglycemia is due to insulin injection, insulin concentrations will be high but the C peptide concentration will be low (Horwitz et al, 1976).

1.10.7. β-Cell Dysfunction in Type 2 Diabetes Mellitus

The storage and metabolism of cellular fuels are regulated through the secretion of insulin by **pancreatic β-cells**. The β-cells function in a highly complex fashion that regulates the timing and overall insulin response to a meal to preserve normoglycemia. The main functions of β-cell are insulin secretion, proinsulin biosynthesis, processing of proinsulin to insulin and β-cell replication. The secreted insulin lowers glycemia by inhibiting hepatic and renal glucose production and by increasing the uptake of glucose into target organs, primarily skeletal muscle. Glucose regulation of insulin secretion occurs directly as glucose induced insulin secretion and through modulation of insulin response to insulinotropic hormones, nutrients and neurotransmitters. These dual aspects of glucose-regulated insulin secretion are a potent modulatory system that ensures the tissues need for insulin in the fasting and postprandial states. The need for insulin is for the most part determined by the sensitivity of tissue to insulin (Kahn et al, 1993).

Insulin resistance induced **increased β-cell demand** and is ultimately associated with progressive loss of β-cell function. This leads to the development of fasting hyperglycemia. The major defect is a loss of glucose-induced insulin secretion which is termed as selective glucose unresponsiveness. Hyperglycemia appears to render the β-cells increasingly unresponsive to glucose called glucotoxicity. The degree of β-cell dysfunction correlates with both glucose concentration and duration of hyperglycemia. Restoration of euglycemia rapidly resolves the defect and increased free fatty acids in serum have also been implicated in β-cell dysfunction (Bell and Polonsky, 2001).

Other insulin secretory abnormalities in type 2 diabetes are the disruption of the normal pulsatile release of insulin and an increased plasma proinsulin to insulin ratio (Leahy, 1990). Recently, evidence obtained from knockout mice reveals that insulin resistance in the β-cells may contribute to changes in insulin secretion, as occur in type 2 diabetes (Kulkarni et al, 1999).

Genetic predisposition is the initial cause of glucose intolerance. Although specific polymorphism or mutated genes are not yet known, many that affect the liver, skeletal muscle, adipose tissues or β-cells will undoubtedly be uncovered. Lifestyle changes in individuals and environmental factors also determine the development of glucose

intolerance (Hamman, 1992). The β-cell dysfunction plays a crucial role in type 2 diabetes mellitus. Alterations in insulin secretion and tissue insulin resistance are of equal importance in the development of type 2 diabetes. The protective effect of exercise is thought to be increased sensitivity to insulin in skeletal muscle and adipose tissue.

1.11. Troponin I

Troponin is a regulatory protein of the thin filament of striated muscle. This protein consists of three subunits I, T and C, which are implicated together in muscle contractions. Troponin I have a cardiac isoform, which rapidly released after acute myocardial infarction (AMI) and is highly specific for the detection of myocardial injury. This isoform can be detected in blood between the 4th and 8th hour after the onset of chest pain, with a peak between the 14th and 36th hour; concentrations in blood remain high for 3 to 7 days (Christenson and Azzazy, 1998). Cardiac troponin is the more specific and sensitive biomarker for the detection of myocardial necrosis than the cardiac enzymes.

Troponin I is an automated quantitative test used for the determination of human cardiac troponin I in human serum or plasma using the ELFA (Enzyme-Linked Fluorescent Assay) technique. Troponin I is intended to be used as an aid in the diagnosis of myocardial infarction. The European Society for Cardiology (ESC) and the American College of Cardiology (ACC) recommended that the diagnosis of myocardial necrosis can be made when the concentration of cardiac troponin in the blood is greater than the 99th percentile of a healthy population, in the clinical setting of acute ischemia. The acceptable imprecision (coefficient of variation) at the 99th percentile for this assay should be defined as ≤10% (Alpert et al, 2000).

1.11.1. Range of Expected Values

The value of troponin I is <0.002µg/l in patients with no cardiac symptoms. But when the concentration of troponin I is 0.002 - 0.1 µg/l, it indicate myocardial injury and >0.1 µg/l is seen in Acute coronory syndrome.

1.11.2. Anemia in Heart Failure

Chronic heart failure (CHF) is a multi-organ syndrome which causes changes in the periphery affecting muscle and blood vessels, neurohormonal activation and metabolic and hormonal changes. It is now recognized that anemia is frequently a part of this syndrome and has been found to affect nearly 25% of heart failure patients. The cause of anemia in chronic heart failure is not known and it also shows a defect in iron utilisation.

Anemia is common in congestive heart failure and is associated with a marked increase in mortality and morbidity and also worsens congestive heart failure (Tang et al, 2008; Anandet al, 2005). Correction of the anemia with subcutaneous erythropoietin and intravenous iron has caused a great improvement in congestive heart failure patient and cardiac function and a marked reduction in the need for hospitalization and for high-dose diuretics. In addition to this, quality of life and exercise capacity of patients also improved with the correction of the anemia (Silverberg et al, 2005; Manolis et al, 2005).

Several studies have showed that the erythropoietin receptor is widely distributed in the cardiovascular system, including endothelial cells, smooth muscle cells and cardiomyocytes. Erythropoietin may have a direct positive effect on the heart by reducing cell apoptosis, and by increasing neovascularization, both of which could prevent tissue damage (Timmer et al, 2009). This could have profound therapeutic implications not only in congestive heart failure but also in the future treatment of myocardial infarction, coronary heart disease and strokes (Roger et al, 2004)

Chronic heart failure is also thought to include bone marrow dysfunction secondary to poor perfusion, impaired renal function and the effects of cytokine activation -especially TNF-alpha- which can induce both reduced erythropoietin production and increased resistance to its effects. In severe heart failure patients, the immune activation and pro-inflammatory cytokines strongly correlate with the severity of anemia.

Patients with CHF apparently suffer from **tissue hypoperfusion**, which causes internal environmental **hypoxia** and subsequently leads to the overexpression of EPO. Tissue hypoperfusion may lead to other types of stimulation such as oxidative stress that

result in the production of oxygen radicals and upregulate the expression of EPO. So in patients with CHF and in those with CHF complicated by anemia, EPO expression is increased and the increase correlates with the severity of CHF. Thus, serum EPO expression performs an important role in the progression of CHF and therefore, is clinically significant for the prediction of the development, outcome and prognosis of CHF (Guo et al, 2013).

1.12. Biostatistics

Biostatistical methods used in this study are defined and described below (Reffenberg, 2012; Gurumani, 2005; Altman, 1991).

1.12.1 Measures of Central Tendency –Averages

Measures that describe the central aspects of a data are called averages. An average summarizes all the characteristics of entire mass of data. Most of the items of the series are clustered around the average, so it is called as measure of central tendency. The main aim of average is to present huge mass of statistical data in a simple and concise manner. Average is used for comparison. The main types of averages are:

1.12.1.1. Arithmetic mean

Arithmetic mean is the sum of all the observations divided by the number of observation

$$\text{Mean} = \frac{\text{sum of all the observations}}{\text{Total number of observations}}$$

1.12.1.2. Median

It is the value of the middle item of a given series of data arranged in ascending or descending order of magnitude.

$$\text{Median} = \frac{\text{value of the item } (n+1)}{2}$$

1.12.1.3. Mode

Mode is the most frequently occurring value in a sample. A sample with a single mode is referred as unimodal. If it has two mode it is called as bimodal and more modes as polymodal or multimodal. If no mode, it is no modal sample.

1.12.2. Measures of Dispersion

A measure of dispersion (also called measure of variation, scatter, spread) is to describe the extent of scattering of items around a measure of central tendency. Different types of measure of dispersion used here are:

1.12.2.1. Range

Range is the minimum and maximum value of the given series of data (Gurumani, 2005).

1.12.2.2. Standard deviation

Standard deviation is defined as the square root of the arithmetic mean of the squared deviation of the various items from the mean. The mean squared deviation is called the variance. Therefore the square root of variance is the standard deviation.

$$\text{Standard deviation (SD)} = \sqrt{\sum_{i=1}^{n} (X_i - \bar{X})^2}$$

Where X_i = variable and $\bar{X}$ = mean of the variable

$X - \bar{X}$ = deviation

$(X - \overline{X})^2$ = squared deviation

$(X - \overline{X})^2/n$ = mean of squared deviation = Variance

1.12.2.3. Coefficient of variation (CV)

CV is also known as relative standard deviation (RSD). It is a measure of dispersion of a probability distribution or frequency distribution. CV is defined as the ratio of standard deviation to the mean and is expressed in percentage. I t is widely used in analytical chemistry to express the precision and repeatability of an assay.

$$\text{Coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \text{ X } 100$$

1.12.3. Skewness and Kurtosis

1.12.3.1. Skewness

Skewness describes the extent of asymmetry of a distribution. Skewness may be positive or negative. In a positively skewed distribution more items are on the right of the highest ordinate and a frequency curve has a steep rise and slow fall with a long tail on the right. In positive skewed distribution is Mean> Median> Mode. In a negatively skewed distribution more items are on the left of the highest ordinate and a frequency curve has a slow rise and deep fall with a long tail on the left. In negative skewed distribution is Mean< Median< Mode.

By these properties we can tests the skewness and it showed as: frequency curve is not perfectly bell shaped; it is tailed either on the right (positive skewness) or on the left (negative skewness). Numerical measure developed to evaluate the skewness of a distribution is Karl Pearson's coefficient of skewness.

$$\text{Karl Pearson's coefficient of skewness} = \frac{(\text{Mean} - \text{Mode})}{\text{Standard deviation}} = (-1 \text{ to } +1)$$

1.12.3.2. Kurtosis

Kurtosis is due to variation in the concentration of items around the mean, the frequency curve may appear peaked or flat when compared to a normal bell-shaped curve. This flatness or peakedness of a frequency curve is kurtosis. A frequency curve that is normal bell-shaped curve is mesokurtic, flat curve is platykurtic and a peaked curve is leptokurtic.

Coefficient of Kurtosis, $\beta_2 = \mu_4/\sigma^4$,

Where $\mu_4 = \Sigma(X - \overline{X})^4/n$ and $\sigma^4 = \{\Sigma(X - \overline{X})^2/n\}^2$.

if $\beta_2 = 3$, the frequency curve is the mesokurtic (normal bell shaped curve).

if $\beta_2 < 3$, the frequency curve is platykurtic (flat),

if $\beta_2 > 3$ the frequency curve is leptokurtic (peaked) (Gurumani, 2005).

1.12.4. Box-Whisker Plot

This plot is a diagrammatic representation of data series to give visual information about measures of central tendency and dispersion, and the extent and direction of skewness. It also provides information on the outliers. A Box-Whisker Plot has a central reference scale on which the data points of the continuous data present is plotted. On this scale, the position of median, first quartile Q1, third quartile Q3, inter quartile range are calculated and plotted. The inner fence points on either side of the median are marked as f1 and f3 are calculated as follows

$f1 = Q1 - 1.5 \times$ Inter quartile Range

$f3 = Q3 + 1.5 \times$ Inter quartile Range

Adjacent value a1 is the value closest to f1 but not less than f1 and adjacent value a3 value is nearest to f3 but not above it. The outer fence f1 and f3 are calculated by the following equations

$F1 = Q1 - 2\ (1.5)$ (Inter quartile Range)

$F3 = Q3 + 2\ (1.5)$ (Inter quartile Range)

The box ending at Q1 and Q3 are drawn. A line cutting the box at the median is also drawn in the box. Adjacent value a1 and a3 are indicated and connected to the box by a dash line. Any value between f1 and F1 are the mild negative outliers and one between the inner and outer fence are indicated by a data point. These are mild negative outliers.Any value between f3 and F3 are the mild positive outliers. Values of the data that lie beyond the outer fences (f1 and F3) are the external outliers.

1.12.4.1. Interpretation of Box-whisker plot

If the distribution is symmetrical, the median is in the middle of the box. If the median is towards the lower value point, away from the center of the box, then it is positively skewed. Similarly if the median point is deviated from the center to a higher value point, then it is negatively skewed.

The inner and outer fences are used to locate the mild and the extreme outliers. The outliers are samples outside the normal distribution scale. Outliers are identified and located for analysis of disease conditions or abnormal values or errors. Outliers may be

removed in one or several steps to identify the normal distribution of the sample or transform a sample to normal distribution.

1.12.5. Centiles

The value, which is below a certain percentage, is called a centile or percentile. This value coresponds to a value with a particular cumulative frequency. For example; if we require 5th and 95th centiles of a distribution, we may arrange the total sample in ascending order. If the total number of sample is 'N', then the 5th and 95th centiles are identified by the following equation.

5th centile = 0.05 X N

95th centile = 0.95 X N

If the centile obtained is an integer, then that value is considered as the 5th and 95th centiles. But if the value calculated is between two numbers, and is a non integer, then the following calculations are done. The adjacent two data points are taken and the following calculations are made.

Lower value + the centile fraction (higher value-lower value) = the particular centile value.

The 25th and 75th centiles are known as **quartiles;** these values together with the median divide the data into four equally populated subgroups. The numerical difference between the 25th and 75th is the **inter-quartile range**, and is occasionally used to describe variability.

1.12.6. Standard Error

Standard error is defined as the standard deviation of the sampling distribution. The standard error of mean is the standard deviation of the sampling distribution of sample means. When population standard deviation (σ) is known, standard error (SE) of mean ($\sigma_{\bar{x}}$),

$$\sigma\,\bar{x} = \frac{\sigma}{\sqrt{n}}$$

If σ is not known, $S\bar{x} = S/\sqrt{n}$; where $S = \sqrt{\frac{\sum(x-\bar{X})^2}{n-1}}$

1.12.7. 95% Confidence Interval of Mean

Confidence interval is a range of values which can be confident including the true values. A confidence interval for the estimated mean extends either sides of the mean by a multiple of the standard error. 95% confidence interval was calculated by multiplying standard error by 1.96 and then identifying the range by adding and subtracting the value from mean.

1.12.8. Frequency Graphs

1.12.8.1. Bar diagram

Bar diagram is a method of presenting data in which frequencies are displayed along one axis and categories of the variable along the other, the frequencies being represented by the bar lengths.

1.12.8.2. Histogram

Histogram is the graphical representation of continuous frequency distribution. The X-axis has the true class intervals, and the Y-axis, the frequencies. The bars are of equal width indicating that the class- intervals are of equal width. The height of the bar is proportional to the respective frequency. Therefore, it may be said that the area (length ×breadth) of each bar is equal to the total of all the frequencies.

1.12.9. Gaussian or Normal Distribution

For the parametric methods of calculations the data must be normally distributed. Normal distribution is derived by its graphical expression called normal curve. Normal distribution is a probability distribution which describes data which have a symmetric distribution and unimodel. The properties of normal curve is unimodel, perfectly bell shaped, symmetrical curve. The tail of the curve is asymptotic, i.e, get closer and closer to the x-axis but they never touch it. Mean, median and mode coincide. The total area under curve is 1. The coefficient of skewness is 0 and measure of kurtosis $\beta_3 = 3$. About 68.27% of items lie between 1SD, 95.46% lie between 2SD and 99.73% between 3SD.

1.12.9.1. Normal quantile-quantile plot

In this method, a plot of the quantiles of the data distribution is plotted against the expected quantiles of the normal distribution. The expected quantiles of the normal distribution is expressed as ± z value of normal distribution. If the sample quantiles of data distribution is normally distributed it will correlate linearly with a theoretical z value of the quantiles. If the plot of quantiles of the data distribution is not normally distributed, the data points will deviate from the straight line.

The plot can be made on a normal probability paper. But at present computer programs such as SPSS will give an output of the distribution of the sample along with the linear relationship of the normal plot for comparison.

The quantile – quantile (Q-Q) plot is a graphical technique for determining if the two data sets come from populations with a common distribution.

1.12.9.2. Test for normality

Kolmogorov – Smirnova (K-S), Shapiro – Wilk (S-W), Anderson – Darling (A-D) and D'Agostino tests were used for normality calculation. P value >0.05 are normal samples.

1.12.9.3. Transformations

When a distribution is not normal, then we transform the data in order to get a normal (Gaussian) distribution. The main transformation methods used are

1. Natural logarithmic transformation – we take the log(e) of the number, where e = 2.718283
2. Logarithmic transformation - we take the log(10) of the number
3. Box-Cox transformation - $X\lambda$ where X is the number and λ is the appropriate value needed for normality.

1.12.10. Hypothesis Testing

1.12.10.1. Student t-test

W.S.Gosset described a distribution called t distribution which enables to make reasonably valid inferences about the population using statistic from small samples. It is

commonly referred to as the t- distribution and the tests of significance based on it are called the t- tests (Altman, 1991). This test requires the variables have Gaussian distribution and equal variances.

1.12.10.2. F test or Variance ratio test

F test is any test in which the test statistic has an F distribution under the null hypothesis. The variance ratio is the ratio of the sample variances or the square of the ratio of the sample standard deviations. The t test is based on the assumption that the two population variances are the same, so we test the null hypothesis by F test (levene's test). Levene's test is used before a comparison of means. This test provides an F statistic and a significant value (P value) and if this value is less than the critical value of 0.05 then the variances are not equal.

1.12.10.3. Mann Whitney U test

It is a non parametric alternative to the t test for comparing data from two independent groups. This test requires all the observations to be ranked as if they were from a single sample. The test involves the calculation of statistic U, and is more complicated and calculated as:

$$U = n1\, n2 + (n1 + 1) - T$$

1.12.11. Correlation

This is a method of analysis to use when studying the possible association between two continuous variables. The relationship between two or more variables is called "correlation", and the variables are said to be correlated. The relationship between two variables is also known as co variation.

1.12.11.1. Types of correlation

Correlation between variables may be either simple or multiple. A simple correlation deals with two variables where as multiple correlation deals with more than two variables. Correlation between two variables may be positive correlation or negative correlation.

A correlation between two variables in which, with an increase in the value of one variable the value of other variable also increases, and with a decrease in the value of one variable the value of the other variable also decreases, is said to be a **positive correlation**. In positive correlation the values of both variables move in the same direction.

A correlation between two variables in which when there is an increase in the values of one variables, the values of the other variable decreases, and when there is a decrease in the values of one variable the other variable increases , is said to be a **negative correlation**. The values of two variables are move in opposite direction in negative correlation.

1.12.11.2. Karl Pearson's coefficient of correlation

Karl Pearson's correlation coefficient (r) is used to measure the degree of association. r can attain any value between -1 to +1. The correlation between two variables is positive if higher values of one variable are associate with higher values of other and negative if one variable tends to be lower as the other gets higher. A correlation of around zero indicates that there is no linear relation between the values of the two variables.

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}}$$

The two method used to study the presence or absence and extend of correlation between variables are scatter diagram and Karl Pearson's coefficient of correlation (Altman, 1991). Karl Pearson's Coefficient of Correlation is a measure of the linear correlation between two variables X and Y, giving a value between +1 and -1 inclusive, where 1 is total positive correlation, 0 is no correlation and -1 is total negative correlation. It is widely used as a measure of the degree of linear dependence between two variables (Altman, 1991).

1.12.11.3. Spearman's rank correlation

The Spearman's rank correlation is a non parametric version of the Pearson correlation. Spearman's correlation coefficient measures the strength and direction of

association between two rank variables. Spearman's rank correlation coefficient is exactly the same as the Pearson correlation coefficient calculated on the ranks of the observations. If the data not have Gaussian distribution for either variable, it is preferale to use rank correlation.

1.12.11.4. Scatter diagram

It is an easy and simple method for studying correlation between two variables. If X and Y are pairs of variables, the values of the variable X are marked in the X – axis and the values of variable Y are marked in the Y axis. A point is plotted against each value of X and the corresponding Y value. A swarm of dots is obtained, and this is called the scatter diagram. From this scatter diagram we can understand about the correlation between the variables whether it is positive correlation or negative correlation.

If the dots are scattered around a straight line running from left to right in an upward direction, then the correlation between the two variables is said to be positive. A scatter diagram in which the plotted dots form a swarm around a straight line that runs from left to right in the downward direction indicates negative correlation between the variables.

1.12.12. Reference Interval

1.12.12.1. Strategies for selection of reference individuals

Some conditions are required to make the comparison of patient's laboratory results with reference values possible and valid (IFCC 1987-1991; Mardia 1980; Solberg & Grasbeck, 1989). The population from which reference data is collected should be defined. All the groups of reference individuals should be clearly defined with inclusion and exclusion criteria. Conditions in which the sample were obtained and processed for analysis should be known. All quantities compared should be of same type. All laboratory results should be produced with the use of adequately standardized methods under sufficient analytical quality control. The diagnostic sensitivity and specificity, prevalence and clinical costs of misclassification should be known for all lab tests used. From the parent population, who fulfill the selection criteria, random sampling is the best method for selecting reference individuals. But strict random sampling is not possible due

to practical reasons. Therefore, using the best reference sample that can possibly be obtained after all practical considerations have been taken into account is necessary (Horowitz, 2012).

1.12.12.2. Partitioning the sample

It is necessary to have separate reference values for sex and age groups, and other criteria if required. The number of criteria for partitioning the reference interval should be kept small and significant, so that sufficient sample sizes for valid statistical estimates must be available. Several analytes vary significantly among different age and gender groups, so age and sex are most frequently used criteria for sub grouping. Other examples of partitioning criteria to be used for possible sub grouping are the genetic factors; the race, ethnic, blood and HLA groups**;** physiological factors such as stage of menstrual cycle, pregnancy, physical condition, and factors such as socio economic, environmental and chronobiological states (Horowitz, 2012). In this study, partitioning based on age and sex was done.

1.12.12.3. Sample collection and data generation

The step of sample collection was essential to minimize bias and variation, standardization of pre analytical preparation of individuals before sample collection, sample collection itself and handling of sample before analysis, etc. were necessary. Venipuncture and skin puncture are standardised procedures for blood sample collection (Grasbeck & Alstrom, 1981). After sample collection its analysis is very important. Same analytical method should be used to ensure comparability between reference and observed value. In the analytical procedures the essential components required specification are: Analysis method, including information on equipment, reagents, calibration type of raw data and calculation methods; quality control; reliability and validity criteria. The specification and validity of the tests should be so clear that another investigator can reproduce the study.

1.12.12.4. Statistical treatment of reference interval

Statistical analysis for reference interval calculations was done for partitioning the sample population for forming the minimum valid groups, analysis of sample distribution

and calculation of reference intervals (Harris & Boyd, 1995; IFCC, 1987; Solberg & Grasbeck, 1989). Baseline reference interval is the reference interval obtained after exclusions by clinical and clinical biochemistry evaluations to remove all clinical and subclinical disease states and deficiencies in the sample population.

1.12.12.5. Methods for calculating reference values

Nonparametric and parametric methods were used to determine reference intervals.

Nonparametric method: When the sample number was above 120, the non parametric method of 95% reference interval calculation was done without any transformation. This was done by calculating the 95% middle percentile values, leaving 2.5% at both ends. 2.5 to 5% of the sample number in the non parametric percentile methods on either are abnormal levels and were excluded. Bootstrap sampling was done to make the reference interval close to population reference interval. The 90% confidence interval of the upper and lower limits of the reference interval was also calculated.

When the sample number was below 30, non parametric range was used to calculate 95% reference interval. This method requires careful analysis of two or three data points near lowest and highest range points. This was to reduce errors at critical lower and upper limits of reference interval (Horowitz, 2012).

Parametric method: Parametric method is much more complicated than simple nonparametric method. Before choosing parametric methods, it was required to verify sample distribution (IFCC, Mardia 1980; Solberg 1986).

1.12.12.6. Analysis of sample distribution

Sample distribution can be analysed visually and by calculating distribution statistic. The visual method of data analysis was done by Anderson–Darling probability plot. The distribution statistic was calculated by Shapiro-Wilk, D'Agostino and Anderson-Darling methods. While Anderson-Darling methods were based on the distribution of the data in relation to Gaussian distribution, D'Agostino methods rely on the skewness and kurtosis character of the sample distribution. Anderson-Darling method works well for analysis of the low sample number distribution (IFCC, 1987; Horowitz, 2012).

When sample distribution was not Gaussian or when the statistic was too low, **data transformations** were done by log_{10} and Box-Cox methods.

1.12.12.7. Robust method

When the sample number was below 120, but above 30, parametric method with **robust** calculation was used to calculate 95% reference interval. Robust method of calculations was used to get better values for the reference interval.The parametric methods require Gaussian distribution of the sample. In robust method instead of using the mean and standard deviation of the sample, it uses robust measures of location (median) and spread (median absolute deviation) (Horowitz, 2012).

1.13. Software Packages

1) SPSS used for calculating distribution characteristics such as the mean, median, mode, range, standard deviation, interquartile range, S.E of mean, 95% confidence interval, coefficient of skewness and kurtosis and plot of normality curve and box-whisker plot. It is also used for normality test such as K-S and S-W. Hypothesis tests such as Student t-test and Mann Whitney U test are done with this software package.

2) MedCalc is used for 95% reference interval calculation by parametric robust method and for D' Agostino method for normality testing. Non-parametric (bootstrap) method for 95% reference interval calculation was also done with this package.

3) Minitab is used for Anderson – Darling probability plot and A-D method for normality testing.

Chapter 2

General Materials and Methodology

2.1. Study Settings and Research Design

Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala institute of Medical sciences and Amala Cancer Research centre who fulfilled the criteria for inclusion and exclusion. Patient samples were also collected**.** Participants were from the near sea level plains of rural Central Kerala, South India, avoiding mountainous regions of Western Ghats. 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 who took part in this study.

This study is an **Observational, Cross sectional study.** Analysis of EPO and related analytes in health and in disease states such as vitamin D deficiency, anemia, iron deficiency, impaired glucose tolerance and myocardial infarction patients with mild to moderate increase of troponin I. There is an Experimental, longitudinal, prospective component in this study**.** Increased or decreased EPO levels and related analytes may see in individuals with a particular disease. This situation may be intervened by treatment of the disease condition or deficiency, such as anemia, iron and vitamin D deficiency. Follow up of the cases are done to evaluate the levels of EPO and the related analytes.

2.2. Selection of Participants, Inclusion and Exclusion Criteria

Clinical evaluation of participants in this study was done in six stages for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. More than **600** individuals were evaluated by clinical history and examination for **exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, alcoholism, or history of such conditions** at the time of examination or 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.**

After exclusion of samples at the first step, blood and urine samples were taken from **411** participants of various age groups and they were also clinically examined, of

which 187 were males and 224 were females (Table 2.1). Informed written consent was obtained from each participant at step 2 who donated blood and urine samples.

Participants 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 >1.3 mg/dl (114.9 µmol/l) in males and >1.2 mg/dl (106.1 µmol/l) in females and total calcium >11 (2.75 mmol/l).** Samples selected after the second stage of exclusion of subclinical disease states by clinical biochemistry evaluation were **302** individuals; of which 116 were males and 186 were females (Table 2.1).

After exclusion of samples at the third stage, participant's aged **18 to 25 years** were selected. Other age groups were excluded to decrease the influence of age, growth phase and environment. There were **199** individuals aged 18 to 25 years, of which 71 were males and 128 were females.

From these 199 individuals, samples with EPO, hemoglobin, iron and ferritin were selected for the **EPO study (n = 159)**; of which males were 49 and females were 110. **PTH samples** selected for the study **after excluding 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, and Total calcium >11 mg/dl (2.75 mmol/l)** were **142;** of which male were 40 and females were 102.

Samples with **C peptide and Insulin** selected for the study after **excluding ferritin >250 ng/ml, osteocalcin >35 ng/ml, ostase (Bone alkaline phosphates) >30µg/l and urine NTx (N-terminal telopeptide) >200 nM BCE (bone collagen equivalents) / mmol urine creatinine** were **102** and **146** respectively. Of which for C peptide there were 27 male and 75 female samples and for insulin there were 42 male and 104 female samples (Table 2.1).

In this study, concentrations of EPO are compared with the concentrations of other plasma or serum analytes in health and disease states. EPO levels along with that of other related analytes will be estimated in diseases such as vitamin D deficiency, anemia, iron deficiency, impaired glucose tolerance and myocardial infarction patients with mild to moderate increase of troponin I.

2.3. Sample Collection

Blood samples (10 ml) 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 hemeolysis and increased the yield of serum which was preferred over plasma for storage. 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.

2.4. Sample Size

Preliminary studies done in our laboratory showed significant relationship between EPO and a number of other parameters. One such parameter was the relationship of EPO with serum ferritin. High and low ferritin groups gave a mean EPO of 7.82±3.14 and 11.69±9.27, respectively ($n_1 = 86$, $n_2 = 87$: p = <0.05, t = 3.65). The mean and standard deviation of EPO was used to calculate the sample size.

$$\text{Sample size, } n = \left[\frac{\left[\left(Z\text{-}\frac{\alpha}{2}\right)+\left(Z\text{-}\beta\right)\right]}{\overline{X}_1 - \overline{X}_2}\right]^2 \times (S_1^2+S_2^2)$$

$Z\text{-}\alpha/2 = 1.96$

$Z\text{-}\beta = 0.842$ (at 80% Power)

A = 0.05

$\overline{X}_1 = 11.69$ $\overline{X}_2 = 7.82$

$S_1 = 9.27$ $S_2 = 3.14$

Minimum Sample Size, $n_1 = 51$, $n_2 = 51$

Rounded Sample Size, n≈102≈110

2.5. Measurement of Body Mass Index

Body mass index (BMI) is a value derived from the person's weight and height. It is accepted as a better estimate of body fat and health risk than body weight.

$$BMI=\frac{\text{Weight (kg)}}{\text{Height(metre)}^2}$$

2.6. Measurement of Waist Circumference

Waist circumference help to assess the health risk associated with excess fat around the waist. Waist circumference measurement is a better indicator of central obesity than BMI. The measurement should be made at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest. Measurement should be taken after the patient exhales while standing without footwear and with feet positioned close together and arms hanging freely at the sides. The measuring tape should be snug around the body, but not pulled so tight that is constricting and measuring tape should be stretch resistant (WHO, 2008). In a research background, WC measurements are typically taken 3 times and recorded to the nearest 0.1 cm.

2.7. Autoanalysers for Chemistry and Immunochemistry Parameters

Biochemical analysis was done by Chemistry autoanalyser Vitros 5, 1 FS (Ortho-Clinical Diagnostics, USA) with both dry chemistry and wet chemistry sections. Immunochemistry autoanalysers used were Access 2 (Beckman Coulter, USA) and Liaison, Diasorin, Italy. Conversion factors used for interconversion between conventionl unit and SI unit of assayed analtytes in this study was given in Table 2.2.

2.8. Detailed Description of an Immunochemistry Assay Eg. EPO Assay

EPO assay was done with Access 2 machine and their reagents, using immunometric assay with magnetic bead coated anti EPO antibody (Procedure manual, Beckman Coulter, 2005). Sandwich or immunometric immune assay was done using mouse monoclonal anti EPO antibody coated on paramagnetic particles and mouse monoclonal anti EPO antibody conjugated with alkaline phosphatase. Detection was by chemiluminescence using chemiluminence reagents and luminometer.

1. A sample was added to a reaction vessel along with the paramagnetic particles coated with mouse monoclonal anti-EPO antibody, mouse monoclonal anti-EPO alkaline phosphatase conjugate and blocking reagent.
2. After incubation in the reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away.
3. Then, the chemiluminiscent substrate Lumi-Phos*530 was added to the vessel and light generated by the reaction was measured with a luminometer. The light production is directly proportional to the concentration of EPO in the sample. The amount of analyte in the sample is determined from a stored, multi-potent calibration curve (Procedure manual, Beckman Coulter, 2005).

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 (Procedure manual, Beckman Coulter, 2005).

The two level internal quality control reagents and the external quality assurance programs were from Biorad Laboratories, Irvine, CA, USA. Claims of the manufacturers of instruments and reagents were validated by internal quality control samples assayed in duplicates for a minimum of ten days or often more. Daily continuous internal quality control data were analyzed according to Westgard rules for acceptance or rejection of

analyte data (Klee and Westgard, 2012). If there is a rejection, appropriate measures were taken to set right errors in machine functioning, reagents or calibration levels.

2.9 Assay of Ferritin

The ferritin assay was a two-site immunoenzymatic ("sandwich") assay in which the sample was added to a reaction vessel with goat anti-ferritin-alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse: mouse anti-ferritin complexes. Serum ferritin binds to the immobilized monoclonal anti-ferritin on the solid phase, while the goat anti-ferritin enzyme conjugate reacts with different antigenic sites on the ferritin molecules. After incubation in a reaction vessel, the solid phase bound materials were held in a magnetic field while unbound materials were washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 is added to the vessel and light generated by the reaction was measured with a luminometer. The light produced is directly proportional to the concentration of ferritin in the sample. The concentration of analyte in the sample is determined from a stored, multi-point calibration curve (Procedure manual, Beckman Coulter, 2005).

2.10 Assay of Intact PTH

The intact PTH assay was done with Access 2 machine and their reagents; (Beckman Coulter, USA) using a two-site immunoenzymatic ("sandwich") assay. The sample is added to a reaction vessel, along with a monoclonal anti-PTH antibody conjugated to alkaline phosphatase, TRIS buffered saline with proteins and paramagnetic particles coated with a goat polyclonal anti-PTH antibody. After incubation in a reaction vessel, materials bound to the solid phase were held in a magnetic field while unbound materials were washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 is added to the reaction vessel and light generated by the reaction is measured with a luminometer and is directly proportional to the concentration of PTH in the sample. The amount of intact PTH in the sample is determined from a stored, multi-point calibration curve (Procedure manual, Beckman Coulter, 2005).

2.11 Assay of Insulin

Ultrasensitive insulin assay was done with Access 2 machine and their reagent, (Procedure manual, Beckman Coulter, 2005) using one-step immunoenzymatic ("sandwich") assay with magnetic bead coated anti-insulin antibody. Sandwich or immunometric immune assay was done using mouse monoclonal anti insulin antibody coated on paramagnetic particles and mouse monoclonal anti-insulin antibody conjugated with alkaline phosphatase. The serum insulin binds to the antibody on the solid phase, while the conjugate reacts with a different antigenic site on the insulin molecule. After incubation in a reaction vessel, materials bound to the solid phase were held in a magnetic field while unbound materials were washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 was added to the vessel. The light generated by the reaction was measured with a luminometer and was directly proportional to the concentration of insulin in the sample. The amount of analyte in the sample was determined from a stored, multi-point calibration curve.

2.12. Assay of Vitamin D

25 OH Vitamin D assay was done with Liaison, Diasorin, Italy, using their reagents. The method for quantitative determination of 25 OH Vitamin D was a direct, competitive chemiluminescence immunoassay (CLIA). Specific antibody to vitamin D was used for coating magnetic particles (solid phase) and vitamin D was linked to an isoluminol derivative. During incubation, 25 OH Vitamin D was dissociated from its binding protein, and competes with labeled vitamin D for binding sites on the antibody. After incubation, the unbound material was removed with a wash cycle. Subsequently, the starter reagents were added and a flash chemiluminescent reaction was initiated. The light signal was measured by a photomultiplier as relative light units (RLU) and was inversely proportional to the concentration of 25 OH Vitamin D present in calibrators, controls, or samples (Procedure manual, Liaison, 2017).

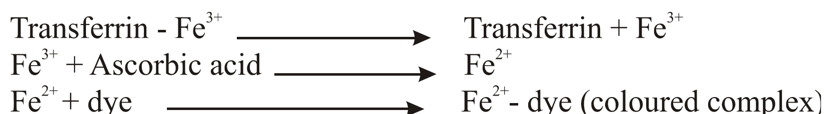
2.13. Assay of C peptide

The autoanalyser used for immunochemistry estimation of serum C peptide was Liaison, Diasorin, using their reagents. Sandwich chemiluminescence immnoassay was done using mouse monoclonal anti C peptide antibody coated on the magnetic particles (Solid Phase) and another monoclonal antibody is linked to an isoluminol-antibody conjugate. During the incubation, C peptide present in samples binds to the solid phase monoclonal antibody, and subsequently the antibody conjugate reacts with C peptide already bound to the solid phase. After incubation the unbound materials was removed with a wash cycle. Subsequently, the starter reagents were added and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of isoluminol-antibody conjugate, was measured by a photomultiplier as relative light units (RLU) and is indicative of C peptide concentration present in samples (Procedure manual, Liaison, 2017).

2.14. Detailed Description of a Chemistry Assay: Eg. Iron Assay

Iron assay was done by Chemistry autoanalyser, Vitros 5, 1 FS, with the dry chemistry section. VITROS iron slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample (10 µl) was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Ferric ion is removed from transferrin at acidic pH and migrates to the reducing layer, where ascorbic acid reduces iron to the ferrous form. The ferrous iron then bound to the dye and forms a colored complex in the reagent layer. Following addition of the sample, the slide is incubated and the reflection density was measured after 1 and 5 minutes. The difference in reflection density is proportional to the concentration of iron in the sample. Assay type was two-point rate and detected by reflectance spectrophotometry at a wavelength of 600 nm. The approximate incubation time for this assay was 5 minutes and temperature of incubation was 37°C (Procedure manual, Ortho-Clinical Diagnostics, 2004).

Reaction sequence



Calibrators were prepared from processed bovine serum and bovine serum albumin to which organic analytes, inorganic salts, electrolytes, stabilizers, and preservatives had been added. The diluents were prepared from processed water. VITROS calibrator Kit 4 was used to calibrate iron. Reportable range: 2 - 600 µg/dl (0.36 – 107.46 µmol/l).

Slide Components are mainly made of six parts in a slide. They are:

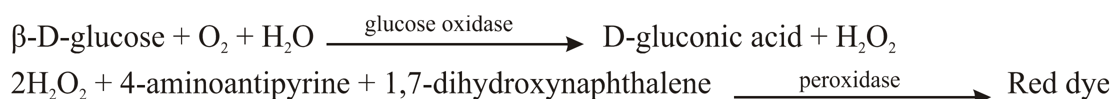
1. Upper slide mount
2. Spreading layer (TiO_2)
3. Reducing layer
    - Ascorbic acid - 160µg
4. Reagent layer
    - Buffer, pH 4.0
    - Dye – 5µg N-(4-(2,4-bis(1,1-dimethylpropyl) phenoxy)butyl)-5-methoxy-6((2,3,6,7-tetrahydro-8-1H, 5H-benzo-(ij)-quinolizin-9-yl)azo)-3-pyridine sulfonamide
    - Other ingredients: Binders, buffer, pigment, surfactants, stabilizers, chelators, dye solubilizer and cross-linking agent.
5. Support layer
6. Lower slide mount

2.15. Assay of Glucose

This assay was done in Vitros 5, 1 FS, Chemistry autoanalyser, with a wet chemistry and dry chemistry sections (Procedure manual, Ortho-Clinical Diagnostics, 2004). VITROS glucose slide is a multilayered, analytical element coated on a polyester support. A drop (10µl) of patient sample was deposited on the slide and was evenly

distributed by the spreading layer to the underlying layers. The oxidation of glucose in the sample was catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction was followed by an oxidative coupling catalyzed by peroxidase in the presence of dye precursors to produce a dye. This colorimetric method required 37°C temperature and an incubation time of 5 minutes. The intensity of the dye is measured by reflected light at 540 nm.

Reaction sequence



Reagents

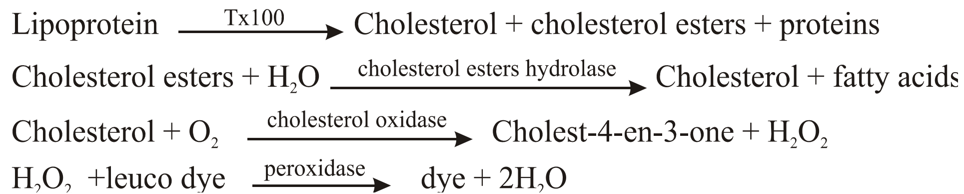
- Glucose oxidase -0.77 U (Aspergillus Niger)
- Peroxidase-3.6 U (horseradish root)
- 1,7 dihydroxynaphthalene – 67µg (dye precursors)
- 4-aminoantipyrine hydrochloride – 0.11 mg (dye precursors)
- Pigment, binders, buffer, surfactance, stabilizers and cross-linking agent.

2.16. Assay of Cholesterol

Cholesterol assay was performed in chemistry autoanalyser Vitros 5, 1 FS using the VITROS dry chemistry slides, which is a multilayered, analytical element coated on polyester support. This is an enzymatic method. A drop of sample (10µl) was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The Triton X-100 (TX100) surfactant in the spreading layer helps in the dissociation of cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol esters to cholesterol was catalyzed by cholesterol ester hydrolase. Free cholesterol was then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The density of the dye formed is proportional to the cholesterol concentration present in the sample and is measured by reflectance spectrophotometry. This colorimetric method requires 37°C

temperature and an incubation time of 5 minutes. The wave length used is 540 nm (Procedure manual, Ortho-Clinical Diagnostics, 2004).

Reaction sequence



Reagents

- Triton X-100 - 0.81mg
- Cholesterol oxidase - 0.4U (Nocardia or Cellulomonas)
- Cholesterol ester hydrolase - 2.0U (Pseudomonas)
- Peroxidase - 5.3 U (horseradish root)
- 2-(3, 5-dimethoxy-4-hydroxyphenyl)-4, 5-bis- (4-dimethylaminophenyl) imidazole (leuco dye) - 0.17mg.
- Pigments, binder, buffer, surfactants, stabilizers and cross-linking agent

2.17. Assay of Direct HDL Cholesterol

HDL (High Density Lipoprotein) cholesterol estimation was performed in chemistry autoanalyserVitros 5, 1 FS using the VITROS dHDL slides which is a multilayered, analytical element coated on polyester support (Procedure manual, Ortho-Clinical Diagnostics, 2004). The method was based on a non-HDL precipitation method followed by an enzymatic method. A drop of sample (10µl) was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. HDL was separated by the precipitation of non-HDL using phosphotungstic acid (PTA) and magnesium chloride ($MgCl_2$) in the spreading layer. The Emulgen B-66 surfactant in the spreading layer helps in the dissociation of cholesterol and cholesterol esters from HDL lipoprotein complexes present in the sample. Hydrolysis of the HDL- derived cholesterol esters to cholesterol was catalyzed by cholesterol ester hydrolase. Free cholesterol was

then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The density of the dye formed is proportional to the HDL cholesterol concentration present in the sample and was measured by reflectance spectrophotometry at 670 nm. This colorimetric method requires 37°C temperature and an incubation time of 5 minutes.

Reaction sequence

Reagents

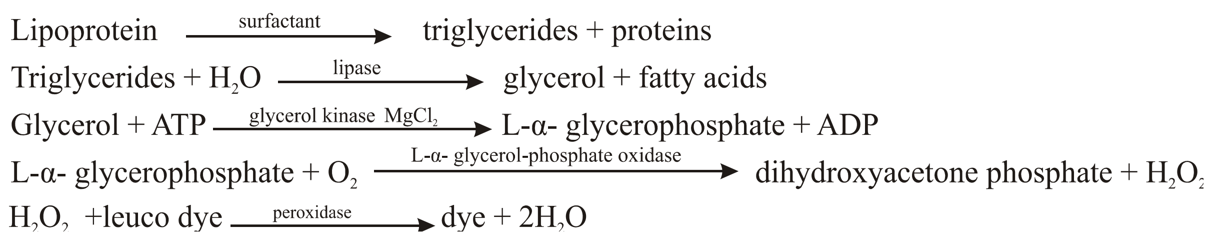
- Emulgen B-66 - 0.7mg
- Phosphotungstic acid - 0.3mg
- Magnesium chloride - 0.2mg
- Cholesterol oxidase - 0.8U (Cellulomonas)
- Cholesterol ester hydrolase - 1.2U (Candida rugosa)
- Peroxidase - 2.2 U (horseradish root)
- 2-(3,5-dimethoxy-4-hydroxyphenyl)-4, 5-bis- (4-dimethylaminophenyl)imidazole(leucodye) - 0.02mg.
- Pigments, binder, buffer, surfactants, stabilizers, scavenger and cross-linking agents.

2.18. Assay of Triglyceride

Triglyceride estimation was performed in chemistry autoanalyser Vitros 5, 1 FS using the VITROS TRIG slides which is a multilayered, analytical element coated on polyester support. This analysis was based on an enzymatic method. A drop of sample (5.5μl) was deposited on the slide and is evenly distributed by the spreading layer to the

underlying layers. The Triton X-100 (TX100) surfactant present in the spreading layer helps in the dissociation of triglyceride from lipoprotein complexes present in the sample. The triglyceride molecules then hydrolysed by lipase to yield glycerol and fatty acids. Glycerol diffuses to the reagent layer, where it was phosphorylated by glycerol kinase in the presence of ATP. In the presence of L-α- glycerol-phosphate oxidase, L-α-glycerophosphate was then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involves the oxidation of a leuco dye, catalyzed by peroxidase to produce a dye. The density of the dye formed is proportional to the triglyceride concentration present in the sample and was measured by reflectance spectrophotometry at 540 nm. This colorimetric method requires 37°C temperature and an incubation time of 5 minutes (Procedure manual, Ortho-Clinical Diagnostics, 2004).

Reaction sequence



Reagents

- Lipase - 0.15U (Candida rugosa)
- Peroxidase - 0.52 U (horseradish root)
- Glycerol kinase - 0.35U (Cellulomonas sp.)
- L-α- glycerol-phosphate oxidase - 0.19U (Pediococcus sp.)
- Triton X-100- 0.62 mg
- 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis-(4 dimethylaminophenyl) imidazole (leuco dye) - 0.04mg.
- Adenosine triphosphate - 0.14mg
- Pigments, binder, buffer, surfactants, stabilizers, scavenger, enzyme cofactor, dye solubilizer and cross-linking agents.

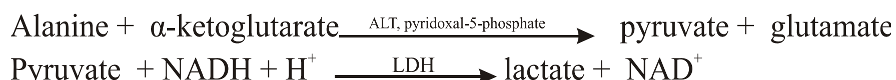
2.19. Estimation of LDL Cholesterol

Both indirect and direct methods were used to measure LDL (Low-Density Lipoprotein) cholesterol. Here we used the indirect method for the estimation of LDL cholesterol. Indirect method assumed that total cholesterol was primarily composed of cholesterol on VLDL, LDL and HDL. LDL cholesterol was then measured indirectly by using Friedewald equation. Here total cholesterol, triglyceride and HDL cholesterol were measured and LDL cholesterol was calculated from the primary measurement by using the empirical Friedewald equation: where all concentrations are given in milligrams per deciliter. The factor [Triglyceride]/5 is an estimate of VLDL cholesterol concentration and is based on the average ratio of triglyceride to cholesterol in VLDL

LDL cholesterol = Total cholesterol – HDL cholesterol – (Triglyceride/5)

2.20. Assay of Alanine Aminotransferase

ALT estimation was performed in Ortho-Clinical Diagnostics 5.1 Vitros instrument, using the VITROS ALT slides. The VITROS ALT slide is a multilayered, analytical element coated on polyester support. A drop of 11µl sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers and incubated at 37°C for 5 minutes. The spreading layer contains the ALT substrates L-alanine and sodium α-ketoglutarate. Alanine aminotransferase catalyzes the transfer of the amino group of L-alanine to α-ketoglutarate to produce pyruvate and glutamate. Lactate dehydrogenase (LDH) then catalyzes the conversion of pyruvate and NADH to lactate and NAD+. The rate of oxidation of NADH is monitored by reflectance spectrophotometry. The rate of change in reflection density is proportional to enzyme activity and is measured at 340nm (Procedure manual, Ortho-Clinical Diagnostics, 2004).



Reagents

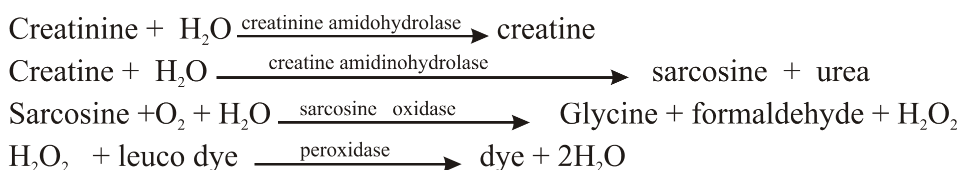
- Lactate dehydrogenase (Porcine muscle) – 0.12 U

- L-alanine - 0.86 mg
- Sodium α-ketoglutarate - 54µg
- Nicotinamide adenine dinucleotide, reduced - 35µg
- Sodium pyridoxal-5-phosphate - 11µg
- Pigments, binder, buffer, surfactants, stabilizers, and cross-linking agents.

2.21. Assay of Creatinine

Creatinine estimation was performed in Ortho-Clinical Diagnostics 5.1 Vitros instrument, using the VITROS CREA slides, and is a multilayered, analytical element coated on polyester support. A drop of sample (6µl) was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Creatinine diffuses to the reagent layer, where it was hydrolyzed to creatine in the rate determining step. The creatine was converted to sarcosine and urea by creatine amidinohydrolase. The sarcosine, in the presence of sarcosine oxidase, was oxidized to glycine, formaldehyde, and hydrogen peroxide. The final reaction involves the peroxidase- catalyzed oxidation of a leuco dye to form a colored product. Following addition of the sample, the slide was incubated at 37°C for 5 minutes. During the initial reaction phase, endogenous creatine in the sample was oxidized. The resulting change in reflection density was measured at 2 time points. The difference in reflection density is proportional to the concentration of creatinine present in the sample and was measure at 670nm (Procedure manual, Ortho-Clinical Diagnostics, 2004).

Reaction sequence



Reagents

- Creatineamidohydrolase (Flavobacterium sp.) – 0.20 U

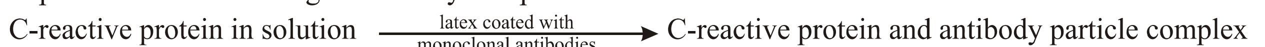
- Creatine amidinohydrolase (Flavobacterium sp.) – 4.7 U
- sarcosine oxidase (Bacillus sp.) – 0.55 U
- Peroxidase (Horseradish root) – 1.6 U
- 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis-(4-dimethylaminophenyl)imidazole(leucodye) - 32 μg.
- Pigments, binder, buffer, surfactants, stabilizers, scavenger, chelator, dye solubilizer, and cross-linking agents.

2.22. Assay of hsCRP

High sensitivity CRP was estimated by immunoturbidimetry in the open channel of VITROS 5.1 FS chemistry analyzer (Procedure manual, Ortho-Clinical Diagnostics, 2004). The quantitative measurement of C-reactive protein (CRP) was performed using the VITROS chemistry products hsCRP reagent in conjunction with the VITROS chemistry products calibrator Kit 17 and VITROS chemistry products FS calibrator 1 on Ortho-Clinical Diagnostics VITROS 5,1 FS chemistry system. hsCRP reagent is a dual chambered package containing ready to use liquid reagents. Samples, calibrators and controls are mixed with Reagent 1 containing a buffer. Addition of anti-CRP antibodies coupled to latex micro particles (Reagent 2) produces an immunochemical reaction yielding CRP antigen/antibody complexes. The turbidity was measured spectrophotometrically at 660 nm. Once a calibration has been performed for each reagent lot, the CRP concentration in each unknown sample can be determined using the stored calibration curve and the measured absorbance obtained in the assay of the sample. This is a two-point rate test type and is incubated at 37°C for 8 minutes. Sample volume used is 16 μl.

Step 1: Dilution of sample

Step 2: Formation of Antigen-antibody complex



Reagents

Reactive ingredients

- Reagent 1(R1): none
- Reagent 2 (R2): latex particles coated with anti- CRP mouse monoclonal antibodies 0.1% [w/w]

Other ingredients

- Reagent 1(R1): buffer, bovine serum albumin, polmer and preservative
- Reagent 2 (R2): buffer and preservative

2.23. Assay of Calcium

Calcium estimation was performed in Ortho-Clinical Diagnostics 5.1 Vitros instrument, using the VITROS Ca slides, which is a multilayered, analytical element coated on polyester support (Procedure manual, Ortho-Clinical Diagnostics, 2004). 10µl of sample was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The bound calcium was dissociated from binding proteins, allowing the calcium to penetrate through the spreading layer into the underlying reagent layer. There, the calcium forms a complex with Arsenazo III dye, causing a shift in the absorption maximum. After incubation at 37°C for 5 minutes, the reflection density of colored complex was measured spectroscopically. The amount of colored complex formed is proportional to the concentration of calcium present in the sample and was measure at 680nm.

$$Ca^{2+} + \text{Arsenazo III} \xrightarrow{\text{pH 5.6}} \text{Colored complex}$$

Reagents

- Arsenazo III dye 60 µg
- Pigment, binders, surfactants, buffer, cross-linking agent and mordant.

2.24. Estimation of Hemoglobin

Blood was diluted with Drabkin’s solution which consists of potassium cyanide and potassium ferricyanide. Potassium ferricyanide converts hemoglobin to methemoglobin, which was further converted to cyanomethemoglobin by potassium cyanide. The absorbance of the solution was measured using colorimeter at a wavelength of 540 nm.

$$\text{Hemoglobin concentration (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 60 \times 0.251$$

OR,

$$\text{Hemoglobin concentration (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 15$$

Take 5ml of Drabkins solution in a test tube and add 20 µl of blood sample. Mix well and incubate at room temperature for 5 minutes. Measure the absorbance of sample against reagent blank and measures the absorbance of standard directly against blank. The wavelength used is 540 nm. In blank Hemoglobin reagent of 5000µl was added, and in sample tube 5000 µl Hemoglobin reagent and 20 µl of blood sample was added.

Reagent composition

Hb reagent

- Potassium phosphate
- Potassium ferricyanide
- Potassium cyanide
- Sodium chloride

Hb Standard

- Cyano methhemoglobin concentration 60 mg/dl.

2.25. Assay of Troponin I

Troponin I is an automated quantitative test done by the VIDAS instruments for the determination of human cardiac troponin I in human serum or plasma using the ELFA (Enzyme-Linked Fluorescent Assay) technique. VIDAS Troponin I Ultra is intended to be used in the diagnosis of myocardial infarction (Procedure manual, VIDAS).

The Solid Phase Receptacle (SPR) serves as the solid phase as well as the pipetting device for the assay. Reagents for this assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps were performed automatically by the instrument. The sample was first transferred into the wells containing anti-cardiac troponin antibodies labeled with alkaline phosphatase (conjugate). The sample/conjugate mixture was then cycled in and out of the SPR several times and this operation enables the troponin I to bind with the immunoglobulins fixed to the interior wall of the SPR and the conjugate to form a sandwich. During washing unbound components were eliminated. Then two detection steps were performed successively. During each step, the substrate (4-Methyl-umbelliferyl phosphate) cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a product (4-Methyl-umbelliferone). The fluorescence of which is measured at 450 nm. The intensity of the fluorescence produced is proportional to the concentration of antigen present in the sample. At the end of the assay, the results were automatically calculated by the instrument in relation to two calibration curves stored in memory corresponding to the two detection steps. A fluorescence threshold value determines the calibration curve to be used for each sample and the results are then printed out.

2.26. Diagnostic criteria and Reference Intervals

Diagnostic criteria **for anemia** according to WHO criteria was **hemoglobin <130 g/l in males and <120 g/l in females** (Khusun et al, 1999). The cut off value of hemoglobin considered for exclusion of hypoxia in both males and females was **125 g/l** (Jelkmann, 2011). The lower cut off considered for deficiency of iron in this study was: iron <9.845 µmol/l (55 µg/dl) and ferritin <20 ng/ml.

Reference intervals for vitamin D metabolite, 1,25$(OH)_2$D are method dependent and is 15 to 60 pg/ml or 36 to 144 pmol/l. Lower limits of the reference interval for **25(OH)D** of 10 or 15 ng/ml (25 to 37 nmol/l) have been inappropriately low, as even above this limit the vitamin D status of the individual is insufficient (Lips et al, 2008). Vitamin D levels **less than 20 to 30 ng/ml (50 to 75nmol/l)** can be associated with increased serum PTH concentrations and reduced calcium absorption.

According to WHO, diagnostic criteria for **diabetes** was with **fasting plasma glucose ≥7 mmol/l (126 mg/dl)** or 2 hour postprandial or 2 hour post glucose load (75 g in 300 ml water for adults) glucose value ≥ 11.1mmol/l (200 mg/dl). The fasting plasma glucose for **Impaired Fasting Glucose (IFG)** was **6.1 to 6.9 mmol/l (110mg/dl to 125mg/dl)** or 2 hour postprandial plasma glucose (if measured) was <7.8mmol/l (140mg/dl). Fasting plasma glucose for Impaired Glucose Tolerance (IGF) was <7.0 mmol/l (126mg/dl) and 2 hour postprandial plasma glucose ≥7.8 and <11.1 mmol/l (140mg/dl and 200mg/dl) (Alberti and Zimmet, 1998).

According to American Diabetes Association (ADA) diagnostic criteria for **diabetes** is same as that of WHO criteria. The fasting plasma glucose for **Impaired Fasting Glucose** was **5.6 to 6.9mmol/l (100 mg/dl to 125 mg/dl).** For Impaired Glucose Tolerance (IGF) the 2 hour postprandial plasma glucose concentration was 7.8-11.0 mmol/l (140 mg/dl and 199 mg/dl) (ADA, 2016).

BMI and waist circumference were defined by the revised criteria for Asian Indians (WPRO.WHO, 2000; Misra et al.2009) as underweight BMI of <18.5kg/m^2, normal range of 18.5 - 22.9kg/m^2, overweight of 23 - 24.9kg/m^2, obese I of 25 - 29.9kg/m^2 and obese II ≥30kg/m^2 for both males and females. Individual with waist circumference ≥ 90 cm in males and ≥80cm in females was considered to have abdominal obesity.

Reference intervals used for the healthy limits in this study were PTH: 10 - 65 ng/l (Roberts et al, 2012), EPO: 4 – 27 U/L (Kratz et al, 2015); 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) (Kratz et al, 2015); Ferritin: Male 29 – 250 ng/ml, Female 10 – 150 ng/ml (Kratz et al, 2015; Roberts et al, 2012).

2.27. Statistical Analysis

Statistical analysis and calculations were done with SPSS, version 23.0 software, Minitab and Medcalc software. Normality of distribution was estimated by graphical and numerical tests. Graphical tests used were histogram, Box-Whisker Plot and normal quantile-quantile plot. Normality of distribution was numerically estimated by Kolmogorov-Smirnova and Shapiro-Wilk test, Anderson - Darling normality test and D'Agostino – Pearson tests. Equality or homogeneity of variances of the groups compared was done by Levene's test. Log_{10} transformations converted most of the positively skewed groups to Gaussian distribution and were of equal variances in the groups compared. 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 significant differences of variables of different groups were compared by 95% confidence interval of mean, two-tailed Student's t test, and by non parametric Mann Whitney U test. (Linnet and Boyd, 2012; Riffenburgh, 2012; Altman, 1991). The relationship between two variables was done by Pearson correlation, only if one of the variables had Gaussian distribution otherwise non parametric Spearman's Rank correlation was done. Correlation between the two variables was plotted graphically by X-Y scatter plot and was used to visually verify the correlations.

Table 2.1. Number of participants (n) after applying exclusion criteria for selection of sample population for this study and partitioning of samples according to age and gender.

Exclusion at various stages for selection of reference sample population			**Sample number at various phases of clinical exclusion**		
			Total n	**Male n**	**Female n**
Stage I (1 to 2)	**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)	411	187	224
Stage II (2 to 3)	**3.**	Samples selected after exclusion of subclinical disease states by Clinical Biochemistry evaluation, and after **exclusion of BMI ≥30, WC ≥100 cm, Fasting glucose >126mg/dl, 2hr Glucose >180mg/dl, ALT>60U/L, Tg>200mg/dl and hsCRP>5mg/l.**	302	116	186
Stage III (3 to 4)	**4a.**	Participants after **exclusion of** growth phase **<18 years** of age	285	105	180
	4b.	Participants after **exclusion of <18 years and >50 years** of age (18 -50 years of age)	263	88	175
	4c.	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 (4c to 5 6, 7 and 8)	**5.**	**EPO samples selected**	159	49	110
	6.	**PTH samples selected**	142	40	102
	7.	**C peptide samples selected**	102	27	75
	8.	**Insulin samples selected**	146	42	104

Table 2.2. Conversion factors for interconversion between conventionl unit and SI unit.

Analytes	Conventional Unit	Conversion factor	SI unit
EPO	mIU/ml	1.0	U/L
Hemoglobin	g/dl	10	g/l
Iron	µg/dl	0.179	µmol/l
Intact PTH	pg/ml	1.0	ng/l
Calcium	mg/dl	0.25	mmol/l
Vitamin D (25 – hydroxyl vitamin D)	ng/ml	2.496	nmol/l
Glucose	mg/dl	0.0555	mmol/l
Insulin	µIU/ml	6.945	pmol/l
C peptide	ng/ml	0.333	nmol/l
Cholesterol	mg/dl	0.0259	mmol/l
Creatinine	mg/dl	88.4	µmol/l
Triglycerides	mg/dl	0.0113	mmol/l
hsCRP	mg/dl	10	mg/l
ALT	Units/L	1.0	U/L
Troponin I	ng/ml	1.0	µg/l

NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 3.

Gender Differences in the Correlations of Erythropoietin with Related Analytes, and their Concentrations in Healthy Young Adults

3.1. Abstract

Regulation of the synthesis of erythropoietin (EPO) by kidneys and absorption of iron by intestine are caused by hypoxia and iron levels. In males, hemoglobin is increased by androgens; in females, hemoglobin, iron and ferritin are decreased by monthly blood loss leading to increase in EPO. EPO increases erythropoiesis to overcome hypoxia. Differences in the concentration of hemoglobin, iron and ferritin influence EPO. The research problems in this section were the influences of these factors and deficiencies which lead to gender differences in EPO and its correlation with the related variables. Participants of this observational cross sectional study, aged 18 to 25 years, from the near sea level plains of rural Central Kerala were evaluated clinically and from quantitative biochemical variables with cut off fixed to include anemia, but exclude other primary and secondary clinical conditions, to obtain the EPO study sample (n = 159). Comparison of the concentrations and correlations of EPO were done with parametric and nonparametric methods. As there were gender differences in EPO, hemoglobin, iron and ferritin, sample was partitioned into males and females. In the female sample, EPO showed strong negative correlation with hemoglobin, iron and ferritin before and after exclusion of anemia/hypoxia. But in the male sample, EPO showed a moderate correlation only with iron before and after exclusion of anemia. After excluding hemoglobin <125g/l, there was no gender difference in EPO levels (P = 0.105) but there were differences when WHO criteria for anemia was used as cut off. Strong gender differences were observed in the negative correlations of EPO with hemoglobin, iron and ferritin in females, with weak correlations in males. The residual correlation of EPO with iron in males, after exclusion of hypoxia/anemia indicated that in addition to hypoxia, iron deficiency also increased EPO.

3.2. Introduction

Erythropoietin production by the peritubular fibroblasts in renal cortex is stimulated by general hypoxia which is sensed by the kidneys through HIF-2 (Mujais et al, 1999; Fisher, 2003). Major contribution of erythropoietin levels in blood plasma is by the kidneys (Mujais et al, 1999), and may be in minor amounts by liver and brain (Fisher, 2003). 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 (Koury & Bondurant, 1998; Manalo et al, 2005). EPO modulates the erythroid progenitor maturation and proliferation (Manalo et al, 2005; Quigley et al, 2014), microenvironment of bone marrow for erythropoiesis (Yamashita et al, 2008) and regulates components of heme synthesis pathway (Hofer et al, 2003; Liu et al, 2004). It takes 3 to 4 days for the reticulocyte counts to increase following the rise of plasma EPO (Jelkmann, 2011).

The higher hemoglobin and RBC counts in men when compared to women are from the augmentation of erythropoiesis by androgens and inhibition by oestrogens (Murphy, 2014). Consequently, will the gender differences in hemoglobin levels inversely affect EPO levels, leading to higher EPO in women? The gender differences of hemoglobin levels are further increased by the monthly loss of blood in women, leading to decrease in hemoglobin, iron and ferritin. Investigating the influences of these on EPO levels in the clinical setting is part of the research problem of this study.

There are major differences in the oxygen sensing mechanism and EPO expression in the hepatic and renal systems: Foetal 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 (Koury et al, 1989) while the hepatic system may respond in a more graded way (Naughton et al, 1984); 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 (Rankin et al, 2007; Haase, 2010). 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 (Bruegge et al, 2007).The proline hydroxylated HIF-α combines with von Hippei-Lindau tumour suppressor protein (VHL) and undergoes proteosomal degradation (Maxwell et al, 1999).PHD-2 and PHD-3 are themselves HIF-target genes, their expression increases and HIF-α levels decline during long term hypoxic periods (Stiehl et al, 2006).This feedback regulation may explain the declining EPO production during chronic anemia 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 hemoglobin, iron and ferritin to EPO production. In healthy young adults at rest, systemic hypoxia may be related to anemia, especially in women. **Is there still an inverse correlation between EPO and the three related variables even after exclusion of anemia?** The second aspect 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 (Peyssonnaux et al, 2008). HIF-2α with PHDs is emerging as potential **iron sensors in the intestinal mucosa. DMT1 and Dcytb** are highly up regulated by both iron deficiency and hypoxia in duodenum, mediated by HIF 2α. **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(Mastrogiannaki et al, 2009; Shah et al, 2009; Yeh et al, 2000; Simpson & McKie, 2009).

Systemic anemia/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 anemia and iron deficiency leading to increased negative correlations of EPO with hemoglobin, iron and ferritin.

3.3. Objectives

3.3.1. Aspects of the Original Objectives Addressed in this Chapter

EPO is a cytokine secreted by kidney, involved in a number of regulatory functions. Therefore, disease states that influence EPO may cause variations in factors that are regulated directly or indirectly by EPO. In this study, we examine

1. Correlation, gender differences and variations in concentrations of EPO with other analytes in healthy individuals.

2. Influences on such correlations and significant variations in disease conditions.

3.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. **EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine**

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment.

3.4. Materials and Methods

3.4.1. Study Setting, Selection of Participants, Inclusion and Exclusion criteria

Please refer study setting, selection of participants, and inclusion and exclusion criteria in Chapter 2 (2.1 and 2.2).

3.4.2. Stages and Steps of EPO Sample Selection by Exclusion of Subclinical Disease States and Deficiencies by Clinical Biochemistry Assays

Informed written consent was obtained from each participant. **In stage I**, volunteers for the study (n > 600) 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. **At stage II,** Clinical Biochemistry laboratory evaluation was done in the sample after exclusion at stage I (n = 411) further exclusion of unhealthy individuals at the subclinical level and to permit inclusion of individuals with anemia 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, anemia and iron deficiency were included in the study. **At stage III**, the samples were partitioned according to age, as <18 years (**Stage IIIa**), 18 – 50 years (**Stage IIIb**) and 18 – 25 years (**Stage IIIc**). **At stage IV**, The lower cut off levels for exclusion of samples with EPO was <3.5 IU/L. After exclusion at stage IV, the sample population was considered as the EPO sample (n = 159) and used in this study. At this stage hemoglobin, iron and ferritin deficient samples were not excluded in this study. **At stage V,** the cut off

levels for exclusion of samples with anemia according to WHO criteria was hemoglobin <130 g/l in males and <120 g/l in females (Khusun et al, 1999). The cut off value of hemoglobin considered for exclusion of hypoxia in both males and females was 125 g/l (Jelkmann, 2011).The lower cut off considered for deficiency of iron were: iron <9.845 μmol/l (55 μg/dl) and ferritin <20 ng/ml (Table 3.1).

3.4.3. Sample Collection and Sample Preparation

Please refer 2.3 for Sample collection in Chapter 2

3.4.4. Reference Intervals Used in this Study

Reference intervals used for the upper healthy limits in this study were EPO: 4 – 27 U/L; Hemoglobin: male 133 – 162 g/l, female 120 – 158 g/l; Iron: 7 – 25 μmol/l (41 – 141 μg/dl); Ferritin: Male 29 – 250 ng/ml, Female 10 – 150 ng/ml (Kratz et al, 2015; Roberts et al, 2012).

3.4.5. 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. Hemoglobin: (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.

3.4.6. Assays 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 (Procedure manual, Beckman Coulter, 2005). The chemistry autoanalyser 5,1 FS, was used for assay of glucose, triglycerides, total cholesterol, HDL cholesterol, serum creatinine, iron and hsCRP (Procedure manual, Ortho Clinical Diagnostics, 2004). Hemoglobin 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. **Please refer detailed description of assays in Chapter 2.**

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 (Procedure manual, Beckman Coulter, 2005).

Daily continuous internal quality control data were analysed according to Westgard rules for acceptance or rejection of analyte data (Klee and Westgard, 2012). If there is a rejection, appropriate measures were taken to set right errors in machine functioning, reagents or calibration levels.

3.4.7. Diagnostic Criteria

Please refer 2.26. Diagnostic criteria and reference interval section in chapter 2.

3.4.8. 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 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 (Altman, 1991).

3.5. Results

3.5.1. Selection of Participants

Selection of participants was done in several steps. The volunteers who were willing to take part in the study were clinically examined and clinical history was taken (Table 3.1, Stage I, Step 1). After exclusion of participants by clinical history and examination (Steps 1 to 2), blood samples were taken from the selected participants for Clinical Biochemistry evaluation for exclusion of diseases at the subclinical level using cut off levels of quantitative biochemical analytes (Stage II). Influence of age and gender increased biological variations and heterogeneity in a healthy sample. The influence of age was decreased to a minimum by selecting young healthy adults aged 18 to 25 years. Participants were partitioned according to age and gender (Stage III). The strict exclusion criteria reduced the EPO sample size to 159 (Table 3.1; Stage IV) from the starting number >600 (Stage I) and further reduced during exclusion of deficiencies to 69 (Stage V, Step 8; male, n = 39; female, n = 30). Exclusions among the male participants from subclinical disease states were more at Stage I and II, and that in female participants from deficiencies were more at Stage V, Steps 6 to 8 (Table 3.1). The EPO sample used in this study was from Stage IV (n = 159).

3.5.2. General Characteristics, Shapiro-Wilk Test for Normality, Levene's test for Equality of Variances and Gender Differences of Variables Used for Sample Selection

After selection of participants at Stage IV, the range (minimum - maximum) of BMI, waist circumference, fasting glucose, 2 h. OGTT glucose, alanine aminotransferase, total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, serum creatinine and hsCRP were within the specified cut off levels (Table 3.2).

As most of the samples were positively skewed, $\log_{10}$ transformation was done before evaluating the distribution by Shapiro Wilk test and Levene's test. Shapiro Wilk test showed that serum creatinine did not have Gaussian distribution in both male and female samples (Table 3.2A). In the male sample, HDL cholesterol was not normally

distributed. All other samples had Gaussian distribution in the male sample. In the female sample, BMI, fasting glucose, ALT and hsCRP did not Gaussian distribution.

Equality of variance of the male and female sample was compared by Levene's test after $\log_{10}$ transformation. HDL cholesterol did not have equality of variance in the male and female samples. All other analytes had equality of variance in males and females.

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 alanine aminotransferase (P = 0.001), triglycerides (P = 0.03), HDL cholesterol (P = 0.001) and serum creatinine ($P < 0.001$) (Table 3.2A). These gender differences were also confirmed by 95% CI of mean. These were the expected gender differences due to increased insulin resistance in males (ALT and TG), lower HDL in males and increased muscle mass in males (serum creatinine).

3.5.3. General Characteristics, Shapiro-Wilk test for Normality, Levene's test for Equality of Variances and Gender Differences of EPO and Variables related it

Deficiencies of hemoglobin, iron, ferritin and increased EPO were permitted in the sample to include subclinical conditions related to EPO, hemoglobin and iron metabolism. EPO and analytes related to it showed that the mean and 95% CI of mean were within the reference range. But there were values outside the cut off for EPO, hemoglobin, iron and ferritin in both males and females. There were EPO values above the reference range in females (Table 3.2B and 3.3).

After $\log_{10}$ transformation, EPO and serum iron did not have Gaussian distribution both in male and female samples. In addition, in the female sample, hemoglobin did not have Gaussian distribution.

Levene's test for equality of variance showed that iron and ferritin did not have equality of variance but EPO and hemoglobin had equal variance.

When the data distribution was not Gaussian, attempts were made to convert the distribution to Gaussian by log_{10} transformation. There were gender differences seen in the concentrations EPO and all the parameters related to it. Student t test or non parametric Mann Whitney U test of EPO (P = 0.006), hemoglobin ($P < 0.001$), iron ($P < 0.001$), ferritin ($P < 0.001$) showed significant differences (Table 3.2B). These gender differences were also confirmed by 95% CI of mean. The gender differences were contributed by androgens which increased hemoglobin in males (Murphy, 2014; Khusun et al, 1999) and monthly blood loss which decreased hemoglobin, iron and ferritin, increased EPO in females.

In the EPO 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 EPO and Iron, the female hemoglobin samples did not have Gaussian distribution and could not be transformed to Gaussian distribution (Table 3.2B).

3.5.4. Visual Analysis of EPO Distribution at Various Stages of Sample Selection

(i) Participants from whom blood samples were taken at Stage I, Step 2 (n = 411)

Visual analysis of EPO distribution at various stages of sample selection was analysed. Histogram of the total EPO sample at Stage I, Step 2 (n = 411) showed a positively skewed appearance; Normal Q-Q plot showed a distribution away from Gaussian distribution and Box- Whisker plot showed 12 far outliers and 18 near outliers (Fig. 3.1, Table 3.3). Histogram of EPO in male (Fig. 3.2) and female (Fig. 3.3) samples showed positively skewed appearance and Normal Q-Q plot showed a non-Gaussian distribution. The male samples had 3 far outliers and 8 near outliers, while, the female samples had 6 far outliers and 14 near outliers (Table 3.3).

(ii) EPO sample selected for this study at Stage IV, Step 5 (n = 159)

Histogram of the total EPO sample at Stage IV, Step 5 (n = 159) showed a positively skewed appearance; Normal Q-Q plot showed a distribution away from Gaussian distribution and Box- Whisker plot showed 4 far outliers and 8 near outliers (Fig. 3.4). Male population (n = 49) in the same sample showed (Fig. 3.5) a histogram with positively skewed appearance, and the Normal Q-Q plot showed a non-Gaussian distribution. The male samples had 0 (zero) far outliers and 3 near outliers. The same male sample after $\log_{10}$ transformation (Fig. 3.6) showed a near normal distribution and had no outliers. Partitioning, exclusion criteria and $\log_{10}$ transformation converted a positively skewed male sample to Gaussian distribution.

The histogram of the female sample (n = 110) did not have Gaussian distribution (Fig. 3.7; Stage IV, Step 5). Normal Q-Q plot showed a distribution that was not Gaussian. There were 3 far and 6 near outliers. The same female sample after $\log_{10}$ transformation (Fig. 3.8) showed a near normal distribution and had 3 near and no far outliers. Partitioning, exclusion criteria and $\log_{10}$ transformation converted a positively skewed female sample to near Gaussian distribution (Table 3.3).

(iii) Sample selected after exclusion of deficiencies at Stage V, Step 8 (n = 69)

Histogram of the male EPO sample at Stage V, Step 8 (n = 39) showed a positively skewed appearance; Normal Q-Q plot showed a distribution away from Gaussian distribution and Box- Whisker plot showed no far and 2 near outliers (Fig. 3.9). The same male sample after $\log_{10}$ transformation (Fig. 3.10), showed a normal distribution and had no outliers. Partitioning, exclusion criteria and $\log_{10}$ transformation converted a positively skewed male sample to Gaussian distribution.

Histogram of the female EPO sample at Stage V, Step 8 (n = 30) showed a near normal distribution; Normal Q-Q plot showed a near Gaussian distribution and Box-Whisker plot showed no far and 3 near outliers (Fig. 3.11). The same male sample after $\log_{10}$ transformation (Fig. 3.12) showed a normal distribution and had only two near outliers. Partitioning, exclusion criteria and $\log_{10}$ transformation converted a positively skewed female sample to Gaussian distribution.

3.5.5. Shapiro-Wilk test for Normality at Various Phases of Sample Selection and Partitioning

As subclinical disease states and deficiencies were excluded and partitioning according to gender and age were done in the sample, the sample was becoming more and more Gaussian distributed and as a consequence outliers were also decreased (Table 3.3). These selection procedures decreased the secondary multiple influences in the sample and brought out the more correct statistical relationships.

Similarly, partitioning improved the Gaussian distribution in the sample and decreased the number of outliers. The male sample partitioned at Stage IV becomes Gaussian distributed by K-S method and the female sample at Stage IV was without outliers. These changes to Gaussian distribution were at an earlier stage than in the total EPO sample and were seen in the total sample without partitioning only at stage V.

3.5.6. Correlations of EPO with Analytes Related to It

In the EPO sample, EPO correlated with all the three variables related to it (Table 3.4A.). In the male sample, EPO had negative correlation with only iron ($P = 0.04$) (Table 3.4B.). But in the female sample, EPO negatively correlated with ferritin, iron ($P < 0.001$) and hemoglobin ($P = 0.001$). In general, correlations were stronger in the female sample due to iron and ferritin deficiencies and anemia (Table 3.4C.).

X-Y scatter diagrams with EPO showed negative correlations which were visually observed to be least in males (Fig. 3.13A to C) and better in females (Fig. 3.13D to F). In the female sample, maximum negative correlation was seen with ferritin and least with hemoglobin. These visual analyses of X-Y scatter diagrams (Fig. 3.13) confirmed the estimation of the correlations (Table 3.4).

3.5.7. Gender Difference of EPO and Its Related Analytes after Excluding Anemia

Exclusion of anaemic/hypoxic samples was done by removing hemoglobin <125g/l in both males and females, assuming that HIF-2 was equally sensitive to hypoxia in males and females (Jelkmann, 2011). There was decreased concentration of

hemoglobin, iron and ferritin in the female sample (*P* <0.001) (Table 3.5A). These decreased levels in females did not cause a difference of EPO in the male and female samples (P = 0.105). These results indicate that a confounding factor is increasing EPO levels in males despite higher hemoglobin. This confounding factor may be increased muscular activity and muscle mass in young adult males leading increased hypoxia.

But when the exclusion of anemia was according to the cut off of hemoglobin proposed by WHO for males (130g/l) and females (120g/l) (Khusun et al, 1999), there was a difference in the level of EPO (P = 0.04). This difference is due to inclusion of 18 samples in females by a lower cut off at 120g/l. When the cut off for hemoglobin 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 3.5B).

When all EPO related deficient analytes involved in iron metabolism were excluded, there was no gender difference in EPO but there were gender differences in hemoglobin, iron and ferritin, with lower levels in females (Table 3.6).

3.5.8. Correlations of EPO and Its Related Analytes after Excluding Anemia

To study the contribution of anemia/hypoxia on the negative correlations of EPO, hemoglobin <125 g/l were excluded. There was no correlation of EPO with hemoglobin and ferritin in the male sample but correlated with hemoglobin (P = 0.028) and ferritin (*P*<0.001) in the female sample. EPO correlated with iron in the male (P = 0.024) and female samples (*P* <0.001), indicating greater iron deficiency in females (Table 3.7A, male and female). When the exclusion of hemoglobin was according to WHO criteria, there was no noticeable variation in the correlation with EPO (Table 3.7B, male & female).

After excluding hemoglobin <125 g/l, iron <9.85 μmol/l and ferritin <20 ng/ml (n = 69), EPO did not correlate with any of the three analytes in males, but correlated with hemoglobin and ferritin in females (Table 3.7C).

3.6. Discussion

EPO shows a diurnal variation with a nadir in the morning (Jelkmann, 2011). 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.

3.6.1. Correlations of EPO

Correlation between two variables arise when one increases or decreases in relation to the other. As an example, hemoglobin, iron or ferritin deficiency increased EPO and they gave rise to negative correlations with EPO (Table 3.4). Correlations may be decreased or lost when there were no deficiencies of hemoglobin, iron or ferritin.

Female sample had lower hemoglobin, iron and ferritin than the male sample (Table 3.2). Lower the hemoglobin, 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.

3.6.2. Gender Differences in the Correlations of EPO

The correlations of EPO with hemoglobin, iron and ferritin were stronger in females (Table 3.4) due to deficiency of hemoglobin, 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 (Fig. 3.13).

Gender differences in the correlations with EPO were due to anemia in females and androgens increasing hemoglobin in males (Murphy, 2014) resulting in higher hemoglobin cut off for anemia in males (130g/l) as compared to females (120g/l). EPO levels increase exponentially when hemoglobin levels decreased below 125 g/l (Jelkmann, 2011; Stiehl et al, 2006).

3.6.3. Residual Gender Differences and Correlations of EPO after Exclusion of Anemia/Hypoxia

When the samples with hemoglobin below 125 g/l were excluded to remove effects of hypoxia/anemia on EPO, there were no gender differences in EPO despite decreased hemoglobin, iron and ferritin in the female sample (Table 3.5A). 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 hemoglobin in anemia were used in males and females (Khusun et al, 1999) to exclude anemia, there were gender differences in EPO, hemoglobin, iron and ferritin. Hemoglobin level was increased in males and this is further supported by the higher maximum and mean of hemoglobin in males (Table 3.5B).

The consequent residual negative correlations of EPO with these variables were more in the female sample due to lower hemoglobin, iron and ferritin (Table 3.7). 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 hemoglobin. 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 (Haase, 2010) in the short term may explain the inverse relationship of hemoglobin, iron and ferritin to EPO production.

3.6.4. Strength, Limitation and Controversies of this Study

The clinical demonstration of the influence of iron on EPO independent of hypoxia/anemia and the gender differences in the correlations with EPO were the strong aspects of this work. But the lack of gender differences in EPO when hemoglobin 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 anemia in the female sample (Khusun et al, 1999).

3.7. Conclusion and Significance

There were gender differences in the correlations of EPO with hemoglobin, iron and ferritin with strong negative correlations in females. The residual correlation of EPO with iron in males, after exclusion of subclinical hypoxia/anemia, 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 hemoglobin <125g/l, iron <9.85 µmol/l and ferritin <20 ng/ml, then a more healthy reference interval for EPO can be determined.

Table 3.1. **N**umber of participants (n) after applying exclusion criteria for selection of the EPO sample population and partitioning of samples according to age and gender.

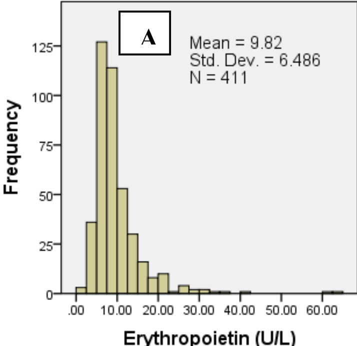
Stages	Steps	Exclusion for selection of reference EPO sample population	Sample number at various phases of clinical exclusion		
			Total, n	**Male, n (% males excluded at each stage)**	**Female, n (% females excluded at each stage)**
I (1 to 2)	**1.**	Participants before exclusion by **clinical history and examination**	>600	~300 (100%)	~300 (100%)
	2.	Participants after exclusion at step 1 and from whom **fasting blood and urine samples** were taken for assays (all age groups)	411	187 (113/300 = 37.67%)	224 (76/300 = 25.33%)
II (2 to 3)	**3.**	EPO samples selected after exclusion of subclinical disease states by **Clinical Biochemistry evaluation**, and after exclusion of **BMI ≥30, WC ≥100 cm, Fasting glucose >126, 2hr Glucose >180, ALT>60, TG>200 and hsCRP>5.**	302	116 (71/187 = 37.97%)	186 (38/224 = 16.96%)
III (3 to 4)	**4a.**	Participants after exclusion of **growth phase <18 years of age**	285	105 (11/116 = 9.48%)	180 (6/186 = 3.23%)
	4b.	Participants after exclusion of <18 years and >50 years of age **(18 - 50 years of age)**	263	88 (28/116 = 24.14%)	175 (11/186 = 5.91%)
	4c.	After exclusion of growth phase <18 years and the influence of age >25 years **(aged 18 to 25 years)**	**199**	**71** (45/116 = 38.79%)	**128** (58/186 = 31.18%)
IV (4c to 5)	**5.**	After exclusion of EPO <3.5 IU/l, and sample without EPO, HB, Iron and ferritin (age 18 -25 years)	159	49 (22/71 = 30.99%)	110 18/128 = 14.06%)
V (5 to 6 and 7)	**6.**	EPO samples after excluding HB <125 g/l	120	46 (3/49 = 6.12%)	74 (36/110 = 32.73%)
	7.	EPO samples after excluding HB in males <130 g/l, females <120 g/l	137	45 (4/49 = 8.16%)	92 (18/110 = 16.36%)
	8.	EPO sample after excluding HB <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml	69	39 (10/49 = 20.41%)	30 (80/110 = 72.73%)

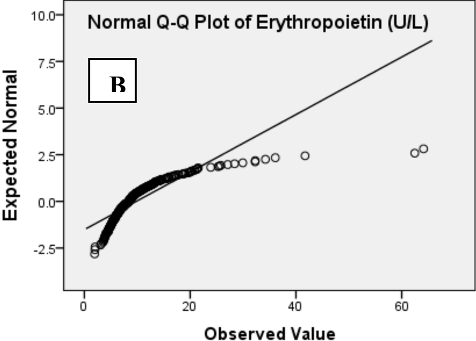
Table 3.2. General characteristics of variables used for sample selection (A) and that of EPO and variables related to EPO (B) (n = 159; male, n = 49; female, n = 110). Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed. The gender differences were compared by 95% CI of mean, Student t test (parametric method) and Mann Whitney U test (non parametric method).

Variables in SI units	EPO sample (n = 159) Mean±SD, Minimum – Maximum, 95% CI of mean	Comparison of male and female in EPO sample					
		Male (n = 49) Mean±SD Minimum – Maximum 95% CI mean	Female (n = 110) Mean±SD Minimum – Maximum 95% CI mean	after $\log_{10}$ transformation			
				Shapiro-Wilk Test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
				Male	Female		
A. Clinical Biochemistry Analytes used in exclusion criteria and sample selection							
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
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)
B. EPO and Clinical Biochemistry Analytes related to EPO							
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 Hemoglobin (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

Fig. 3.1. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **sample population (n = 411)** of individuals of all age groups **after exclusion by clinical history and examination at Stage I, Step 2.**





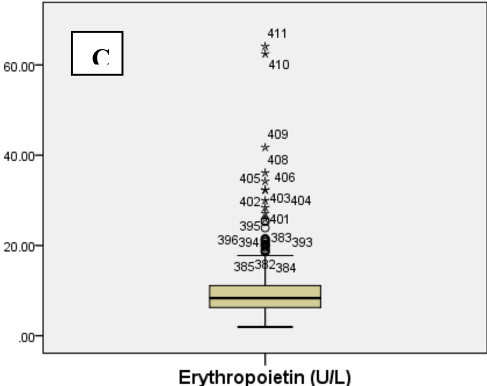


Fig. 3.2. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in **male sample population (n = 187)** of individuals of all age groups after **exclusion by clinical history and examination at Stage I, Step 2.**

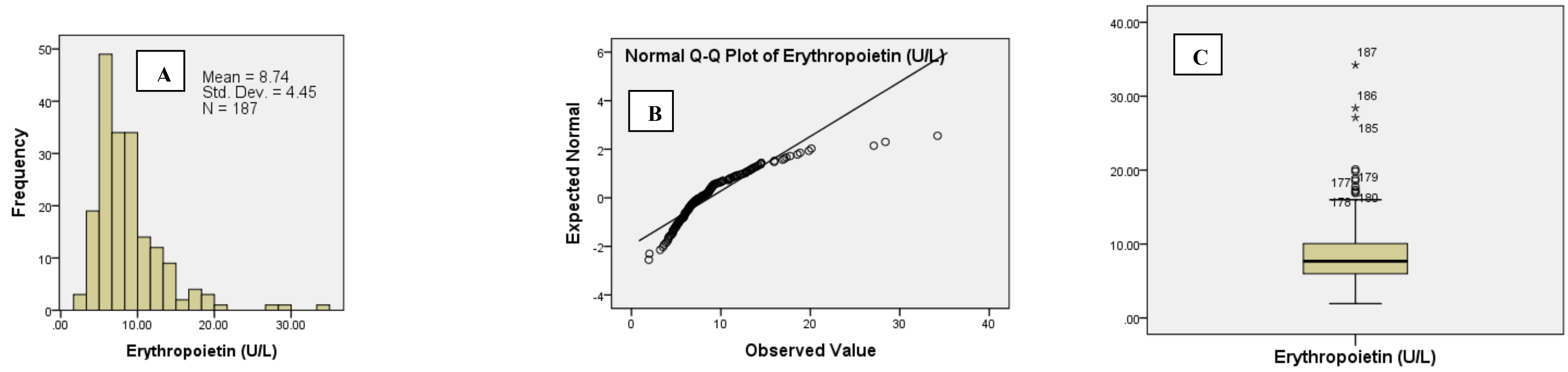


Fig. 3.3. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in **female sample population (n = 224)** of individuals of all age groups after **exclusion by clinical history and examination at Stage I, Step 2**.

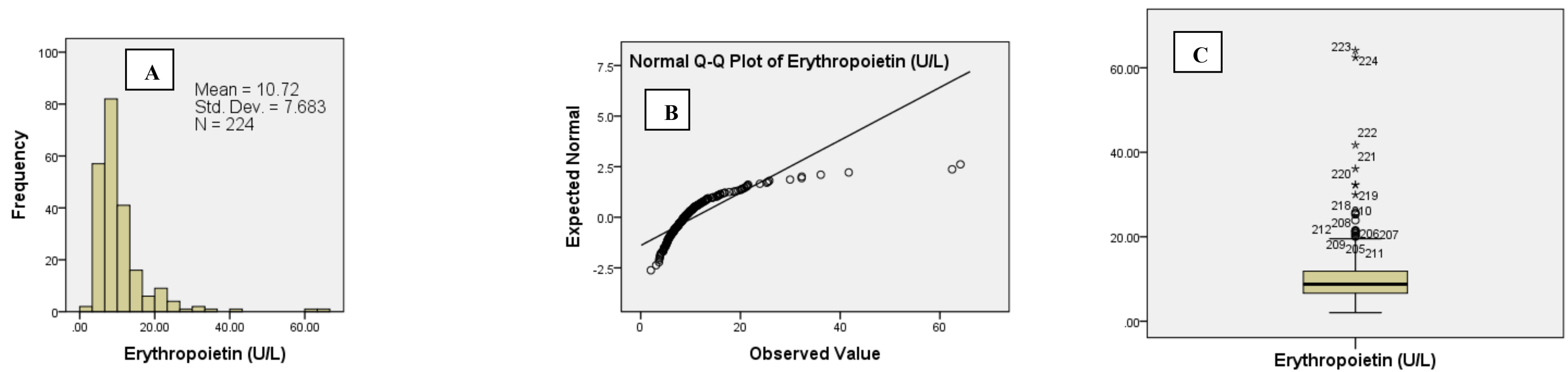
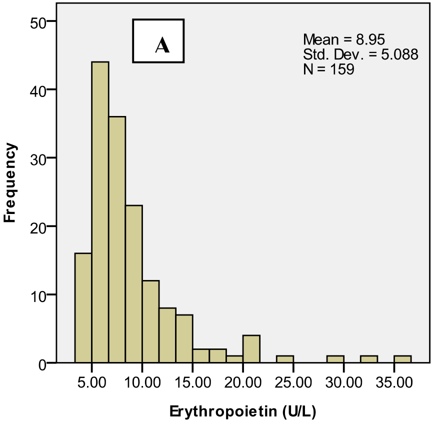
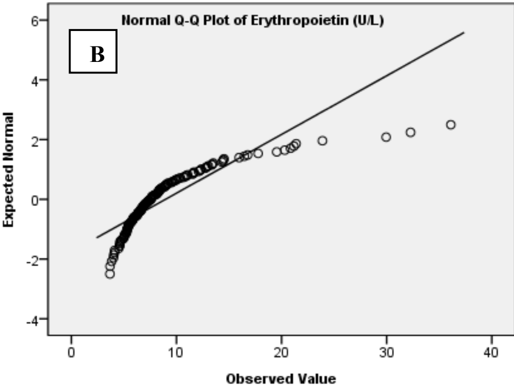


Fig. 3.4. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **EPO sample population (n = 159)** aged **18 to 25 years** after **exclusion by Clinical examination and Clinical Biochemistry assays at Stage IV, Step 5.**





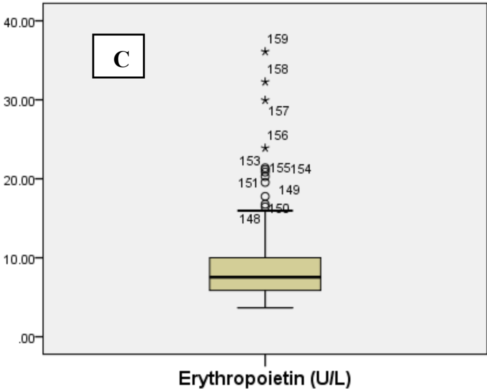


Fig. 3.5. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **male EPO sample population (n = 49)** aged **18 to 25 years** after exclusion by **Clinical examination and Clinical Biochemistry assays at Stage IV, Step 5.**

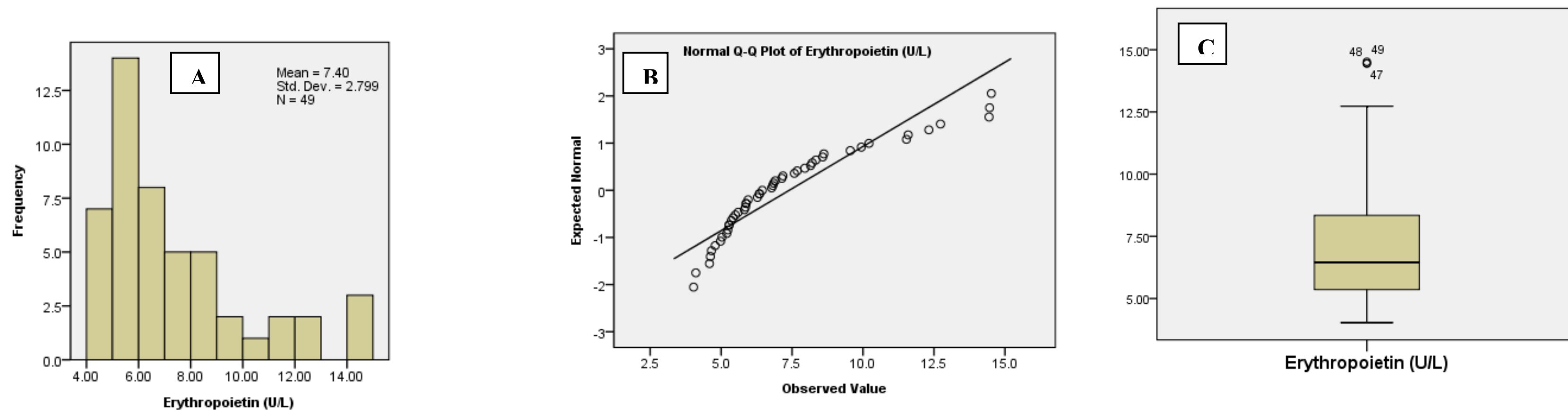


Fig. 3.6. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of **$\log_{10}$ transformed** EPO concentrations in the **male EPO sample population (n = 49)** aged **18 to 25 years** after exclusion by **Clinical examination and Clinical Biochemistry assays at Stage IV, Step 5.**

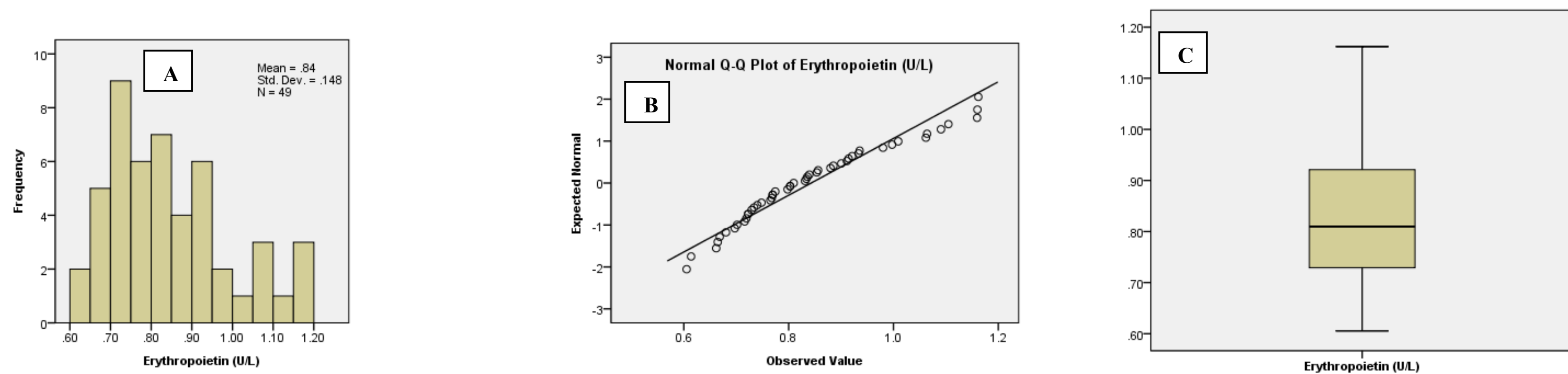


Fig. 3.7. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **female EPO sample population (n = 110)** aged **18 to 25 years** after exclusion by **Clinical examination and Clinical Biochemistry assays at Stage IV, Step 5.**

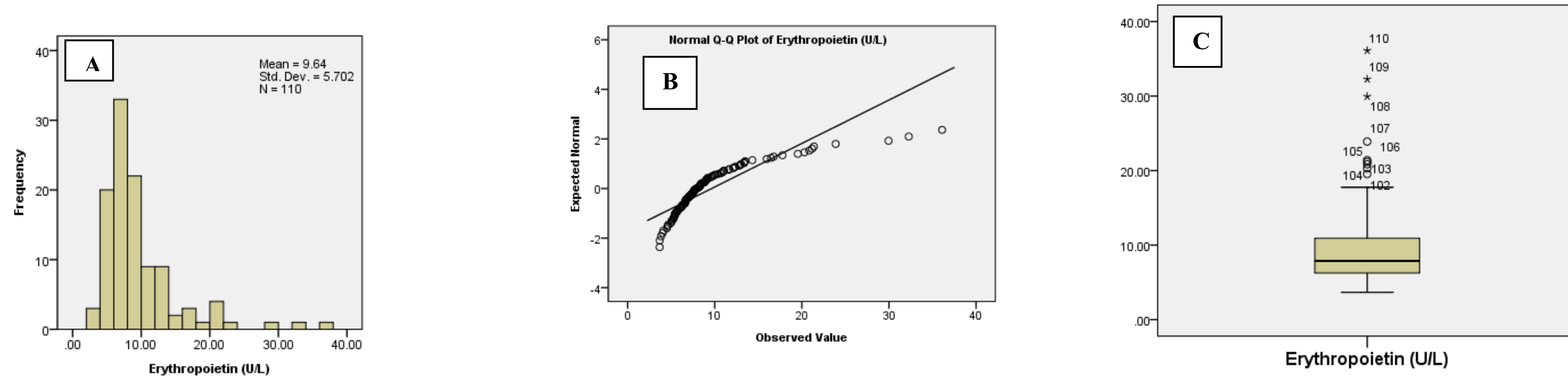


Fig. 3.8. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of **$\log_{10}$ transformed** EPO concentrations in the **female EPO sample population (n = 110)** aged 18 to 25 years after exclusion by **Clinical examination and Clinical Biochemistry assays** at **Stage IV, Step 5.**

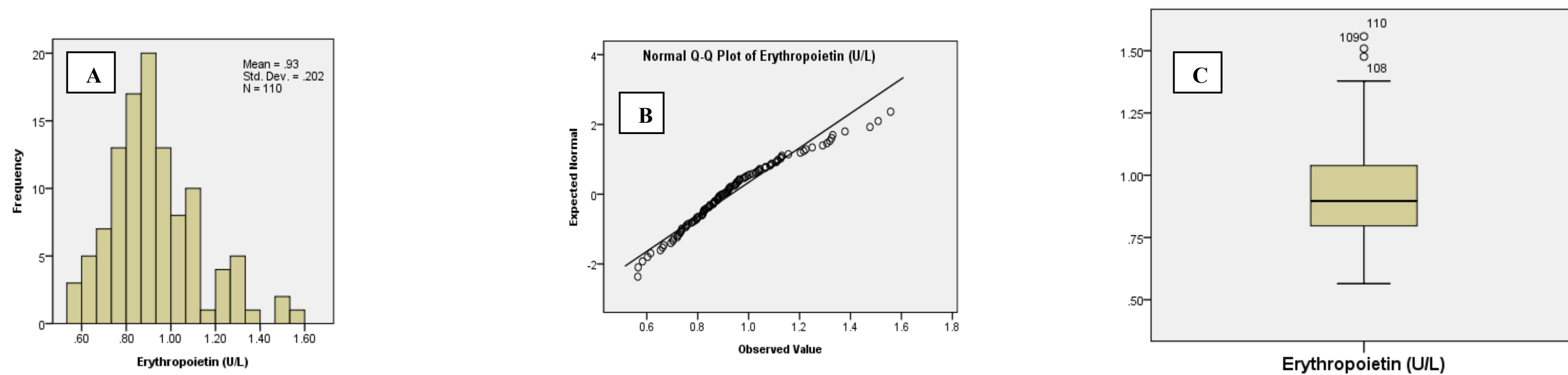


Fig. 3.9. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **male EPO sample population (n = 39)** aged **18 to 25 years after excluding hemoglobin<125 g/l, iron <9.85 µmol/l and ferritin <20ng/ml at Stage V, Step 8**.

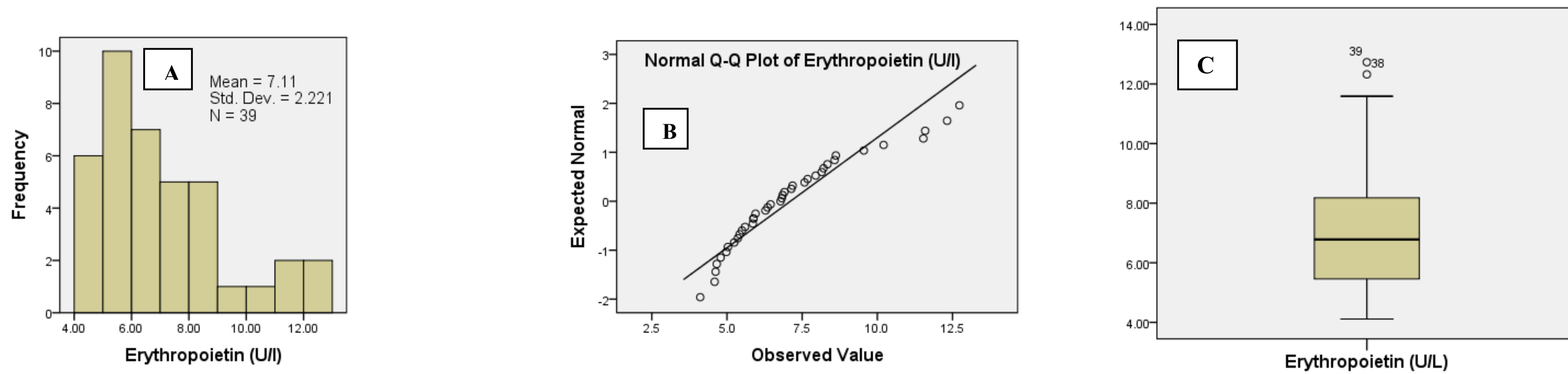


Fig. 3.10. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **$\log_{10}$ transformed male EPO sample population (n = 39)** aged **18 to 25 years** after **excluding hemoglobin<125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml at Stage V, Step 8.**

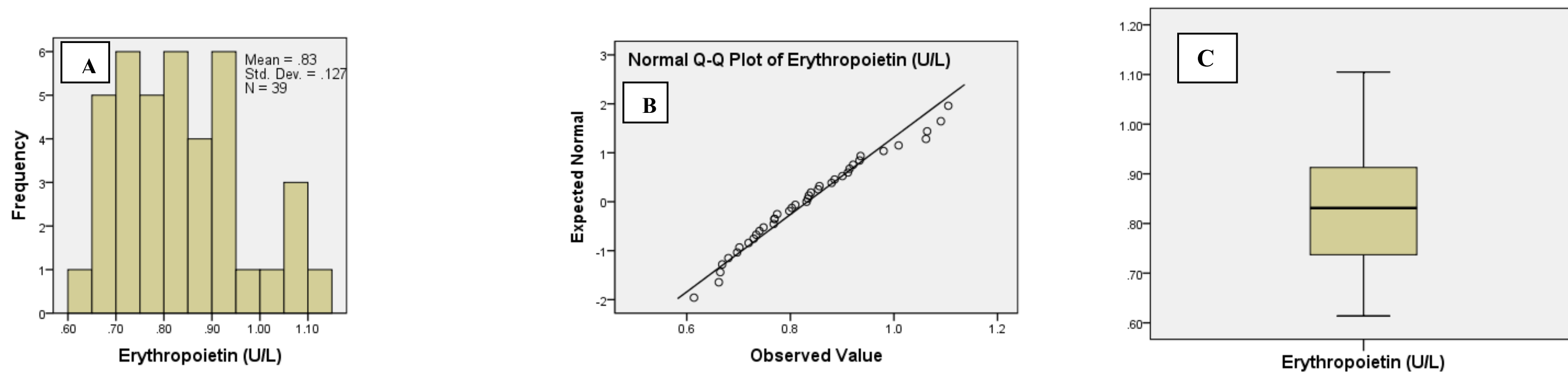


Fig. 3.11. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **female EPO sample population** (n = 30) aged **18 to 25 years** after **excluding hemoglobin<125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml at Stage V, Step 8.**

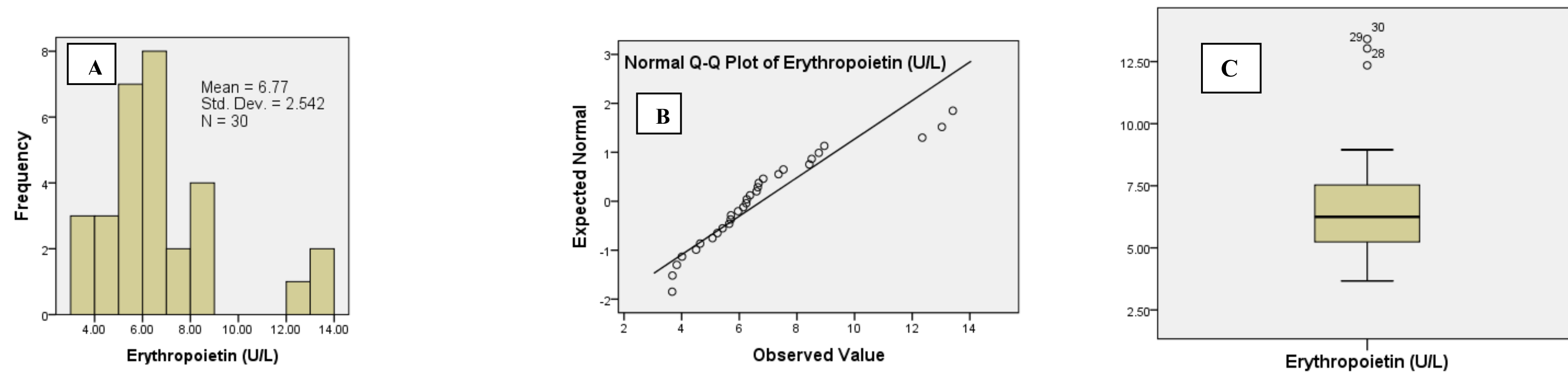


Fig. 3.12. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of EPO in the **$\log_{10}$ transformed female EPO sample population (n = 30)** aged **18 to 25 years** after **excluding hemoglobin<125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml at Stage V, Step 8.**

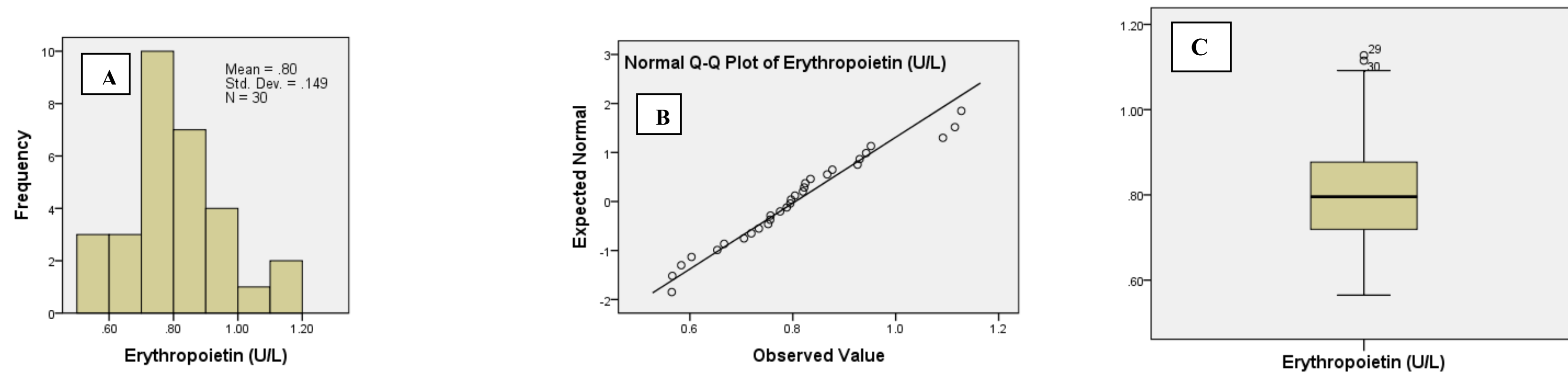


Table 3.3. Summary of histogram data in figures 1 to 12, estimated for Normality of distribution for EPO at various Stages and steps of sample selection. KS (Kolmogorov-Smirnova), SW (Shapiro-Wilk), LT (Log_{10} transformed).

Stages	Steps	Total				Male				Female			
		K-S P	S-W P	Outliers		K-S P	S-W P	Outliers		K-S P	S-W P	Outliers	
				Near	Far			Near	Far			Near	Far
I	2.	<0.001	<0.001	18	12	<0.001	<0.001	8	3	<0.001	<0.001	14	6
II	3.	<0.001	<0.001	13	10	<0.001	<0.001	6	1	<0.001	<0.001	11	5
III	4a.	<0.001	<0.001	13	8	<0.001	<0.001	6	1	<0.001	<0.001	11	6
	4b.	<0.001	<0.001	13	7	<0.001	<0.001	5	1	<0.001	<0.001	11	6
	4c.	<0.001	<0.001	10	5	<0.001	<0.001	3	1	<0.001	<0.001	8	4
IV	5	<0.001	<0.001	8	4	0.002	0.001	3	0	<0.001	<0.001	6	3
	LT	0.003	<0.001	4	0	0.200	0.019	0	0	0.003	0.001	3	0
V	8	0.001	<0.001	5	0	0.105	0.003	2	0	0.007	0.001	3	0
	LT	0.200	0.118	0	0	0.200	0.242	0	0	0.200	0.214	2	0

Table 3.4. Correlations of EPO with hemoglobin, iron and ferritin in the **EPO sample (A)** and after partitioning into **males (B)** and **females (C)**, by parametric (Pearson's, r) and non parametric (Spearman's, ρ) methods. Iron was not log_{10} transformed as it had Gaussian distribution before transformation. P values after log_{10} transformation and Spearman's rho (ρ) are in brackets.

Correlation of EPO with related analytes	**A. EPO Sample (n = 159)**		
	Shapiro Wilk test before (or after) log_{10} transformation, P	**Pearson's, r (Spearman's rho ρ)**	**Significance of correlation P**
S. EPO	(<0.001)	--	--
Blood Hemoglobin	(0.014)	(-0.290)	<0.001
S. Iron	0.288	-0.455	<0.001
S. Ferritin	(0.026)	(-0.414)	<0.001
B. Male (n = 49)			
S. EPO	(0.019)	--	--
Blood Hemoglobin	(0.177)	-0.071	0.629
S. Iron	(0.375)	-0.295	0.040
S. Ferritin	(0.149)	-0.215	0.138
C. Female (n = 110)			
S. EPO	(0.001)	--	--
Blood Hemoglobin	(0.001)	(-0.324)	0.001
S. Iron	0.151	-0.454	<0.001
S. Ferritin	(0.126)	-0.557	<0.001

Fig. 3.13. X-Y scatter diagram, **in males (A – C; n = 49)** and **females (D – F; n = 110)**, of EPO with hemoglobin (A, D), iron (B, E) and ferritin (C, F).

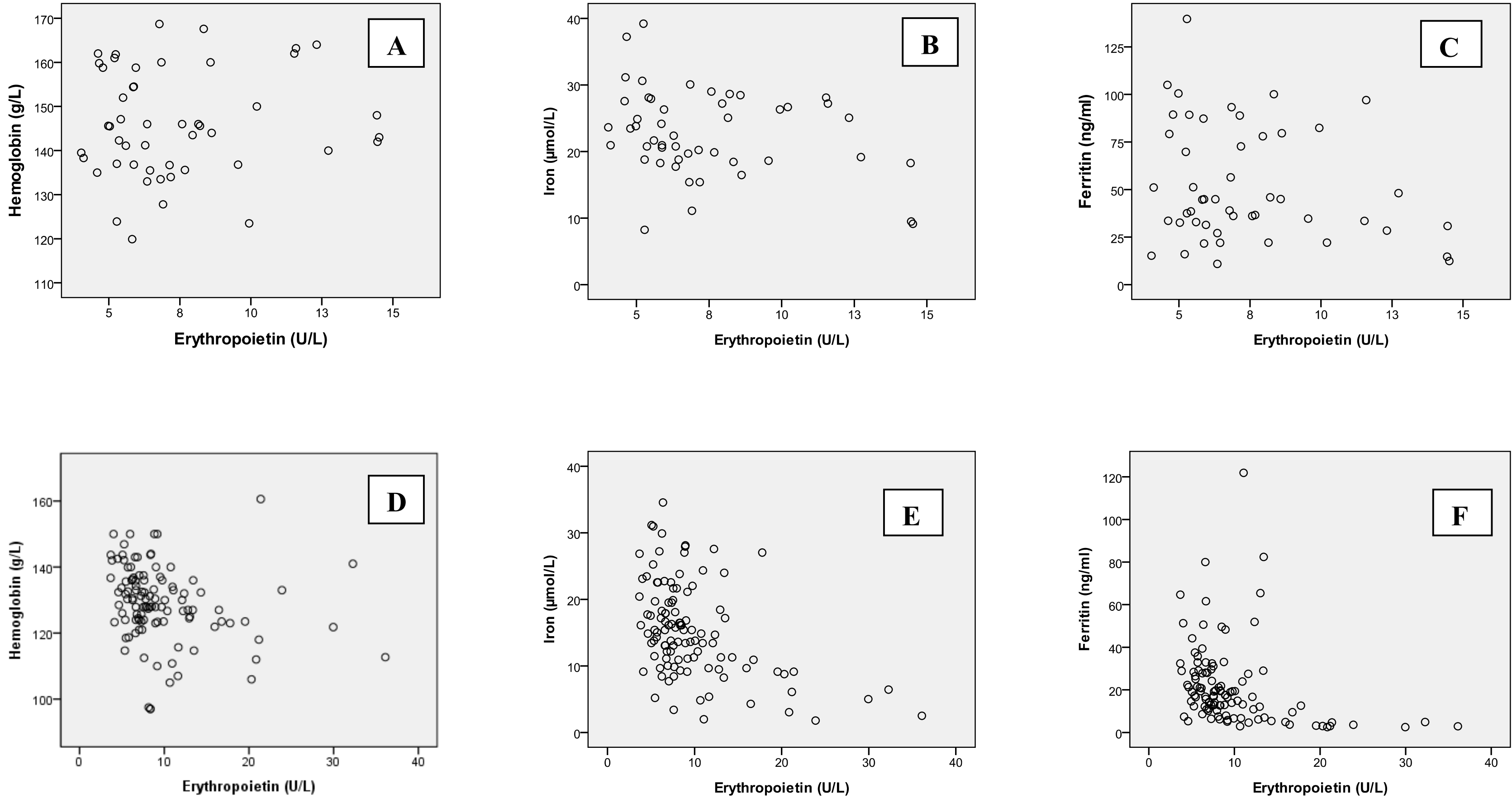


Table 3.5. Comparison of EPO, hemoglobin (HB), iron and ferritin in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding anemia/hypoxia. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed. The cut off levels for exclusion of hemoglobin were <125g/l in male and females (A), <130g/l in males and <120g/l in females (B).

Comparison between males (n = 46) and females (n = 74) after excluding hemoglobin <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 HB (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	<0.001
			(before $\log_{10}$ transformation)			
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 hemoglobin 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 HB (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	<0.001
			(before $\log_{10}$ transformation)			
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 3.6. Comparison of EPO, hemoglobin (HB), iron and ferritin in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding hemoglobin <125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Male Mean ±SD Minimum – Maximum 95%CI of mean (n = 39)	Female Mean ±SD Minimum – Maximum 95%CI of mean (n = 30)	After log_{10} transformation			
			Shapiro-Wilk test for normality P		Levene's test for equality of variances P	Student t test (or Mann-Whitney U test) P
			male	Female		
S. EPO (U/L)	7.11±2.22 4.11 – 12.73 6.39 – 7.83	6.77±2.54 3.67 – 13.41 5.82 – 7.72	0.242	0.214	0.650	0.398
Blood HB (g/l)	149.24±12.81 127.80 – 181.80 145.09 – 153.39	135.97±6.79 125.00– 150.00 133.44 – 138.50	0.182	0.179	0.002	(<0.001)
S. Iron (µmol/l)	23.78±5.80 11.10 – 39.20 21.90 – 25.66	19.86±6.17 11.28– 34.55 17.56 – 22.17	0.452	0.207	0.126	0.005
S. Ferritin (ng/ml)	55.02±26.86 21.60 – 105.00 46.32 – 63.73	39.17±17.72 20.80 – 82.40 32.56 – 45.79	0.015	0.046	0.216	(0.008)

Table 3.7. Correlation of EPO with hemoglobin (HB), iron and ferritin after partitioning into males and females, (A) after excluding hemoglobin <125g/l, (B) after excluding hemoglobin in males at <130g/l and in females at <120g/l, and (C) after excluding hemoglobin <125 g/l, iron <9.85 μmol/l and ferritin <20 ng/ml by parametric (Pearson's, r) and non parametric (Spearman's, ρ) methods. Iron was not $\log_{10}$ transformed as it had Gaussian distribution before transformation.

Correlation of EPO with related analytes	**(A). After excluding HB <125g/l, (n = 120)**		**(B). After excluding HB male <130g/l and female <120g/l, (n = 137)**		**(C). After excluding HB <125 g/l, iron <9.85 μmol/l, ferritin <20 ng/ml (n = 69)**	
	Pearson's, r (Spearman's rho ρ)	**P**	**Pearson's, r (Spearman's rho ρ)**	**P**	**Pearson's r**	**P**
	Male (n = 46)		**Male (n = 45)**		**Male (n = 39)**	
Blood HB	(0.028)	0.855	(0.040)	0.796	0.090	0.588
S. Iron	-0.333	0.024	-0.353	0.017	-0.151	0.358
S. Ferritin	-0.220	0.141	-0.221	0.145	-0.191	0.243
	Female (n = 74)		**Female (n = 92)**		**Female (n = 30)**	
Blood HB	(-0.256)	0.028	(-0.283)	0.006	-0.485	0.007
S. Iron	-0.443	<0.001	-0.417	<0.001	-0.277	0.139
S. Ferritin	-0.384	<0.001	-0.469	<0.001	0.347	0.060

Fig. 3.14. X-Y scatter diagram, **in males (A – C; n = 46)** and **females (D – F; n = 74)**, of EPO with hemoglobin (A, D), iron (B, E) and ferritin (C, F) after **excluding hemoglobin <125g/l**.

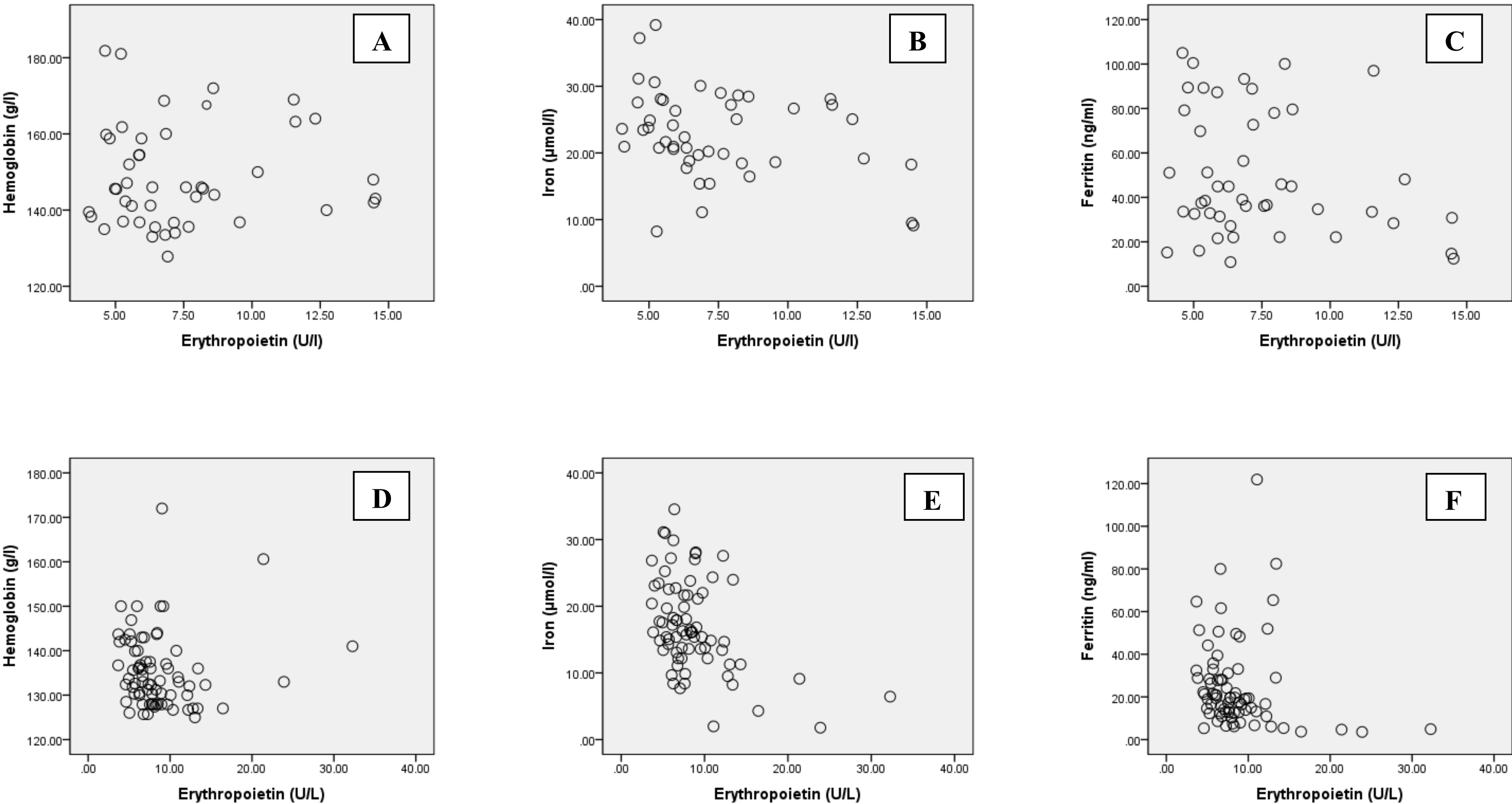


Fig. 3.15. X-Y scatter diagram, **in males (A – C; n = 45)** and **females (D – F; n = 92)**, of EPO with hemoglobin (A, D), iron (B, E) and ferritin (C, F) after **excluding hemoglobin in males <130g/l and females <120g/l**.

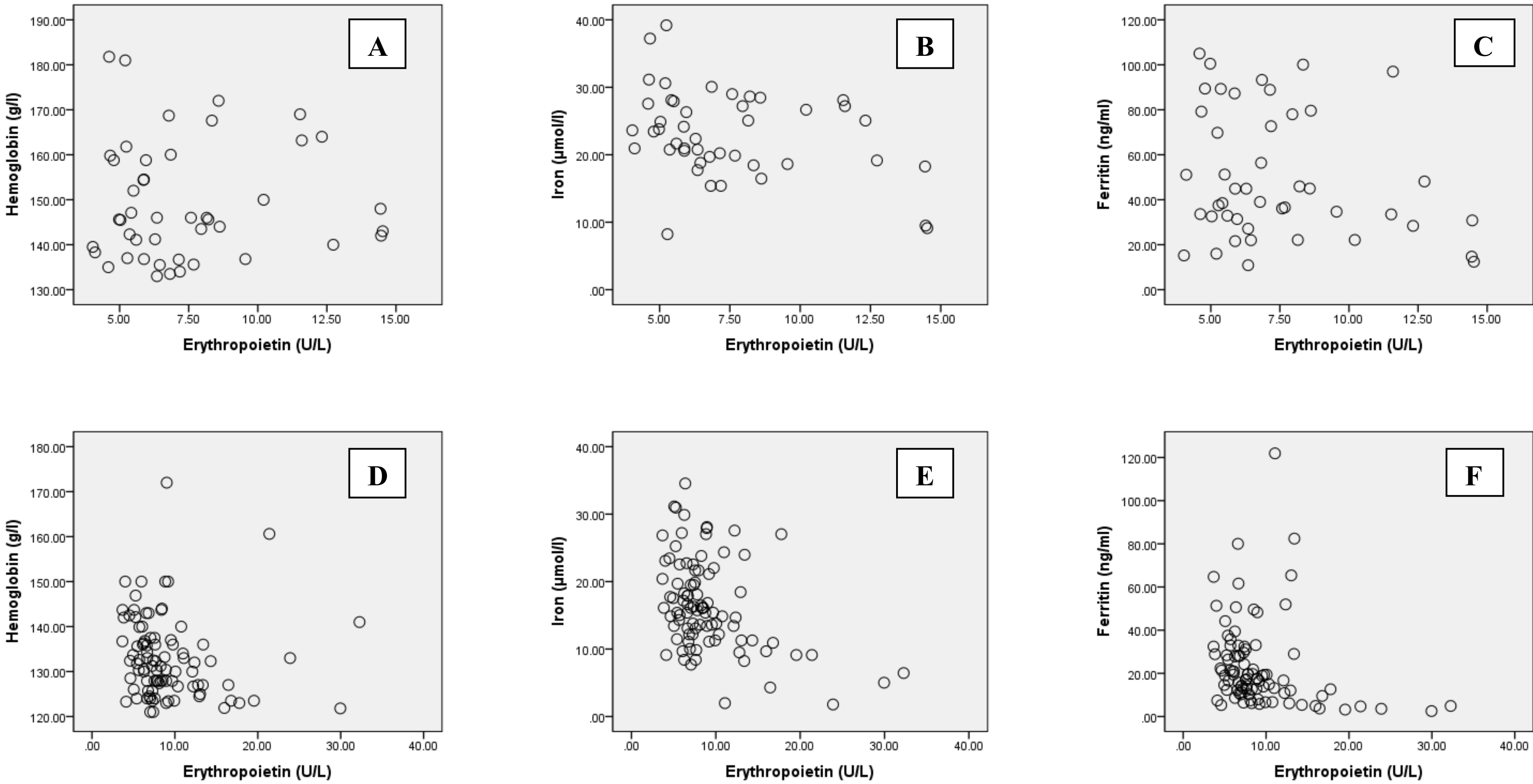
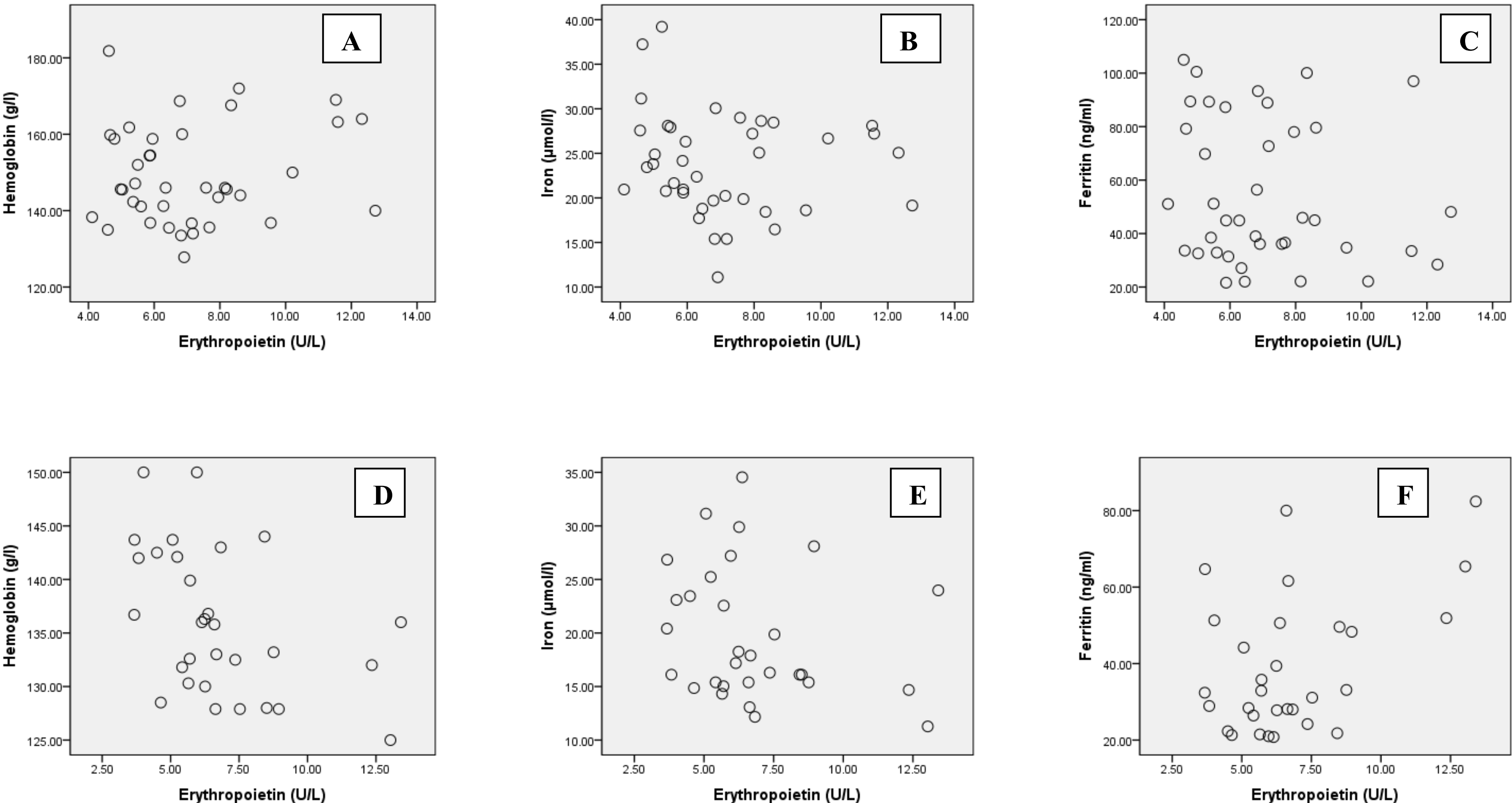


Fig. 3.16. X-Y scatter diagram, **in males (A – C; n = 39)** and **females (D – F; n = 30)**, of EPO with hemoglobin (A, D), iron (B, E) and ferritin (C, F) after **excluding hemoglobin <125g/l, iron <9.85 µmol/l and ferritin <20 ng/ml**.



NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 4.

The Gender Differences in Parathyroid Hormone and the Related Analytes in Healthy Young Adults

4.1. Abstract

Erythropoietin had shown positive correlations with parathyroid hormone in several studies done for this dissertation. Analytes related to parathyroid hormone functions, such as vitamin D, hemoglobin, iron and ferritin, may have deficiencies that can influence the clinical interpretations of smaller changes in concentrations of parathyroid hormone and its correlations. Parathyroid hormone resistance are known in diseases and during deficiencies. In an earlier study, we had reported gender differences in erythropoietin even after exclusion of Anemia. In this study, gender differences in parathyroid hormone, erythropoietin and analytes related to them were evaluated after excluding the deficiencies. Participants aged 18 to 25 years from rural, near sea level plains of central Kerala, who were evaluated clinically and by quantitative biochemical analytes, with cut off levels fixed to exclude common subclinical diseases states, but include deficiencies of analytes related to intact parathyroid hormone, took part in this observational cross sectional study. Before the exclusion of deficiencies, gender differences were seen in all selected analytes related to parathyroid hormone: in females, concentrations of parathyroid hormone and erythropoietin increased, while vitamin D, calcium, hemoglobin, iron and ferritin decreased. Exclusion of deficiencies of vitamin D, hemoglobin, iron and ferritin resulted in loss of gender differences in all analytes except hemoglobin, markedly decreased sample size, especially in females. Correlations of parathyroid hormone with analytes were difficult to interpret in the presence of deficiencies. But after the exclusions, there was a negative correlation of parathyroid hormone with hemoglobin in males and females, resulting in positive correlation of parathyroid hormone with erythropoietin for erythropoiesis in females and its negative correlation with iron due its consumption for higher hemoglobin in males. Higher hemoglobin in men and gender differences in the correlations may be due to androgens in males; in females the lower hemoglobin may be due to inhibition by oestrogens resulting in higher levels of erythropoietin and parathyroid hormone. Exclusion of deficiencies in analytes related to parathyroid hormone brought out the gender differences that may indicate differences in regulation of hemoglobin concentrations in males and females.

4.2. Introduction

Primary regulation of bone mineral metabolism and erythropoiesis are by parathyroid hormone (PTH), 1,25 dihydroxy cholecalciferol (1,25$(OH)_2D_3$) and erythropoietin (EPO). Immediate and short term regulator for synthesis and secretion of PTH is plasma free ionic calcium. PTH, 1,25$(OH)_2D_3$ and phosphate, increase plasma free calcium and decreases plasma phosphate by promoting bone resorption, calcium uptake by kidneys, by phosphaturia and intestinal absorption of calcium (Fitzpatrick & Bilezikian, 2006; Nissenson & Jüppner, 2008; Risteli et al, 2012). Inverse Relationship of PTH and calcitriol by activation and inhibition, respectively of 1α-hydroxylase may lead to a secondary inverse relationship of PTH with Vitamin D. This relationship may be more pronounced and may cause PTH resistance when there is vitamin D deficiency. Subclinical vitamin D deficiency, a common life style disorder, may contribute to increase in PTH resulting from decreased 1,25$(OH)_2D_3$ formation (Fitzpatrick & Bilezikian, 2006; Nissenson & Jüppner , 2008; Risteli et al, 2012; Brown, 2000). 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 Anemia (Nesheera et al, 2017).

Hormone resistance are known in disease conditions but may also be involved in apparently healthy individuals who may have subclinical deficiencies. In dialysis patients with hyperparathyroidism, high serum PTH is associated with low concentration of EPO, decrease in the formation of erythroid progenitors and shorter survival of red cells (Meytes et al, 1981; Dunn & Trent, 1981; Bogin et al, 1982) resulting in Anemia (Goicoechea et al, 1996; Barbour, 1979; Mandolfo, 1998). Deficiency of EPO secretion from kidney damage in hemodialysis patients is the most important cause of Anemia (Erslev, 1991; Nissenson et al, 1991) and is corrected by recombinant human EPO (Eschbach et al, 1989). 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 (Stivelman, 1989). Of these, the most important is iron deficiency (Van Wyck et al, 1989). Vitamin D deficiency may also decrease the effectiveness of PTH. In some of these patients, parathyroidectomy and control of secondary hyperparathyroidism

increased hemoglobin, reduced the requirement of EPO stimulating agents and reduced fibrosis of bone marrow (Goicoechea et al, 1996; Barbour, 1979; Mandolfo et al, 1998; Sudhaker Rao et al, 1993). A major cause of EPO resistance is bone marrow fibrosis and there was significant reduction in osteoclastic activity (Sudhaker Rao et al, 1993).

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 (McGee et al, 2012; Bab et al 2015). A relationship between PTH and EPO may be required for altering the bone microenvironment during increased erythropoiesis that is seen in Anemia. Hematopoiesis also requires bone formation and EPO has been reported to be involved (Shiozawa et al, 2010). 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 (Murphy, 2014). Will the higher hemoglobin levels in males lead to gender differences in EPO and PTH? The gender differences of hemoglobin levels are further increased by the monthly loss of blood in women, leading to decrease in hemoglobin, iron and ferritin.

Earlier we had shown that in healthy young females, the concentration of EPO was increased, when that of hemoglobin, iron and ferritin was decreased. There were also gender differences in the negative correlation of EPO with hemoglobin, iron and ferritin which persisted even after excluding Anemia (Nesheera et al, 2017). In this study, we are further evaluating the cause of the residual gender differences in the absence of Anemia, after excluding the subclinical deficiencies of vitamin D, hemoglobin, iron and ferritin that may increase PTH and EPO levels, and may cause EPO and PTH resistance in otherwise healthy young adults.

4.3. Objectives

4.3.1. Aspects of the Original Objectives Addressed in this Chapter

Intact PTH concentrations are related to a number of regulatory functions and including circulating EPO. Therefore, disease states that influence PTH may cause variations in factors that are regulated directly or indirectly by PTH and EPO. In this study, we examine

1. Correlation, gender differences and variations in concentrations of PTH with other analytes in healthy individuals.

2. Influences on such correlations and significant variations in disease or deficiency states.

4.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. **EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine**

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment.

4.4. Materials and Methods

4.4.1. Study Setting and Research Design

Please refer 2.1. Study setting and Research design section in chapter 2

4.4.2. Exclusion of Subclinical Disease States and Deficiencies by Clinical Biochemistry Assays for PTH Sample

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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Table 2.1; Stage I).

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. 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 (Table 2.1; 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 4.1, 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, , serum creatinine 1.2 mg/dl (106.08 µmol/l) total calcium >11 mg/dl (2.75 mmol/l), EPO <3.5 IU/L (Table 4.1; 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 4.2) and after (Table 4.3, 4.4 and 4.5) 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.

4.4.3. Sample Collection and Sample Preparation

Please refer 2.3. Sample collection section in chapter 2

4.4.4. Analytical Control and Assays

Analytical measurements of **ALT, triglycerides, creatinine, high sensitive CRP, fasting glucose, total calcium and iron** were done with the chemistry autoanalyser 5,1 FS, (Procedure manual, Ortho Clinical Diagnostics, 2004). **EPO, intact PTH and ferritin** assays were done with Access 2 machine and their reagents, using immunometric assay with magnetic bead coated anti EPO, anti PTH or anti ferritin antibody (Procedure manual, Beckman Coulter, 2005).**Vitamin D** assay was done by Diasorin Liaison (Italy) (Procedure manual, Liaison, 2017). **Hemoglobin** estimation was done manually by Drabkin's method using colorimeter. **Please refer detailed description of these assays in chapter 2.**

There were twice a day internal quality control programs and once a month external quality assurance programs (Biorad, USA). 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 with 95% confidence was 1 ng/l

(0.1 pmol/l). It was also far below the lowest linear six point intact PTH calibrator value (eg. PTH: 10.7 ng/l). Example of the actual linear six point calibration values for intact PTH in ng/l from a particular lot of calibrators was 0, 10.7, 61.2, 303, 1467, 3369.5 (Procedure manual, Beckman Coulter, 2005).

4.4.5. Diagnostic criteria and Reference Interval

Please refer 2.26. Diagnostic criteria and reference interval section in chapter 2.

4.4.6. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer 2.27. Statistical analysis section in chapter 2

4.4.7. Inter conversion of Units of variables

Please refer Table 2.2. Conversion factors for interconversion between conventionl unit and SI unit.

4.5. Results

4.5.1. Selection of Participants

Participants of this study were partitioned into males and females at all stages of sample selection. Selection of participants by clinical history and examination was followed by exclusion of diseases at the subclinical level using cut off levels of quantitative Clinical Biochemistry analytes (Chapter 2 and 3, Table 4.1, Stage I and II). The sample number in male participants decreased much more that in females at these stages. Sample was again partitioned according to age and participants selected were between ages 18 and 25 years to exclude influence of growth phase and age (Table 4.1, Stage III). Selection of participants was done after excluding some diseases related to analytes used in this study such as samples with high ferritin, osteocalcin, ostase, NTx and low EPO (Table 4.1, Stage IV). The sample at this state was referred to as the PTH sample. Strict exclusion criteria reduced the PTH sample size to 142 (Table 4.1; Stage IV) from the starting number >600 (Stage I) and further reduced during exclusion of different deficiencies to 40 (Stage V and VI).

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 (Chapter 3, Table 4.1).

4.5.2. Visual Evaluation of Distribution of PTH in the Sample at Various Phases of Exclusion

Distribution of total PTH sample (n = 142) was found to be positively skewed by histogram (Fig. 4.1). The normal Q-Q plot showed that the distribution was away from Gaussian distribution. There were only four near outliers.

The total PTH sample was partitioned in to male and female samples. The male sample (n = 40) was found to have near Gaussian distribution and there were only two outliers (Fig. 4.2). But after $\log_{10}$ transformation, the male sample was found to have Gaussian distribution by histogram (Fig. 4.3). The normal Q-Q plot also showed Gaussian distribution and there were no outliers by Box-Whisker plot.

The female sample (n = 102) showed a positively skewed appearance by histogram (Fig. 4.4). Normal Q-Q plot was away from Gaussian distribution. There was only one near outlier. After $\log_{10}$ transformation, both histogram and normal Q-Q plot showed Gaussian distribution and there were no outliers by Box-Whisker plot (Fig. 4.5)

Histogram of intact PTH of the male sample (n = 22) after excluding hemoglobin <125 g/l, ferritin <20 ng/l, iron <9.85 μmol/l and vitamin D <50 nmol/l was plotted. The sample distribution was found to be near normal and there were no outliers (Fig. 4.6). After $\log_{10}$ transformation, the sample was found to be almost normal by histogram and by normal Q-Q plot. There were no outliers by Box-Whisker plot (Fig. 4.7).

The female sample (n = 18) was found to have a distribution different from Gaussian distribution (Fig. 4.8). The normal Q-Q plot showed some deviation from Gaussian distribution. Box-Whisker plot showed one far outlier and three near outliers. The female sample after $\log_{10}$ transformation (Fig. 4.9), after exclusion of all four deficiencies showed a distribution that was near normal by histogram and normal Q-Q plot. There were three near positive outliers and three negative outliers.

4.5.3. Characteristics and Gender Differences in the Quantitative Analytes Used for Sample Selection and those Related to PTH

After selection of participants at Stage II, the range of BMI, waist circumference, serum creatinine, fasting glucose, alanine aminotransferase (ALT), triglycerides and hsCRP were within the specified cut off levels (Chapter 3; Stage II; Table 4.2). The PTH sample at Stage IV in Table 4.1, partitioned into males (n = 40) and females (n = 102) were used for this study.

Gender differences in the concentrations of analytes at Stage IV were used for this study (Table 4.2). Among the biochemical analytes used for sample selection at stage II, there were higher levels of triglycerides (P = 0.002), alanine aminotransferase (P <0001) and serum creatinine (P <0.001) in the male sample (Table 4.2A). These were the expected gender differences and may be due to higher insulin resistance and muscle mass in young males.

All intact PTH-related analytes showed gender differences (Table 4.2B). 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 Anemia and iron deficiency resulting from monthly blood loss (Nesheera et al, 2017) and due to increased vitamin D deficiency and hypocalcaemia.

4.5.4. Gender Difference in the Concentration of EPO and PTH Decreased and that of Hemoglobin Persisted after Exclusion of Deficiencies

4.5.4.1. After exclusion of vitamin D <50 nmol/l

Gender difference in the concentration of EPO was lost, and that of intact PTH and iron decreased after exclusion of vitamin D deficiencies. Hemoglobin, calcium and ferritin continued to show significant gender differences even after exclusion of deficiencies (Table 4.3).

4.5.4.2. After exclusion of hemoglobin<125 g/l and iron <9.85 µmol/l

EPO levels were almost equal in males and females and gender difference in their concentrations was removed (Table 4.4). Other analytes related to intact PTH continued to show significant changes in their concentrations.

4.5.4.3 After exclusion of hemoglobin <125 g/l, Ferritin <20 ng/ml, Iron <9.85 µmol/l and vitamin D <50 nmol/l

Anemia and deficiencies in Vitamin D, iron and ferritin were more in females (Table 4.1, Stage V and VI). The gender difference in most of the analytes related to PTH and EPO were lost when all the deficiencies were excluded (Table 4.5). When all deficiencies were excluded, EPO and PTH were decreased, contributing to the loss of its gender difference and may be attributed to the increase in hemoglobin, iron, ferritin and vitamin D in females. PTH was found to decrease when vitamin D was excluded. Surprisingly, even after all exclusions, gender difference in hemoglobin persisted. This may be attributed to the effect of androgen to increase hemoglobin in males (Nesheera et al, 2017; Murphy, 2014).

4.5.4.4. Comparison of Concentrations before and after exclusion of deficiencies in males and in females

The effects of deficiencies on the concentrations of analytes related to EPO and PTH can be summarised as follows. Exclusion of deficiencies increased hemoglobin, iron and ferritin in the female sample resulting in decrease of EPO in females, thus removing the gender differences (Table 4.6). Exclusion of vitamin D decreased PTH in males and females.

4.5.5. 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 4.7A and 4.7C) but not after exclusion of vitamin D deficiency (Table 4.7B and 4.7D), 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 absence of significant

correlation was 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 4.7C, r = 0.367) and further increased when vitamin D, hemoglobin, iron and ferritin deficiencies were excluded (Table 4.7D, r = 0.577). Results indicated that deficiencies in the female sample influenced the direct correlation of PTH and EPO, decreasing hemoglobin levels and erythropoiesis. Exclusion of these deficiencies increased the correlation statistic, hemoglobin concentrations and erythropoiesis. In none of these circumstances PTH correlated with EPO in the male sample, indicating strong gender differences in the regulation of erythropoiesis.

4.5.6. 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 4.7D). 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 4.7D). 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 Anemia in the female sample may lead to increase in EPO which in turn increased PTH levels to increase erythropoiesis 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 Anemia and iron deficiency or

vitamin D deficiency or both. Oestrogens decrease the concentration of hemoglobin in females.

4.6. Discussion

In an earlier report we had shown gender difference in EPO concentrations and correlations after exclusion of deficiencies in hemoglobin, iron and ferritin (Chapter 3; Nesheera et al, 2017). Later we identified that vitamin D deficiency and correlations of PTH, in addition to EPO, are involved in erythropoiesis. These observations, along with the gender difference in concentrations of PTH and its related analytes, and the correlations of PTH are reported here.

4.6.1. Exclusion Criteria and Partitioning for Selection the PTH Sample

Very strict inclusion and exclusion criteria were imposed on participants of this study to decrease the primary and secondary influences on parathyroid hormone secretion. 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. Exclusion criteria were implemented at stages I, II and IV for selection of PTH sample, and partitioning according to age at stage III and gender at all stages (Table 4.1). The decrease in the male sample was more than that in the female sample. In males, the major causes for decrease in sample size were insulin resistance and life style disorders; in females, it was hypothyroidism.

4.6.2. Subclinical Deficiencies Influenced the Gender Differences in Analytes Related to Intact PTH

Gender differences in the concentration of all seven analytes (Table 4.2B) 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 intact PTH (Risteli et al, 2012; Nesheera et al, 2017). Hemoglobin, iron and ferritin deficiencies increased EPO. EPO directly correlated with PTH in the female sample (Risteli et al, 2012; Meytes, 1981; Tables 4.7A to D) irrespective of the presence or

absence of deficiencies, indicating the strength of the relationship. The deficiencies influenced the concentrations all seven analytes (Tables 4.2B to 4.6) and the correlations of intact PTH (Table 4.7) leading to difficulties in interpretation of the results. This is further complicated by the heterogeneity in the sample resulting from presence and absence of deficiencies of the four analytes. When the deficiencies were excluded, the gender differences in the concentrations of EPO (Table 4.3 to 4.6) were absent.

4.6.3. Gender Differences after Exclusion of Deficiencies

When all four deficiencies in the PTH sample were excluded (Table 4.1; Steps 8) the interpretations were easier. The higher hemoglobin concentration in males, even after exclusion of all four deficiencies, may be attributed to the effect of androgen to increase hemoglobin in males (Nesheera et al, 2017; Murphy, 2014) (Table 4.5 and 4.6C). The gender differences in correlations of PTH may be due to the greater probabilities of hypoxia in females from lower concentrations of hemoglobin due to inhibition by oestrogens ((Nesheera et al, 2017; Murphy, 2014)(Table 4.7C). Hypoxia leads to PTH – EPO system being more active in females. In males, although PTH is inversely related to hemoglobin, the correlation with EPO may be confounded by the influence of androgens which may statistically confound the EPO – PTH correlation.

4.6.4. Interpretations from Correlations of PTH, Deficiencies and Gender Differences

Correlation between two variables arise when one increases or decreases in relation to the other. Some analytes are related even in their reference intervals after exclusion of deficiencies. When variables are related, deficiencies may increase correlations. For example, iron deficiency may decrease hemoglobin and cause resistance to the action of EPO, increase EPO and its negative correlations with hemoglobin. Sometimes deficiencies increase, restrict or remove correlations. As an example, vitamin D deficiency decreased the correlation of PTH with hemoglobin. When variables are not related, gender differences may be present due to other influences.

4.7. Conclusion

There are gender differences in the levels of PTH and EPO, and the parameters that are related to them. In women, the lower levels of hemoglobin and iron may be due to monthly blood loss, and the lower levels of vitamin D may be due to decreased exposure to sunlight. These deficiencies may be contributing to higher levels of PTH and EPO in women. There was correlation of PTH and hemoglobin only after exclusion of hemoglobin, iron and vitamin D deficiency. Thereby, indicating the roles of these causing both PTH and EPO resistance, inhibiting erythropoiesis. In men, after excluding all deficiencies, hemoglobin was much higher than in women and may be due to androgens. In men, PTH did not correlate with EPO but negatively correlated with hemoglobin and iron after excluding vitamin D deficiency or all four deficient analytes. This may be due to PTH resistance resulting from vitamin D deficiency.

Table 4.1. Number of participants (n) at various stages (III to VI) and steps (4 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)**
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	57	118
IV (4 to 5)	**5.**	PTH sample after excluding 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 Anemia 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 Anemia and deficiencies of iron, ferritin and vitamin D.	40 (10.39%)	22 (18/40 = 45%)	18 (84/102 = 82.35%)

Fig.4.1. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of Intact parathyroid hormone (PTH) in the PTH sample population aged **18 to 25** years after excluding **ferritin >250 ng/ml, osteocalcin>35 ng/ml, ostase>30µg/l and NTx>200 nM BCE/mmol urine creatinine** (n = 142).

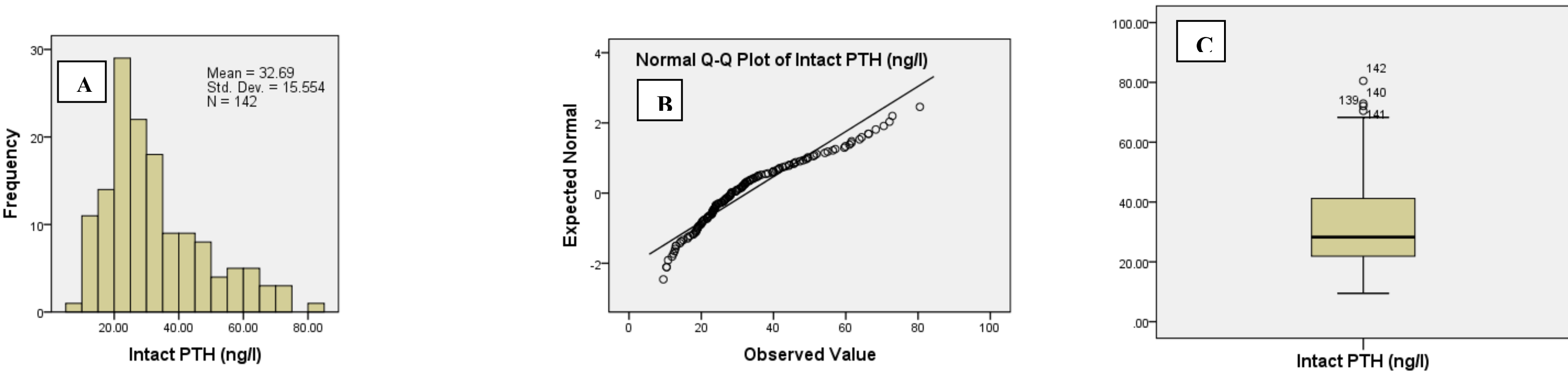


Fig. 4.2. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot (C) of Intact parathyroid hormone (PTH) in the **male** PTH sample population aged **18 to 25** years after excluding **ferritin >250 ng/ml, osteocalcin>35 ng/ml, ostase>30µg/l and NTx>200 nM BCE/mmol urine creatinine** (n = 40).

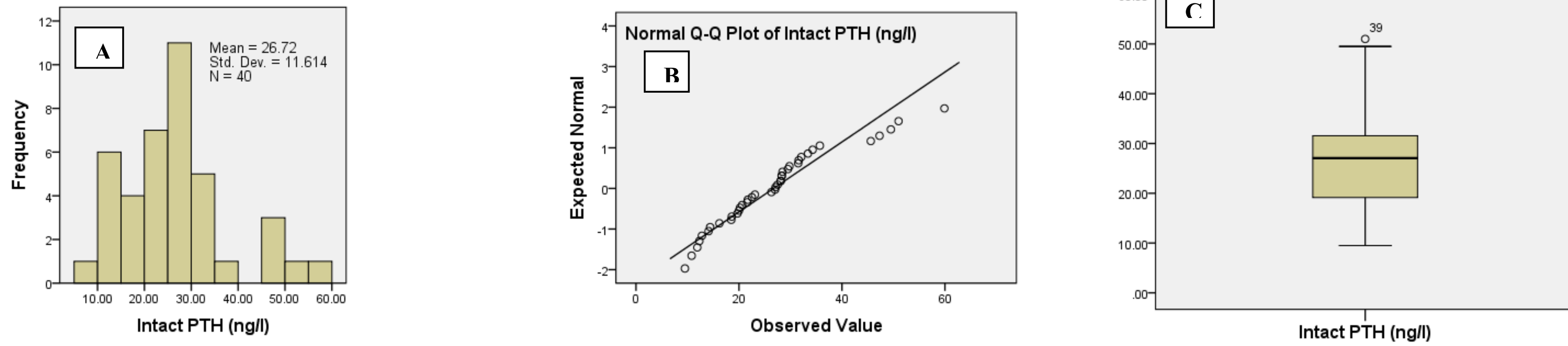


Fig. 4.3. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot (C) of **$\log_{10}$ transformed** Intact parathyroid hormone (PTH) concentrations in the **male** PTH sample population aged **18 to 25** years after excluding **ferritin >250 ng/ml, osteocalcin>35 ng/ml, ostase>30µg/l and NTx>200 nM BCE/mmol urine creatinine** (n = 40).

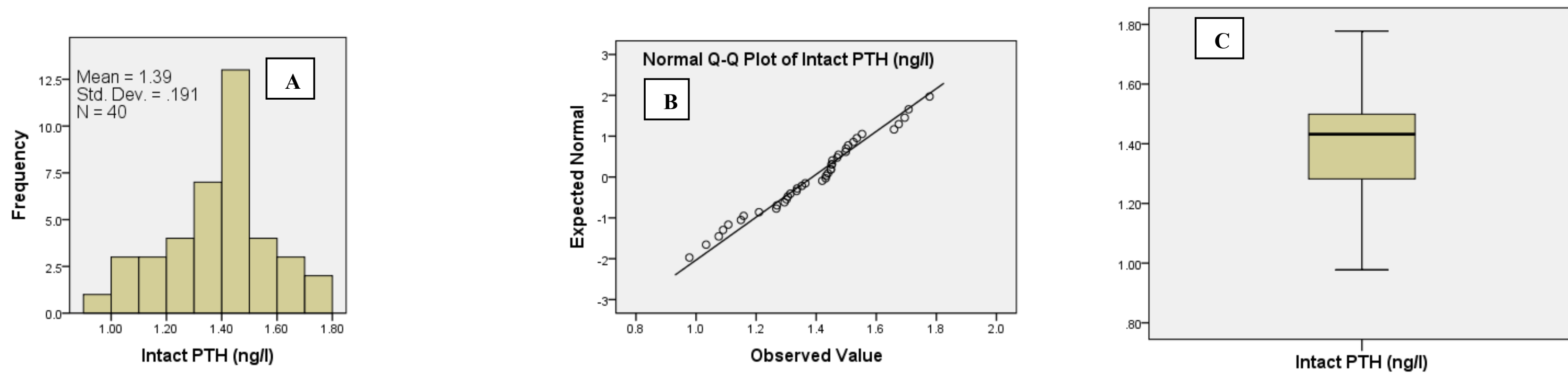
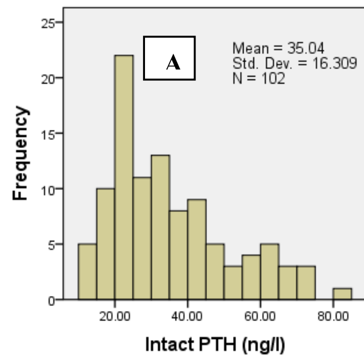
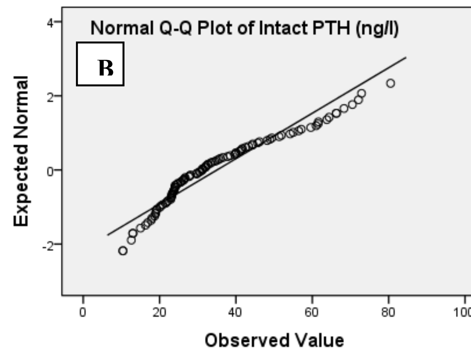


Fig. 4.4. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of Intact parathyroid hormone (PTH) in the **female** PTH sample population aged **18 to 25** years after excluding **ferritin >250 ng/ml, osteocalcin>35 ng/ml, ostase>30µg/l and NTx>200 nM BCE/mmol urine creatinine** (n = 102).





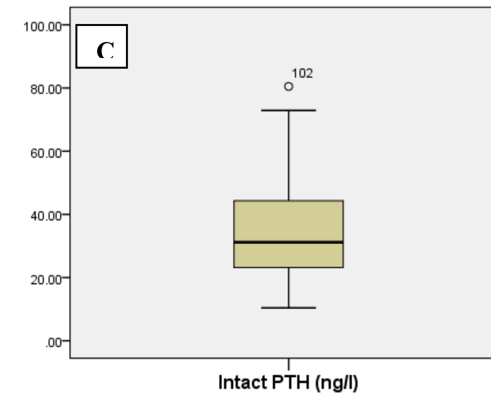
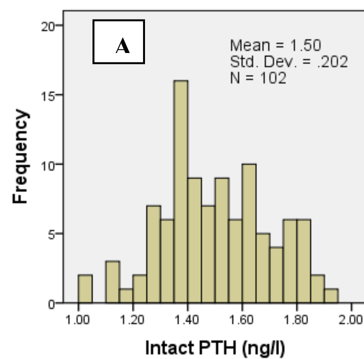
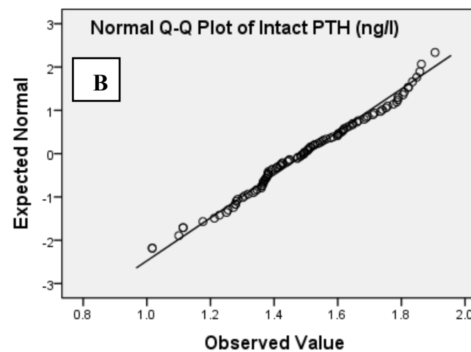


Fig. 4.5. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of $\mathbf{log_{10}}$ **transformed** Intact parathyroid hormone (PTH) concentrations in the **female** PTH sample population aged **18 to 25** years after excluding **ferritin >250 ng/ml, osteocalcin>35 ng/ml, ostase>30µg/l and NTx>200 nM BCE/mmol urine creatinine** (n = 102).





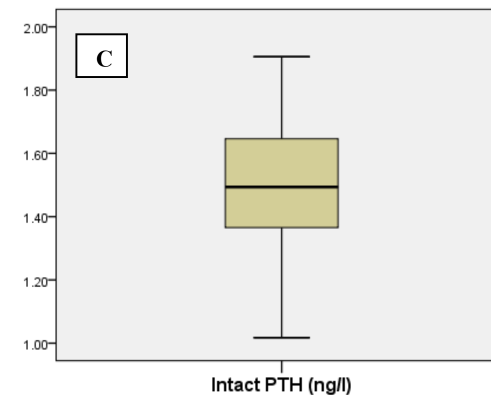


Fig. 4.6. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of Intact parathyroid hormone (PTH) in the **male** PTH sample population aged **18 to 25** years after excluding **Hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l** (n = 22).

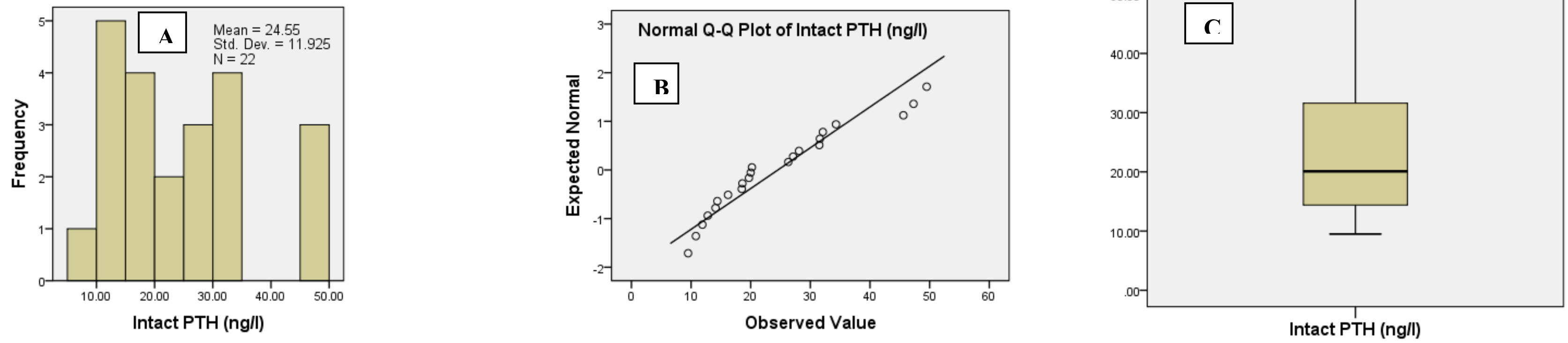


Fig. 4.7. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of **$\log_{10}$ transformed** Intact parathyroid hormone (PTH) in the **male** PTH sample population aged **18 to 25** years after excluding **Hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l** (n = 22).

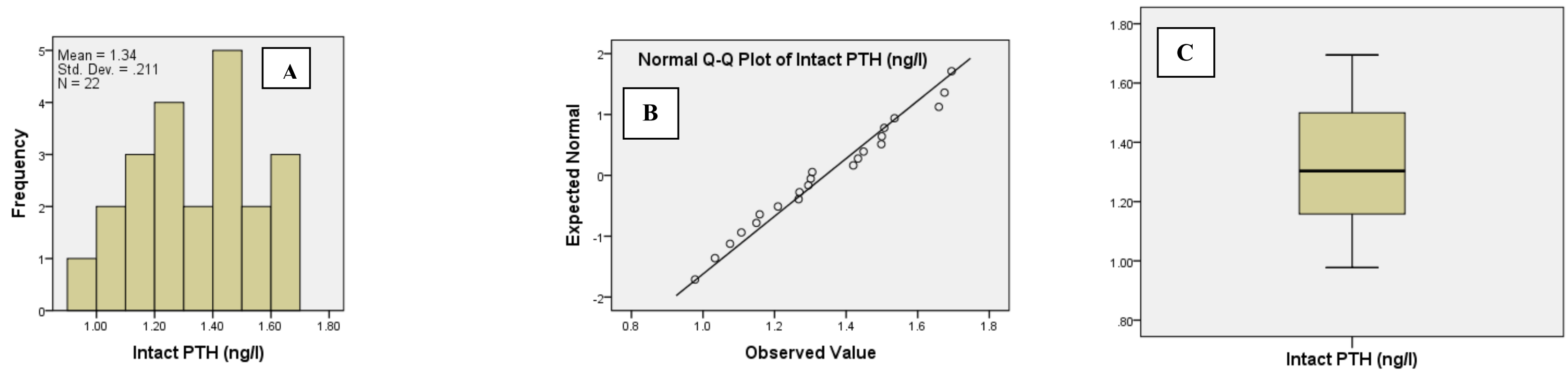


Fig. 4.8. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of Intact parathyroid hormone (PTH) in the **female** PTH sample population aged **18 to 25** years after excluding **Hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l** (n = 18).

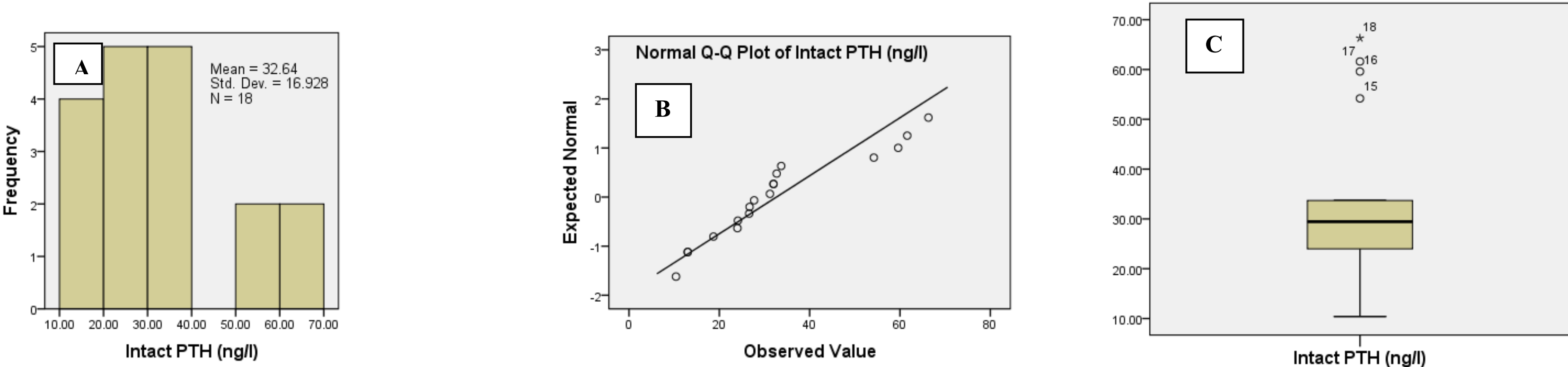


Fig. 4.9. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of **$\log_{10}$ transformed** Intact parathyroid hormone (PTH) in the **female** PTH sample population aged **18 to 25** years after excluding **Hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l** (n = 18).

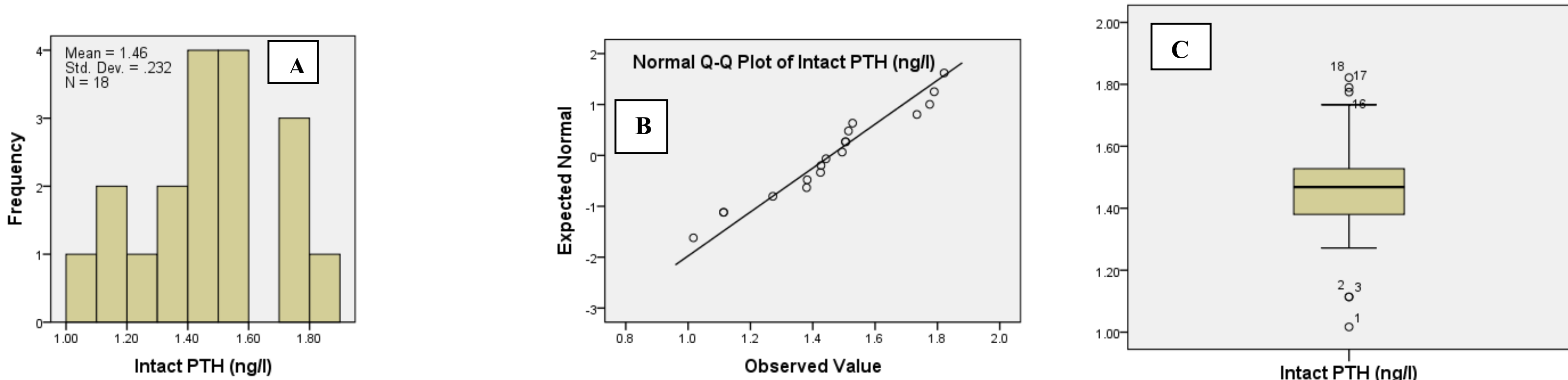


Table 4.2. General characteristics and gender differences of analytes used in exclusion criteria for sample selection (A) and those related to intact PTH (B) in the sample population. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male (M) and female (F) samples were analysed. The 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	Total sample (n = 142) Mean±SD Minimum - Maximum 95% CI of mean	Comparison of gender differences of analytes in the total sample					
		M (n = 40) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 102) Mean±SD Minimum - Maximum 95% CI of mean	After log_{10} transformation			
				Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
				M	F		
A. Quantitative analytes used in exclusion criteria for sample selection							
Age (18-25 years)	22.2±1.75	22.05±1.54	22.28±1.85	---	---	---	---
BMI (kg/m^2)	21.3±3.07 15.1 – 28.1 20.8 – 21.8	21.38±3.21 15.05 – 27.18 20.35 – 22.41	21.28±3.06 16.87 – 28.08 20.69 – 21.88	0.305	<0.001	0.854	(0.713)
Waist circum-ference (cm)	77.1±7.81 55.0 – 100.0 75.8 – 78.4	78.59±8.36 63.00 – 100.00 75.92 – 81.27	76.47±7.55 55.00 – 99.00 74.99 – 77.95	0.627	0.340	0.473	0.149
S. Creatinine (µmol/l)	68.2±14.98 44.2 – 106.1 65.8 – 70.7	85.09±13.68 53.04 – 106.08 80.71 – 89.46	61.62±9.18 44.20 – 97.24 59.82 – 63.42	0.012	<0.001	0.048	(<0.001)
Fasting Glucose (mmol/l)	4.9±0.459 4.01 – 6.7 4.8 – 4.97	4.91±0.417 4.01 – 5.94 4.78 – 5.05	4.89±0.476 4.05 – 6.70 4.80 – 4.98	0.120	0.001	0.242	(0.471)
ALT (U/L)	25.7±9.1 11.0 – 60.0 24.2 – 27.2	30.00±10.71 16.00 – 60.00 26.57 – 33.43	24.00±7.81 11.00 – 60.00 22.47 – 25.53	0.322	0.063	0.101	<0.001

Triglycerides (mmol/l)	0.873±0.329 0.37 – 2.12 0.82 – 0.93	0.991±0.353 0.37 – 2.07 0.878 – 1.10	0.826±0.309 0.42 – 2.12 0.766 – 0.887	0.451	0.018	0.611	(0.002)
High sensitive CRP (mg/l)	0.89±0.95 0.10 – 5.0 0.73 – 1.04	0.830±0.888 0.10 – 3.70 0.546 – 1.11	0.907±0.979 0.10 – 5.00 0.715 – 1.10	0.304	<0.001	0.231	(0.973)
B. Quantitative analytes related to intact PTH							
Intact PTH (ng/l)	32.69±15.55 9.50 – 80.50 30.11 – 35.27	26.72±11.61 9.50 – 59.90 23.01 – 30.44	35.04±16.31 10.40 – 80.50 31.83 – 38.24	0.534	0.196	0.535	0.003
S. EPO (U/L)	8.64±4.96 3.67 – 36.10 7.82 – 9.47	7.18±2.02 4.03 – 12.73 6.54 – 7.82	9.22±5.62 3.67 – 36.10 8.11 - 10.32	0.664	<0.001	0.017	(0.052)
Vitamin D (nmol/l)	58.20±34.50 12.46 – 187.20 52.47 – 63.92	76.48±46.80 12.46 – 187.20 61.52 – 91.45	51.03±25.12 13.20 – 125.10 46.09 – 55.96	0.099	0.179	0.078	0.001
Total Calcium (mmol/l)	2.44±0.132 2.13 – 2.75 2.42 – 2.46	2.51±0.122 2.20 – 2.75 2.47 – 2.54	2.42±0.128 2.13 – 2.70 2.39 – 2.44	0.253	0.242	0.384	<0.001
Blood Hemoglobin (g/l)	132.80±13.18 97.00 – 172.00 130.62 – 134.99	142.53±11.69 123.23 – 167.60 138.80 – 146.27	128.99±11.73 97.0 – 172.0 126.68 – 131.29	0.302	<0.001	0.891	(<0.001)
S. Iron (µmol/l)	17.00±7.31 1.79 – 39.20 15.79 – 18.21	21.47±6.64 7.34 – 39.20 19.34 – 23.59	15.25±6.82 1.79 – 34.55 13.91 – 16.59	0.061	<0.001	0.032	(<0.001)
S. Ferritin (ng/ml)	31.96±30.08 2.50 – 193.00 26.97 – 36.95	60.26±37.21 11.40 – 193.00 48.36 – 72.16	20.86±16.95 2.50 – 82.40 17.53 – 24.19	0.597	0.190	0.063	<0.001

Table 4.3. Comparison of analytes related to intact PTH in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding vitamin D <50 nmol/l. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of male and female after excluding vitamin D <50 nmol/l					
	Male Mean±SD Minimum - Maximum 95% CI of mean (n = 26)	Female Mean±SD Minimum - Maximum 95% CI of mean (n = 42)	after $\log_{10}$ transformation			
			Shapiro-Wilk test for normality, P		Levene's test for equality of variances, P	Student t test (or Mann-Whitney U test), P
			Male	female		
Intact PTH (ng/l)	24.89±11.02 9.50 – 49.50 20.44 – 29.34	31.64±14.41 10.40 – 70.50 27.15 – 36.13	0.600	0.340	0.652	0.039
S. EPO (U/L)	6.93±2.00 4.03 – 12.73 6.12 – 7.74	8.32±3.53 3.67 – 21.17 7.22 – 9.43	0.505	0.912	0.071	0.112
Vitamin D (nmol/l)	98.50±43.41 50.00 – 187.20 80.97 – 116.04	74.62±21.07 50.00 – 125.10 68.05 – 81.18	0.047	0.026	0.020	(0.024)
Total calcium (mmol/l)	2.51±0.113 2.30 – 2.68 2.47 – 2.56	2.40±0.113 2.15 – 2.60 2.36 – 2.43	0.178	0.337	0.775	<0.001
Blood HB (g/l)	140.19±10.34 123.50 – 162.00 136.02 – 144.37	130.00±13.54 97.05 – 172.00 125.78 – 134.22	0.482	0.020	0.330	(0.001)
S. Iron (µmol/l)	21.20±6.88 10.38 – 39.20 18.42 – 23.98	16.16±6.37 4.30 – 31.15 14.17 – 18.14	0.732	0.004	0.421	(0.005)
S. Ferritin (ng/ml)	59.14±39.51 11.40 – 193.00 43.18 – 75.10	26.87±19.13 2.90 – 82.40 20.91 – 32.83	0.890	0.070	0.415	<0.001

Table 4.4. Comparison of Intact PTH and analytes related to intact PTH in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding hemoglobin (HB) <125 g/l and Iron <9.85 µmol/l. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of male and female after excluding hemoglobin<125 g/l and iron <9.85 µmol/l					
	Male Mean±SD Minimum - Maximum 95% CI of mean (n = 37)	Female Mean±SD Minimum - Maximum 95% CI of mean (n = 60)	after log_{10} transformation			
			Shapiro-Wilk test for normality, P		Levene's test for equality of variances, P	Student t test (or Mann-Whitney U test), P
			Male	female		
Intact PTH (ng/l)	26.16±11.34 9.50 – 59.90 22.38 – 29.94	35.12±16.42 10.40 – 72.90 30.88 – 39.37	0.572	0.131	0.394	0.008
S. EPO (U/L)	7.11±2.00 4.03 – 12.73 6.44 – 7.77	7.52±2.42 3.67 – 14.32 6.90 – 8.15	0.660	0.846	0.163	0.515
Vitamin D (nmol/l)	76.85±47.88 12.46 – 187.20 60.88 – 92.81	53.49±26.05 13.20 – 117.56 46.76 – 60.22	0.110	0.202	0.193	0.020
Total Calcium (mmol/l)	2.52±0.117 2.20 – 2.75 2.48 – 2.55	2.43±0.129 2.13 – 2.70 2.40 – 2.47	0.303	0.371	0.356	0.002
Blood HB (g/l)	144.07±10.74 127.56 – 167.60 140.49 – 147.66	134.91±8.18 125.70 – 172.00 132.80 – 137.02	0.161	<0.001	0.034	(<0.001)
S. Iron (µmol/l)	21.79±6.42 10.38 – 39.20 19.65 – 23.93	18.75±5.63 11.10 – 34.55 17.29 – 20.20	0.247	0.101	0.697	0.020
S. Ferritin (ng/ml)	55.95±34.63 11.40 – 193.00 44.40 – 67.49	26.45±18.88 5.30– 82.40 21.57 – 31.33	0.435	0.460	0.174	<0.001

Table 4.5. Comparison of Intact PTH and analytes related to intact PTH in male and female samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of male and female after excluding hemoglobin<125 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l					
	Male Mean±SD Minimum - Maximum 95% CI of mean (n = 22)	Female Mean±SD Minimum - Maximum 95% CI of mean (n = 18)	after log_{10} transformation			
			Shapiro-Wilk test for normality, P		Levene's test for equality of variances, P	Student t test (or Mann-Whitney U test), P
			Male	female		
Intact PTH (ng/l)	24.55±11.92 9.50 – 49.50 19.26 – 29.84	32.64±16.93 10.40 – 66.30 24.22 – 41.06	0.568	0.281	0.891	0.106
S. EPO (U/L)	6.81±1.72 4.62 – 12.73 6.05 – 7.58	6.92±2.81 3.67 – 13.41 5.52 – 8.32	0.112	0.739	0.016	(0.638)
Vitamin D (nmol/l)	101.37±45.32 50.00 – 187.20 81.28 – 121.47	78.52±20.46 50.00 – 117.56 68.34 – 88.70	0.025	0.522	0.040	(0.180)
Total Calcium (mmol/l)	2.52±0.115 2.30 – 2.68 2.47– 2.57	2.43±0.101 2.15 – 2.55 2.38 – 2.48	0.188	0.033	0.568	0.020
Blood HB (g/l)	142.05±9.77 127.56 – 162.00 137.71 – 146.38	135.48±6.45 127.71 – 150.00 132.27 – 138.68	0.226	0.176	0.089	0.019
S. Iron (µmol/l)	21.28±7.23 10.38 – 39.20 18.07 – 24.48	17.79±4.93 12.17 – 31.15 15.34 – 20.24	0.778	0.116	0.134	0.113
S. Ferritin (ng/ml)	58.59±36.37 21.60 – 193.00 42.46 – 74.71	42.29±19.09 20.80 – 82.40 32.80 – 51.79	0.376	0.230	0.740	0.060

Table 4.6. Influence of exclusion of samples deficient in vitamin D (<50 nmol/l), Hemoglobin (Anemia, <125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml) (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 in males (M & M) and in females (F & F). Comparisons were done by Student t test (or Mann Whitney U test). Increase (↑) and decrease (↓) of mean is shown.

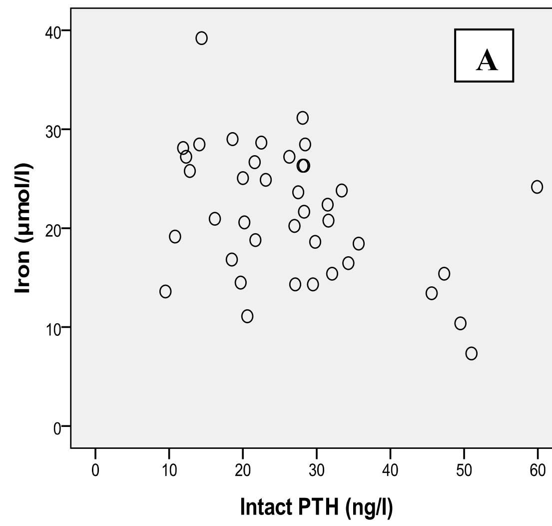
PTH and its related variables	A. Before and after exclusion of Vitamin D deficient samples. M (n = 26), F (n = 42)		B. Before and after exclusion of HB and Iron deficient samples. M (n = 37), F (n = 60)		C. Before and after exclusion of vitamin D, HB, iron, ferritin deficiencies. M (n = 22), F (n = 18)	
	Comparisons before and after sample exclusions by Student t test (Mann-Whitney U test), P					
	M & M	**F & F**	**M & M**	**F & F**	**M & M**	**F & F**
Intact PTH (ng/l)	0.508	0.267	0.836	0.918	0.394	0.430
S. EPO (U/L)	0.611	(0.755)	0.874	**(0.160)** ↓	0.540	**(0.041)** ↓
Vitamin D (nmol/l)	**0.018**↑	**(<0.001)** ↑	0.980	0.610	**(0.016)** ↑	**(<0.001)** ↑
Total calcium (mmol/l)	0.762	0.363	0.713	0.470	0.713	0.746
Hemoglobin (g/l)	0.435	(0.568)	0.525	**(0.001)** ↑	0.978	**(0.007)** ↑
S. Iron (µmol/l)	0.901	(0.345)	0.761	**(0.001)** ↑	0.905	**(0.071)** ↑
S. Ferritin (ng/ml)	0.839	**0.040**↑	0.641	**0.015**↑	0.916	**(<0.001)** ↑

Table 4.7. Correlation (r) and significance of correlation (P) of Intact PTH with EPO, vitamin D, total calcium, hemoglobin, iron and ferritin in males and in females, before (A) and after (B, C and D) exclusion of samples deficient in vitamin D (<50 nmol/l), hemoglobin (anemia, <125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml). Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after $\log_{10}$ transformation.

Variables related to PTH	Pearson's, r	P	Pearson's, r	P
A. Correlations of PTH (before exclusion of deficiencies).				
	Male (n = 40)		**Female (n = 102)**	
S. EPO	-0.019	0.909	**0.302**	**0.002**
Vitamin D	**-0.311**	**0.051**	0.166	0.095
Total Calcium	**-0.476**	**0.002**	-0.157	0.114
Blood Hemoglobin	-0.248	0.122	-0.086	0.388
S. Iron	**-0.365**	**0.020**	-0.040	0.687
S. Ferritin	0.055	0.735	-0.040	0.692
B. Correlations of PTH after exclusion of vitamin D deficiency.				
	Male (n = 26)		**Female (n = 42)**	
S. EPO	0.056	0.784	**0.301**	**0.052**
Vitamin D	-0.129	0.531	0.126	0.428
Total Calcium	-0.236	0.246	-0.005	0.977
Blood Hemoglobin	**-0.411**	**0.037**	-0.059	0.709
S. Iron	**-0.415**	**0.035**	0.107	0.614
S. Ferritin	-0.093	0.652	0.107	0.500
C. Correlations of PTH after exclusion of anemia and iron deficiencies				
	Male (n = 37)		**Female (n = 60)**	
S. EPO	-0.093	0.583	**0.367**	**0.004**
Vitamin D	**-0.304**	**0.067**	-0.136	0.299
Total Calcium	**-0.448**	**0.005**	-0.206	0.114
Blood Hemoglobin	-0.185	0.274	-0.185	0.158
S. Iron	**-0.298**	**0.073**	0.167	0.202
S. Ferritin	-0.007	0.967	0.078	0.552
D. Correlations of PTH after exclusion of vitamin D, hemoglobin, iron and ferritin deficiencies				
	Male (n = 22)		**Female (n = 18)**	
S. EPO	0.014	0.950	**0.577**	**0.012**
Vitamin D	-0.094	0.677	0.065	0.799
Total Calcium	-0.282	0.204	-0.141	0.578

Blood Hemoglobin	**-0.426**	**0.048**	**-0.494**	**0.037**
S. Iron	**-0.443**	**0.039**	-0.144	0.570
S. Ferritin	-0.018	0.936	0.316	0.202

Fig. 4.10. X-Y scatter diagram of PTH with iron in male (A; n = 40) and PTH with EPO in females (B; n = 102), in the PTH sample population.



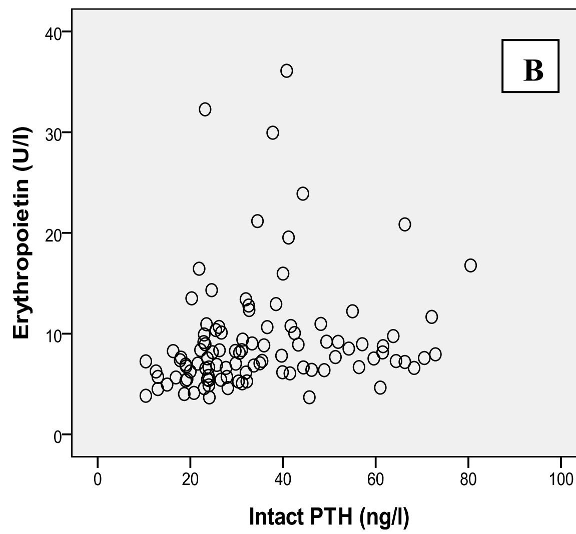
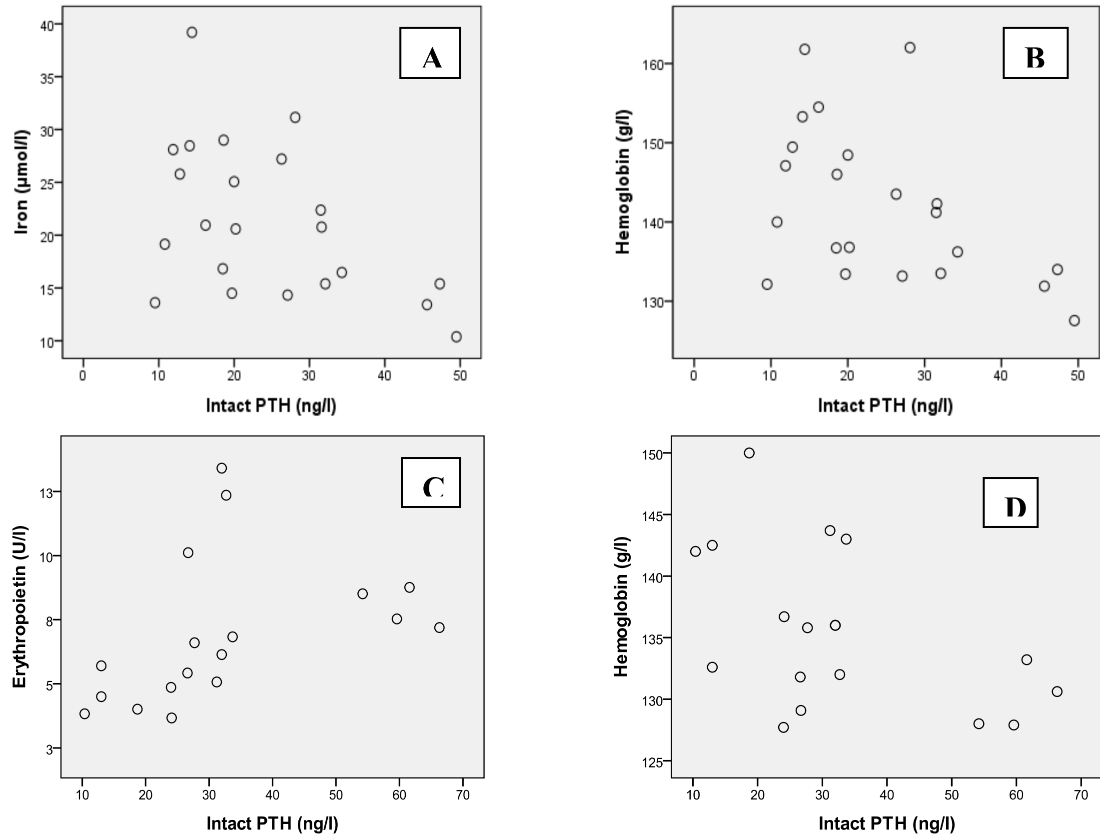


Fig. 4.11. X-Y scatter diagram, in males (A,B; n = 22) and females (C,D; n = 18), of PTH with iron (A), Hemoglobin (B,D), and EPO (C) after excluding hemoglobin<12.5 g/l, ferritin <20 ng/ml, iron <9.85 µmol/l and vitamin D<50 nmol/l.



NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 5.

Gender Difference in the Relationship of Erythropoietin with C Peptide

5.1. Abstract

Introduction: In the presence of increased insulin resistance in males, insulin secretion is higher. C peptide is closely related to insulin resistance. EPO has been shown to have a beneficial role in type 2 diabetes mellitus and was proposed to be due to decrease in insulin resistance.

Results: The concentrations of hemoglobin, iron and ferritin were lower in females resulting in higher EPO in females. But the concentration of C peptide was higher in male sample due to increased insulin resistance. The relationships of EPO with C peptide were analysed in the male and female sample after exclusion of samples with deficiencies. The higher hemoglobin in males will not increase EPO and, therefore, EPO was not related to C peptide. In the female sample, insulin resistance is lower. Therefore, EPO directly correlated with PTH and with C peptide.

Conclusion and Significance: The increased EPO resulted in increased insulin secretion in the female sample. The direct correlation of EPO with C peptide in females with decreased in insulin resistance indicated increase in insulin secretion. As insulin resistance is lower in females, EPO increased insulin secretion.

5.2. Introduction

Connecting peptide (C peptide), a cleavage product of proinsulin, is secreted by pancreatic β-cells in equimolar amounts along with insulin. This phenomenon has been exploited to assess prehepatic insulin secretion in humans (Orci et al, 1988). Although a considerable amount of insulin is extracted by the liver, C peptide is subjected to negligible first pass metabolism by the liver, thereby serving as a surrogate marker for endogenous insulin secretion. Therefore, it has been suggested that peripheral C peptide levels more closely reflect pancreatic insulin secretion than do peripheral insulin levels (Polonsky et al, 1986). As it is considered as an inert by-product of insulin synthesis, C peptide has been has also been of great value in the understanding of the characteristics of type 1 and type 2 diabetes mellitus.

However, C peptide has recently been re-evaluated as a bioactive peptide in its own right. It was believed that C peptide was important for ensuring correct folding insulin. Studies have also showed that C peptide exerts direct effect on renal function, to augment glucose utilization and to improve autonomic nervous function in insulin dependent diabetes mellitus, as well as insulin secretion. All these effects could be mediated by direct impact of C peptide on $Na^{+}K^{+}$-ATPase activity in various tissues (Wahren et al, 2000). Although insulin and C peptide are secreted into the portal circulation in equimolar amounts, fasting concentration of C peptide are 5-10 fold higher than those of insulin due to longer half life of C peptide (about 35 minutes). C peptide is removed from circulation by the kidneys and degraded with a fraction exerted unchanged in urine.

EPO is under investigation for the treatment of variety of diseases, but provides exciting prospects for the treatment of diabetes mellitus (Zhang et al, 2014). In experimental animal models of DM, EPO reduce blood glucose levels and obesity (Katz, 2010). EPO also protect against the detrimental effects of obesity (Zhang et al, 2014) and can treat diabetic peripheral neuropathy (Chattopadhyay et al, 2009) in animal models. EPO has been shown to reduce oxidative stress in renal tubular cells due to high glucose level (Dang et al, 2010). Choi et al through their studies showed that recombinant human EPO treatment can protect against diabetes development in type 1 (streptozotocin model) and type 2 (db/db mouse model) diabetes. Protection in these models was mediated by JAK2 signalling pathways directly in pancreatic β cells, which exert antiapoptotic, proliferative, anti-inflammatory, and angiogenic effects (Choi et al, 2010). In nonerythroid cells EPO can signal via a heterodimeric receptor composed of an EPOR monomer chain and CD131 (Brines and Cerami, 2006). EPO deficiency and a higher incidence of anemia in diabetic patients suggests the potential beneficial effects of EPO in the setting of diabetes (McGill and Bell, 2006; Thomas, 2006).

As EPO was found to have potential beneficial role in type 2 diabetes, the role EPO in relation to insulin sensitivity and secretion were studied. In this study, the correlations of EPO with fasting C peptide and the related analytes were examined.

5.3 Objectives

5.3.1. Aspects of the Original Objectives Addressed in this Chapter

C peptide, EPO, PTH and their related analytes which are regulated in the healthy state, may vary in relation to their regulatory factors in diseases states. Insulin, EPO and PTH are involved in a number of regulatory functions. Secretion of insulin, measured as C peptide, is regulated by a number of factors. They may cause or result in variations in the factors that are regulated by EPO, PTH and C peptide. In this study, we examine

1. Correlation and significant variations in the concentrations of C peptide, EPO and PTH along with other analytes in healthy individuals,

2. Identification of the influence of such correlations and significant variations in disease conditions and deficiencies.

5.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. **These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine**

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment.

5.4 Materials and Methods

5.4.1. Study Setting and Research Design

Please refer 2.1. Study setting and Research design section in chapter 2

5.4.2. Selection of Participants, Inclusion Criteria and Exclusion of Subclinical Disease States and Deficiencies by Clinical Biochemistry Assays

Healthy cross section of participants (n = 102) 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Table 2.1; Stage I).

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. 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 (Table 2.1; Stage II).

C peptide samples were selected after excluding growth phase at <18 years and influence of age at >25 years (18 to 25 years) (Table 5.1, 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, EPO <3.5 IU/L (Table 5.1; Stage IV). These stringent exclusion criteria reduced the sample number from over 600 to 102. Even after selection of C peptide 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 C peptide sample (n = 102) was studied before (Table 5.2) and after (Table 5.3, 5.4 and 5.5) 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.

5.4.3. Sample Collection and Sample Preparation

Please refer section 2.3. Sample collection in chapter 2

5.4.4. Analytical Control and Assays

Immunochemistry autoanalyser Access 2 (Beckman Coulter, USA) and their reagents were used for intact PTH, EPO, insulin and ferritin assays using immunometric method with magnetic bead coated anti PTH, anti EPO or anti ferritin antibodies (Procedure manual, Beckman Coulter, 2005). The chemistry autoanalyser Vitros 5,1 FS (Ortho Clinical Diagnostics, USA) and their reagents were used for assay of glucose and iron (Procedure manual, Ortho Clinical Diagnostics, 2004). Vitamin D and C peptide assays were done by Diasorin Liaison (Italy) (Procedure manual, Liaison, 2017). Hemoglobin estimation was done manually by Drabkin's method using colorimeter. **Please refer detailed description of these assays in chapter 2.**

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 (Klee & Westgard, 2012). 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 was done on the days of C peptide, EPO and intact PTH assays.

5.4.5. Diagnostic Criteria and Reference Interval

Please refer section 2.26. Diagnostic criteria and reference interval in chapter 2.

5.4.6. Inter conversion of Units of variables

Please refer Chapter 2, Methodology. **Table 2.2**. Conversion factors for interconversion between conventionl unit and SI unit.

5.4.7. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer 2.27. Statistical analysis section in chapter 2

5.5. Results

5.5.1. Selection of Participants

Participants of this study were partitioned into males and females at all stages of sample selection. 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 (Chapter 2 and 3, Tables 2.1 & 3.1, Stage I and II). The sample number in the male participants decreased much more that in females at these stages. Sample was partitioned according to age. Participants selected were between ages 18 and 25 years to exclude influence of growth phase and age (Table 5.1, Stage III). Selection of participants was done after excluding some diseases related to analytes used in this study such as samples with high ferritin, osteocalcin, ostase, NTx and low EPO (Table 5.1, Stage IV). The strict exclusion criteria reduced the C peptide sample size to 102 from the starting number >600 (Stage I) and further reduced during exclusion of different deficiencies to 24 (Table 5.1; Stage V, Step 8).

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, Step 8 (Chapter 5, Table 5.1).

5.5.2. Visual Evaluation of Distribution of C Peptide in the Sample at Various Phases of Exclusion

Distribution of C peptide in the C peptide sample (n = 102) was found to be positively skewed by histogram (Fig. 5.1A). The normal Q-Q plot showed that the distribution was not Gaussian. There was only one outlier (Fig. 5.1B and C).

The C peptide sample was partitioned in to male and female groups. The male sample (n = 27) was found to have near Gaussian distribution (Fig. 5.2A) but after $\log_{10}$ transformation was found to have Gaussian distribution by Q-Q plot and Shapiro-Wilk test (Fig. 5.2B and Table 5.2.). The female sample (n = 75) showed Gaussian distribution before and after $\log_{10}$ transformation (Fig. 5.2C and D; Table 5.2). Fasting Insulin and PTH also had Gaussian distribution after $\log_{10}$ transformation both in the male and female sample (Table 5.2.).

5.5.3. Gender Differences in the Concentration of Analytes before and after Exclusion of Deficiencies

There were borderline gender differences in fasting C peptide, with increased concentration in males. Iron was lower in the female sample resulting in increased EPO in the female sample (Table 5.2).

After excluding vitamin D deficiency, gender difference in iron decreased resulting in borderline gender difference in EPO, and loss of gender difference in C peptide (Table 5.3).

After excluding hemoglobin, iron and ferritin deficiencies, gender difference in iron decreased resulting in loss of gender difference in EPO, and there were gender differences in C peptide and insulin (Table 5.4), with higher concentrations in males.

After excluding all four deficiencies, gender difference was seen only in iron. Fasting insulin showed borderline gender difference with higher concentrations in males. There was loss in the gender difference in C peptide (Table 5.5).

When deficiencies of hemoglobin iron and ferritin were excluded, EPO levels decreased and iron levels increased (Table 5.6).

5.5.4. Deficiencies Influenced the Correlations of EPO and C Peptide with Analytes and Showed Complex Gender Differences

In the female sample, in the presence of deficiencies, EPO positively correlated with PTH, C peptide and insulin, but not in the male sample. Also, in the female C peptide sample, EPO negatively correlated with hemoglobin, iron and ferritin (Table 5.7A).

After exclusion of vitamin D deficiency, the correlations of EPO in the female sample did not change. But in the male sample, EPO showed a negative correlation with hemoglobin and there was no significant positive correlation with PTH and ferritin (Table 5.7B).

After exclusion of hemoglobin, iron and ferritin deficiencies, the correlations of EPO in the female sample with C peptide and insulin did not change. But in the male sample, EPO did not show any significant correlations (Table 5.7C).

After exclusion of all four deficiencies, the correlations of EPO in the female sample correlated with C peptide and PTH. In both male and female sample, EPO showed a negative correlation with hemoglobin and iron, there was a positive correlation with PTH (Table 5.7D).

The above observations were confirmed by the X-Y scatter of C peptide with EPO before and after exclusion of deficiencies (Fig. 5.3A to H).

As above, the correlations of C peptide with EPO were consistent in the presence and absence of deficiencies. But in the presence of deficiencies, the correlations were complex and could not be explained easily (Table 5.8).

5.6. Discussion

C peptide is a marker of insulin secretion (Orci et al, 1988, Polonsky et al, 1986) and is more reliable than serum insulin levels. Hyperinsulinemia is more related to insulin resistance, but insulin secretion increases in hyperinsulinemia. Therefore, all these issues are interrelated. EPO was found to be having beneficial in type 2 diabetes mellitus (McGill and Bell, 2006; Thomas, 2006). These beneficial effects of EPO have been

reported to be mediated through the decrease in insulin resistance (Mak 1996; Pan et al, 2013). In this study the relationship of EPO and C peptide with each other and with related analytes evaluated.

5.6.1 Gender Difference in C Peptide

The higher C peptide in the male sample was expected, as insulin resistance was higher in the male sample (Table 5.2). The gender difference in the C peptide persisted after excluding hemoglobin, iron and ferritin deficiencies. But the gender difference was lost after excluding vitamin D deficiency. At present the role of vitamin D in decreasing gender difference of C peptide cannot be explained. But when hemoglobin and iron deficiencies were excluded EPO level decrease (Table 5.6). This could be explained as EPO levels are inversely related to hemoglobin and iron (Nesheera et al, 2017).

5.6.2 Correlations of EPO and C Peptide

In the female sample there was a strong direct correlation between EPO and C peptide and this relationship was not influenced by the presence or absence of any of the four deficiencies (Table 5.6). This result indicates that EPO was involved in increase in insulin secretion. The absence of correlation of EPO with C peptide in the male sample may be due to the interference of insulin resistance which also increases insulin secretion probably independent of EPO.

EPO also correlates positively with PTH in the female sample independent of the presence and absence of deficiencies (Table 5.7). In the male sample EPO directly correlates with PTH after exclusion of all deficiencies. This gender difference in the correlation may be due to the differences in the relationship between EPO and PTH in the male and female sample.

As insulin secretion is increased by insulin resistance and by EPO, C peptide should be correlating with insulin. C peptide should be positively correlating with insulin in both males and females (Table 5.8A). This correlation which was present in the presence of deficiencies, was absent when all deficiencies were excluded (Table 5.8D). These results indicate that deficiencies may be related to the correlation of C peptide and insulin.

But the correlation of C peptide with EPO was present only in the female sample. In the female sample after exclusion of all deficiencies C peptide was also directly related to fasting glucose concentration with border line significance (Table 5.8 C and D).

These results indicate the correlations of C peptide and EPO are complex but there is an indication of increased insulin secretion by EPO.

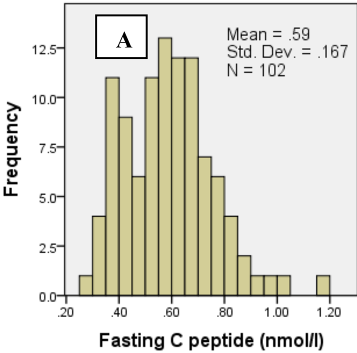
5.7. Conclusion

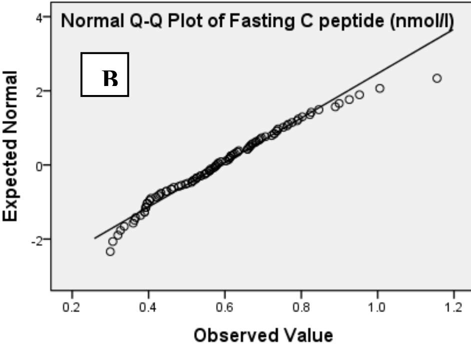
Insulin secretion is increased in the presence of insulin resistance. EPO has been shown to have a beneficial role in type 2 diabetes and was proposed to be due to decrease in insulin resistance. Insulin resistance is higher in the male sample. Hemoglobin was also higher in the male sample resulting in lower EPO. The lower EPO in the male sample did not decrease C peptide and was not inversely related to C peptide. In the female sample, it was observed that EPO directly correlated with C peptide which indicated increased C peptide with increased insulin secretion and EPO. As insulin resistance is lower in females, EPO increased insulin secretion.

Table 5.1. Number of participants (n) at various stages (III to V) and steps (4 to 8) of implementation of exclusion criteria for selection of the C peptide sample population (n = 102; at Stage IV) and partitioning of samples according to age (18 – 25 years) and gender (males, M & females, F).

Stages	**Steps**	**Exclusion at various stages for selection of reference C peptide sample population**	**Sample number at various phases of clinical exclusion**		
			Total (M+F), n	**Male, n (% males excluded at each stage)**	**Female, n (% females excluded at each stage)**
III (3 to 4)	**4c.**	Participants after exclusion of growth phase <18 years and the influence of age >25 years (aged 18 to 25 years)	**136**	**45**	**91**
IV (4 to 5)	**5.**	C peptide samples selected for this study after excluding ferritin >250, osteocalcin>35, ostase>30, NTx>200. Age 18 -25 years	102	27 (18/45 = 40%)	75 (16/91 = 17.58%)
V (5 to 6, 5 to 7 5 to 8)	**6.**	C peptide samples after excluding vitamin D deficiency <50nmol/l	42	14 (13/27 = 48.15%)	28 (47/75 = 62.67)
	7.	C peptide sample after excluding hemoglobin<125 g/l, Iron <9.85 µmol/l and ferritin <20 ng/ml,	47	24 (3/27 = 11.11%)	23 (52/75 = 69.33)
	8.	C peptide sample after excluding hemoglobin<125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml, and vitamin D<50 nmol/l	24	11 (16/27 = 59.26%)	13 (62/75 = 82.67)

Fig. 5.1. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of fasting C peptide in the C peptide sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 102)





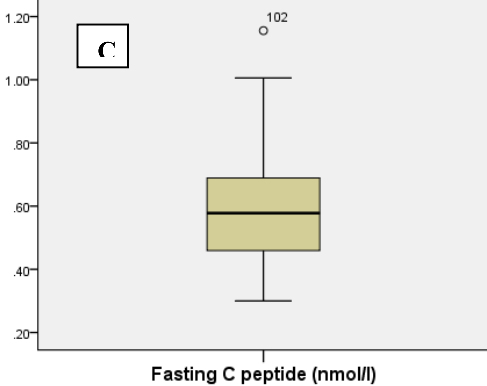


Fig. 5.2. Normal probability Quantile-Quantile (Q-Q) Plot of fasting C peptide in **male (n = 27) (A, B)** and **female (n = 75) (C, D)** C peptide sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays. Before **$\log_{10}$ transformation** (A, C), and after **$\log_{10}$ transformation (B, D)**

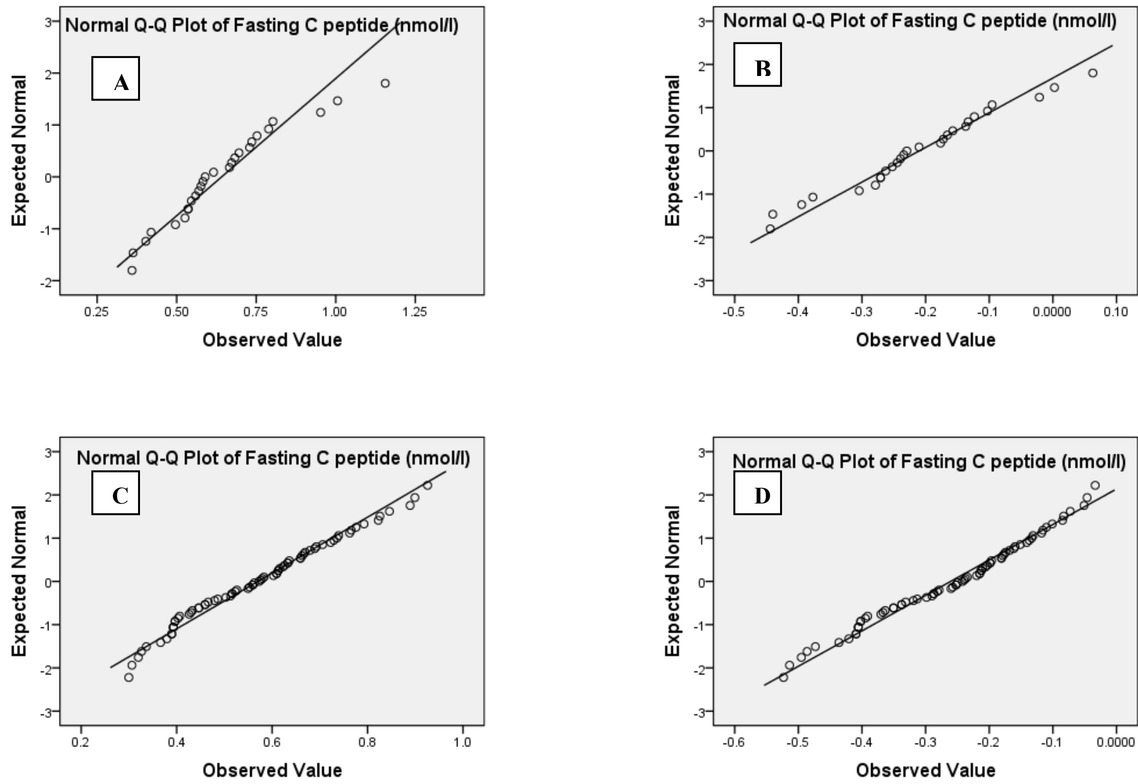


Table 5.2. Comparison of gender differences in analytes related to fasting C peptide and EPO in the C peptide sample. The Shapiro-Wilk test for normality and Levene's test for equality of variances of male (M) and female (F) samples were analysed. The gender differences were compared by 95% CI of mean, Student t test (parametric method) or Mann Whitney U test (non parametric method).

Variables in SI units	**Gender differences in concentration of analytes related to C peptide and EPO in the C Peptide sample**					
	M (n = 27) Mean±SD Minimum - Maximum 95% CI mean	**F (n = 75) Mean±SD Minimum - Maximum 95% CI mean**	**After $\log_{10}$ transformation**			
			Shapiro - Wilk test, P		**Levene's test, P**	**Student t test (Mann-Whitney U test), P**
			M	F		
S. EPO (U/L)	6.76±1.94 4.03 – 11.59 6.00 – 7.53	8.86±5.78 3.67 – 36.10 7.53 – 10.19	0.434	<0.001	0.105	**(0.060)**
S. Iron (µmol/l)	23.46±5.64 11.10 – 39.20 21.23 – 25.69	16.39±6.78 2.51 – 34.55 14.83 – 17.95	0.305	0.005	0.010	**(<0.001)**
Fasting Glucose (mmol/l)	4.81±0.393 4.01 – 5.94 4.66 – 4.97	4.84±0.500 4.05 – 6.70 4.72 – 4.95	0.073	0.002	0.146	(0.799)
Fasting Insulin (pmol/l)	41.76±14.78 17.92 – 85.22 35.91 – 47.61	39.07±16.26 14.17 – 88.90 35.33 – 42.81	0.934	0.957	0.199	0.291
Fasting C peptide (nmol/l)	0.642±0.189 0.36 – 1.16 0.567 – 0.716	0.570±0.155 0.30 – 0.93 0.534 – 0.606	0.770	0.147	0.754	**0.070**
Intact PTH (ng/l)	27.22±10.18 11.90 – 59.90 23.11 – 31.34	33.75±16.39 10.40 – 80.50 29.98 – 37.52	0.223	0.398	0.045	(0.147)
Vitamin D (nmol/l)	54.08±25.21 12.46 – 115.1 44.11 – 64.05	48.51±25.74 13.2 – 125.1 42.59 – 54.43	0.030	0.074	0.657	0.341

Table 5.3. Comparison of fasting C peptide and analytes related to fasting C peptide in male (M) and female (F) samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding vitamin D <50nmol/l. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of gender differences of analytes after excluding Vitamin D < 50 nmol/l					
	M (n = 14) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 28) Mean±SD Minimum - Maximum 95% CI of mean	after $\log_{10}$ transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			M	F		
S. EPO (U/L)	6.05±1.53 4.03 – 9.94 4.03 – 9.94	7.64±2.57 3.67 – 12.94 6.65 – 8.64	0.812	0.383	0.067	0.058
S. Iron (µmol/l)	23.81±6.35 15.39 – 39.20 20.14 – 27.47	17.94±6.08 5.37 – 31.15 15.59 – 20.30	0.883	0.086	0.343	0.007
Fasting Glucose (mmol/l)	4.86±0.364 4.44 -5.94 4.65 – 5.07	4.69±0.408 4.05 – 5.66 4.53 – 4.85	0.007	0.412	0.318	(0.210)
Fasting Insulin (pmol/l)	39.64±9.11 24.31 – 54.17 34.37 – 44.90	38.96±17.25 16.25 – 82.09 32.27 – 45.65	0.862	0.810	0.055	0.515
Fasting C peptide (nmol/l)	0.603±0.115 0.40 – 0.79 0.537 – 0.670	0.562±0.156 0.30 – 0.90 0.501 – 0.623	0.382	0.593	0.060	0.289
Intact PTH (ng/l)	25.93±9.34 11.90 – 47.30 20.29 – 31.57	31.96±15.64 10.40 – 72.10 25.90 – 38.03	0.557	0.376	0.385	0.329
Vitamin D (nmol/l)	72.06±19.57 50.00 – 115.07 60.77 – 83.36	75.81±20.59 50.00 – 125.10 67.83 – 83.79	0.466	0.332	0.745	0.579

Table 5.4. Comparison of fasting C peptide and analytes related to fasting C peptide in male (M) and female (F) samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding Hemoglobin<125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of gender differences of analytes after exclusion of anemia, iron and ferritin deficiencies					
	M (n = 24) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 23) Mean±SD Minimum - Maximum 95% CI of mean	after log_{10} transformation			
			Shapiro - Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			M	F		
S. EPO (U/L)	6.81±1.84 4.62 – 11.59 6.03 – 7.58	6.20±2.05 3.67 – 12.35 5.31 – 7.09	0.249	0.692	0.528	0.191
S. Iron (µmol/l)	23.53±5.89 11.10 – 39.20 21.04 – 26.02	19.88±5.96 13.07 – 34.55 17.31 – 22.46	0.389	0.050	0.386	0.032
Fasting Glucose (mmol/l)	4.77±0.343 4.01 – 5.74 4.63 – 4.91	4.88±0.442 4.27 – 5.92 4.69 – 5.07	0.395	0.153	0.258	0.365
Fasting Insulin (pmol/l)	41.96±15.58 17.92 – 85.22 35.38 – 48.54	34.98±13.42 18.06 – 66.39 29.18 – 40.78	0.940	0.419	0.498	0.084
Fasting C peptide (nmol/l)	0.645±0.198 0.36 – 1.16 0.561 – 0.728	0.546±0.163 0.30 – 0.90 0.476 – 0.616	0.862	0.288	0.502	0.068
Intact PTH (ng/l)	27.41±10.79 11.90 – 59.90 22.74 – 32.07	33.37±17.17 10.40 – 61.60 25.94 – 40.80	0.415	0.131	0.052	0.381
Vitamin D (nmol/l)	51.32±23.39 12.46 – 105.08 41.44 – 61.20	57.59±25.64 13.20 – 100.09 46.51 – 68.68	0.030	0.203	0.948	(0.419)

Table 5.5. Comparison of fasting C peptide and analytes related to fasting C peptide in male (M) and female (F) samples by 95% CI of mean and Student t test (Mann Whitney U test), after excluding hemoglobin<125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml, and vitamin D<50 nmol/l. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male and female samples were analysed.

Variables in SI units	Comparison of gender differences of analytes after exclusion of vitamin D, anemia, iron and ferritin deficiencies					
	M (n = 11) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 13) Mean±SD Minimum - Maximum 95% CI of mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			M	F		
S. EPO (U/L)	5.95±1.03 4.62 – 7.95 5.25 – 6.64	6.31±2.47 3.67 – 12.35 4.82 – 7.81	0.869	0.768	0.031	(0.908)
S. Iron (µmol/l)	24.05±7.01 15.39 – 39.20 19.34 – 28.76	18.71±4.82 14.68 – 31.15 15.80 – 21.63	0.722	0.022	0.649	**(0.034)**
Fasting Glucose (mmol/l)	4.78±0.218 4.44 – 5.14 4.63 – 4.93	4.83±0.423 4.27 – 5.66 4.57 - 5.08	0.307	0.377	0.130	0.794
Fasting Insulin (pmol/l)	39.49±9.94 24.31 – 54.17 32.81– 46.17	31.36±11.17 18.06 – 51.18 24.62 – 38.11	0.737	0.391	0.279	**0.059**
Fasting C peptide (nmol/l)	0.600±0.122 0.40 – 0.79 0.518 – 0.682	0.520±0.173 0.30 – 0.90 0.415 – 0.624	0.506	0.962	0.126	0.146
Intact PTH (ng/l)	25.96±10.65 11.90 – 47.30 18.34 – 33.58	31.14±17.32 10.40 – 61.60 20.68 – 41.60	0.636	0.435	0.487	0.608
Vitamin D (nmol/l)	70.95±16.19 50.00 – 105.08 60.07 – 81.83	75.32±18.07 50.00 – 100.09 64.39 – 86.24	0.980	0.286	0.448	0.571

Table 5.6. Influence of exclusion of samples deficient in vitamin D (<50 nmol/l), hemoglobin (HB <125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml) (A to C) on the concentrations of fasting C peptide, EPO and their related parameters. Concentrations were compared before (Table 2) and after exclusion of deficiencies in males (M & M) and in females (F & F). Comparisons were done by Student t test (or Mann Whitney U test). Increase (↑) and decrease (↓) of mean is shown.

Variables in SI units	**A. Before M (n = 27), F (n = 75) and after exclusion of vitamin D deficient samples. M (n = 14), F (n = 28)**		**B. Before M (n = 27), F (n = 75) and after exclusion of HB, Iron, ferritin deficient samples. M (n = 24), F (n = 23)**		**C. Before M (n = 27), F (n = 75) and after exclusion of vitamin D, HB, iron, ferritin deficiencies. M (n = 11), F (n = 13)**	
	Comparisons before and after sample exclusions by Student t test (Mann-Whitney U test), P					
	M & M	**F & F**	**M & M**	**F & F**	**M & M**	**F & F**
S. EPO (U/L)	0.241	(0.772)	0.879	**(0.005)** ↓	0.243	**(0.034)**↓
S. Iron (µmol/l)	0.879	(0.223)	0.995	**(0.025)** ↑	0.853	(0.148)
Fasting Glucose (mmol/l)	(0.710)	(0.313)	0.686	(0.508)	0.844	(0.764)
Fasting Insulin (pmol/l)	0.828	0.912	0.992	0.315	0.791	0.113
Fasting C peptide (nmol/l)	0.653	0.809	0.991	0.473	0.624	0.232
Intact PTH (ng/l)	0.704	0.587	0.996	0.736	0.658	0.438
Vitamin D (nmol/l)	**(0.012)** ↑	**(<0.001)** ↑	(0.734)	0.125	**(0.019)** ↑	**(<0.001)**↑

Table 5.7. Correlation (r) and significance of correlation (P) of EPO with fasting glucose, fasting insulin, fasting C peptide, intact PTH, vitamin D, hemoglobin, iron and ferritin in males and in females, before (A) and after (B, C and D) exclusion of samples deficient in vitamin D (<50 nmol/l), hemoglobin (anemia, <125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml) by parametric (Pearson's, r) and non parametric (Spearman's, ρ) methods.

Variables	Pearson's, r (Spearman's rho ρ)	P	Pearson's, r (Spearman's rho ρ)	P
A. Correlations of EPO (before exclusion of deficiencies)				
	Male (n = 27)		**Female (n = 75)**	
Fasting Glucose	-0.290	0.142	(0.078)	0.504
Fasting Insulin	-0.097	0.630	**(0.274)**	**0.017**
Fasting C peptide	-0.030	0.881	**(0.319)**	**0.005**
Intact PTH	-0.061	0.768	**(0.410)**	**<0.001**
Vitamin D	-0.192	0.338	(-0.138)	0.239
Hemoglobin	-0.057	0.779	**(-0.341)**	**0.003**
S. Iron	-0.064	0.753	**(-0.330)**	**0.004**
Ferritin	0.155	0.441	**(-0.509)**	**<0.001**
B. Correlations of EPO after exclusion of vitamin D deficiency				
	Male (n = 14)		**Female (n = 28)**	
Fasting Glucose	-0.258	0.373	-0.088	0.657
Fasting Insulin	-0.061	0.836	**0.443**	**0.018**
Fasting C peptide	0.054	0.855	**0.449**	**0.016**
Intact PTH	**0.346**	0.226	**0.563**	**0.002**
Vitamin D	-0.228	0.432	0.133	0.501
Hemoglobin	**-0.531**	**0.050**	**-0.376**	**0.049**
S. Iron	-0.197	0.501	**-0.418**	**0.027**
Ferritin	**0.394**	0.163	**-0.397**	**0.037**
C. Correlations of EPO after exclusion of anemia, iron and ferritin deficiencies				
	Male (n = 24)		**Female (n = 23)**	
Fasting Glucose	-0.123	0.568	0.154	0.484
Fasting Insulin	-0.098	0.650	0.180	0.412
Fasting C peptide	-0.006	0.977	**0.476**	**0.022**
Intact PTH	-0.091	0.681	**0.410**	**0.052**

Vitamin D	-0.152	0.479	-0.091	0.679
Hemoglobin	-0.058	0.789	**-0.637**	**0.001**
S. Iron	-0.129	0.549	-0.263	0.226
Ferritin	0.015	0.946	0.179	0.414
D. Correlations of EPO after exclusion of vitamin D, anemia, iron and ferritin deficiencies				
	Male (n = 11)		**Female (n = 13)**	
Fasting Glucose	0.429	0.188	0.179	0.558
Fasting Insulin	-0.041	0.905	0.237	0.435
Fasting C peptide	0.231	0.495	**0.525**	**0.065**
Intact PTH	**0.438**	0.206	**0.691**	**0.009**
Vitamin D	-0.105	0.758	0.160	0.603
Hemoglobin	**-0.740**	**0.009**	**-0.698**	**0.008**
S. Iron	**-0.494**	0.122	**-0.494**	**0.086**
Ferritin	0.166	0.626	0.313	0.297

Fig. 5.3. X-Y scatter diagram, of EPO with fating C peptide in **males (A – D)** and **females (E – H)**, before (A, E), after exclusion of vitamin D <50 nmol/l (B, F), after exclusion of Hemoglobin <125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml (C, G) and after exclusion of Hemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50 nmol/l (D, H).

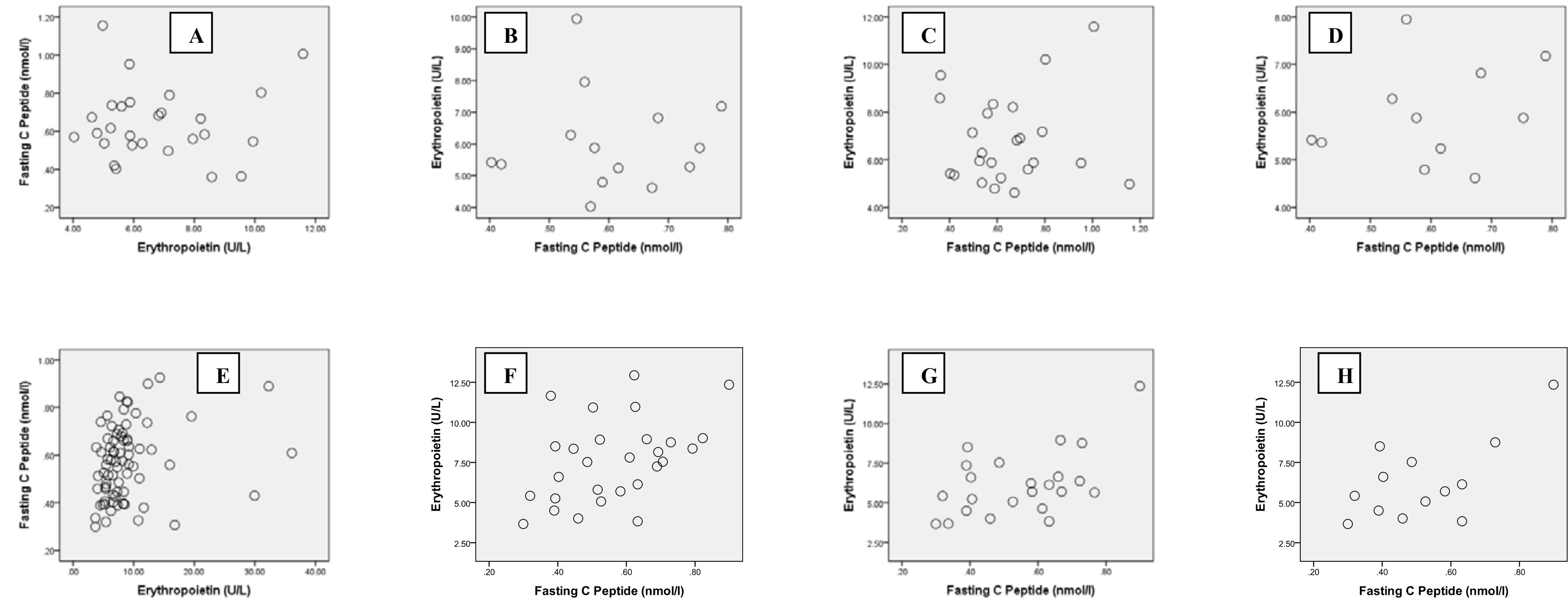


Table 5.8. Correlation (r) and significance of correlation (P) of fasting C peptide with EPO, fasting glucose, fasting insulin, intact PTH, vitamin D, hemoglobin, iron and ferritin in males and in females, before (A) and after (B, C and D) exclusion of samples deficient in vitamin D (<50 nmol/l), hemoglobin (anemia, <125 g/l), iron (<9.85 µmol/l) and ferritin (<20 ng/ml). Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Pearson's, r	P	Pearson's, r	P
A. Correlations of C peptide (before exclusion of deficiencies)				
	Male (n = 27)		**Female (n = 75)**	
EPO	-0.030	0.881	0.280	**0.015**
Fasting Glucose	0.427	**0.026**	0.009	0.940
Fasting Insulin	0.673	**<0.001**	0.615	**<0.001**
Intact PTH	0.082	0.690	-0.003	0.982
Vitamin D	-0.101	0.617	0.069	0.558
Hemoglobin	0.015	0.943	0.077	0.511
S. Iron	-0.079	0.694	-0.017	0.883
Ferritin	0.202	0.312	-0.112	0.340
B. Correlations of C peptide after exclusion of vitamin D deficiency				
	Male (n = 14)		**Female (n = 28)**	
EPO	0.054	0.855	**0.449**	**0.016**
Fasting Glucose	-0.017	0.953	0.015	0.938
Fasting Insulin	0.195	0.505	**0.534**	**0.003**
Intact PTH	0.111	0.704	-0.084	0.669
Vitamin D	0.395	0.162	**0.351**	**0.067**
Hemoglobin	-0.001	0.998	0.110	0.577
S. Iron	-0.356	0.211	-0.049	0.803
Ferritin	0.001	0.999	-0.144	0.466
C. Correlations of C peptide after exclusion of anemia, iron and ferritin deficiencies				
	Male (n = 24)		**Female (n = 23)**	
EPO	-0.006	0.977	**0.476**	**0.022**
Fasting Glucose	**0.541**	**0.006**	**0.348**	**0.103**
Fasting Insulin	**0.667**	**<0.001**	**0.464**	**0.026**
Intact PTH	0.102	0.644	0.118	0.593
Vitamin D	-0.140	0.513	-0.124	0.573

Hemoglobin	0.025	0.908	-0.264	0.223
S. Iron	-0.052	0.808	-0.155	0.479
Ferritin	0.186	0.385	-0.077	0.726
D. **Correlations of C peptide after exclusion of vitamin D, anemia, iron and ferritin deficiencies**				
	Male (n = 11)		**Female (n = 13)**	
EPO	0.231	0.495	**0.525**	**0.065**
Fasting Glucose	0.022	0.950	**0.450**	**0.123**
Fasting Insulin	0.116	0.733	0.216	0.477
Intact PTH	0.332	0.349	0.096	0.754
Vitamin D	0.257	0.445	0.357	0.231
Hemoglobin	0.104	0.760	-0.043	0.889
S. Iron	-0.298	0.374	-0.266	0.379
Ferritin	-0.230	0.497	0.024	0.937

NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 6.

Gender Differences in Concentrations and Correlations of Fasting Insulin, Before and After Exclusion of Deficiencies in Hemoglobin, Iron and Ferritin

6.1. Abstract

Deficiencies in hemoglobin, iron and ferritin were more in females, resulting higher concentrations of EPO in females, which increased insulin secretion. Increased fasting insulin secretion increased fasting C peptide. One of the causes of increase in fasting insulin or hyperinsulinaemia is insulin resistance. Insulin resistance increases insulin secretion as a compensatory mechanism. In males insulin resistance is higher than in females resulting in increased fasting insulin in males. EPO has been shown to be beneficial for type 2 diabetes, probably due to decrease in insulin resistance. In this study we have shown that there was no gender difference in the fasting insulin concentration, and fasting insulin correlated with EPO in the female sample and not in the male sample. This gender difference may be due to EPO decreasing insulin resistance in males, resulting in decrease of insulin secretion. But increase in insulin secretion directly by EPO may be confounding the correlation between insulin, C peptide and EPO in the male sample. This confounding was less in the female sample where EPO correlated with insulin before exclusion of deficiencies. But exclusion of deficiencies EPO decreased in the female sample resulting in loss of gender differences in the correlation of EPO with fasting insulin.

6.2. Introduction

6.2.1. Insulin Resistance and Hyperinsulinemia

Insulin resistance is a condition in which insulin produces a biological response which is lower than expected. The compensatory increase in insulin secretion results in hyperinsulinemia during insulin resistance. Insulin resistance also refers to impaired sensitivity to insulin mediated glucose disposal. The compensatory hyperinsulinaemia is to maintain normal blood glucose levels in the setting of peripheral insulin resistance in muscle and adipose tissue (Wilcox, 2005). Insulin resistance can be divided into two – decreased insulin sensitivity (normal response but increased hormonal level) and decreased responsiveness (normal level of hormone but less response).

6.2.2. Causes of Insulin Resistance

Insulin resistance can be seen in type 2 diabetes, obesity, hypertension, polycystic ovarian disease, a variety of genetic syndromes, and in physiologic conditions such as puberty and pregnancy (Kahn, 1978). Insulin resistance is also present in many states of stress, in association with infection and secondary to treatment with a variety of drugs, particularly glucocorticoids. Insulin resistance plays an important role in type 2 diabetes and metabolic syndrome. In type 2 diabetes studies showed that there is a decrease in insulin receptor concentration, in receptor kinase activity, in the concentration and phosphorylation of IRS-1 and 2, in PI3-kinase activity, in glucose-transporter translocation and defects in activity of intracellular enzyme; but there is no reduction in the insulin action on MAP-kinase pathway (Reusch, 2002).

6.2.3. Beneficial Effects of EPO on Type 2 Diabetes Mellitus

Though the primary action of EPO in adults is on erythropoiesis, there are actions of EPO on non erythropoietic cells (Brines and Cerami, 2006; Hand and Brines, 2011). These multiple targets of EPO have been mediated through different cell signalling pathways (Broxmeyer, 2013).

When EPO was used in patients with anemia from renal diseases, it has been shown to decrease insulin resistance (Khedr et al, 2009; Tuzcu et al, 2004). In transgenic mice EPO lowers blood sugar, insulin and HBA1c (Katz, et al, 2010; Hojman, et al, 2009). EPO receptor in null mice develops insulin resistance (Teng, et al, 2011). Therefore, it was assumed that EPO may regulate glucose tolerance and insulin sensitivity. EPO was also found to regulate glucose metabolism (Saltiel and Kahn, 2011). The potential mechanism by which EPO improves glucose tolerance and regulates glucose metabolism was found to be by improving glucose intolerance by inhibiting gluconeogenesis and the inflammatory response from liver (Cornier, et al, 2008). EPO was also found to protect against diabetes by cytoprotection of pancreatic B-cells (Choi, et al, 2010).

6.2.4. Consequences of Insulin Resistance

Studies showed that insulin resistance in muscle leads to increased accumulation of fat and secondary Insulin resistance, hypertriglyceridemia and increased levels of free fatty acids. Insulin resistance in liver leads to increased hepatic glucose output. Insulin resistance in brain leads to increased appetite, more obesity and further defect in hepatic glucose output. Finally Insulin resistance in β-cell leads to defect in glucose sensing and thereby leads to relative insulin deficiency. Thus Insulin resistance in multiple tissues could produce all of the defects associated with type 2 diabetes and treatment that improves insulin sensitivity would be expected to improve all defects.

6.2.4.1. Free fatty acids and intracellular triglycerides in insulin resistance

Central obesity is associated with insulin resistance. Central adipocytes are more resistant to insulin-inhibition of lipolysis, and thus cause an increase in the delivery of free fatty acids to liver. These results in accumulation of triglycerides, which in turn increases hepatic glucose output, reduced hepatic extraction of insulin and thus hepatic insulin resistance. This increased free fatty acids and insulin resistance in type 2 diabetes could be the cause for accumulation of triglycerides in muscles.

6.2.4.2. Effect of obesity on insulin resistance

Obesity is an important factor in the pathogenesis of insulin resistance and substantially increases the risk of type 2 diabetes (Vague, 1996). Lifestyle and environmental factors also determine the development of glucose intolerance (Hamman, 1992). Beta cell dysfunction plays a crucial role in type 2 diabetes mellitus. Insulin secretion and tissue insulin resistance are of equal importance in the development of the disease. Type 2 diabetes is a progressive disease.

In this chapter, the gender differences in the concentrations of various analytes and relationships of EPO with fasting insulin and their related analytes were investigated. This was done to study the possible influence of EPO on fasting insulin levels and insulin resistance in healthy young adults.

6.3. Objectives

6.3.1. Aspects of the Original Objectives Addressed in this Chapter

Insulin, EPO, PTH and their related analytes which are regulated in the healthy state, may vary in relation to their regulatory factors in diseases states. Insulin, EPO and PTH are involved in a number of regulatory functions. Concentration of circulating insulin is regulated by a number of factors, including insulin resistance. Disease states may be the cause or result of variations in EPO, PTH and Insulin. In this study, we examine

1. Correlation and significant variations in the concentrations of Insulin, EPO and PTH along with other analytes in healthy individuals,

2. Identification of the influence of such correlations and significant variations in disease conditions and deficiencies.

6.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. **These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine**

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment.

6.4. Materials and Methods

6.4.1. Study Setting and Research Design

Please refer 2.1. Study setting and Research design section in chapter 2

6.4.2. Selection of Participants, Inclusion Criteria and Exclusion of Subclinical Disease States and Deficiencies by Clinical Biochemistry Assays

Healthy cross section of participants (n = 146) 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Table 2.1; Stage I).

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. 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 (Table 2.1; Stage II).

Insulin samples were selected after excluding growth phase at <18 years and influence of age at >25 years (18 to 25 years) (Table 6.1, Stage III), and after excluding certain EPO and 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, EPO <3.5 IU/L (Table 6.1; Stage IV). These stringent exclusion criteria reduced the sample number from over 600 to 146. Even after selection of insulin samples, they were again subjected to exclusion of deficiencies of serum 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 = 33 males and 30 females). The selected fasting insulin sample (n = 146) was studied before (Table 6.2) and after (Table 6.3) exclusion of these 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.

6.4.3. Sample Collection and Sample Preparation

Please refer Chapter 2 Section 2.3. for sample collection.

6.4.4. Analytical Control and Assays

Immunochemistry autoanalyser Access 2 (Beckman Coulter, USA) and their reagents were used for intact PTH, EPO, insulin and ferritin assays using immunometric method with magnetic bead coated anti PTH, anti EPO or anti ferritin antibodies (Procedure manual, Beckman Coulter, 2005). The chemistry autoanalyser Vitros 5,1 FS (Ortho Clinical Diagnostics, USA) and their reagents were used for assay of ALT, triglycerides, glucose and iron (Procedure manual, Ortho Clinical Diagnostics, 2004). C peptide assay was done by Diasorin Liaison (Italy) (Procedure manual, Liaison, 2017). Hemoglobin estimation was done manually by Drabkin's method using colorimeter. **Please refer detailed description of these assays in Chapter 2.**

Limit of detection of insulin was taken as the lowest insulin concentration distinguishable from zero (calibrator as 0 pmol/L insulin) with 95% confidence, and was 0.21 pmol/l. it was far below, the lower limit of range of fasting insulin in this study. It was also far below the lowest calibrator after zero, of the linear six point insulin calibrator value (example 5.88 pmol/L). An example of actual linear six point calibration values for insulin in pmol/l for a particular lot of calibrators were 0, 5.88, 58.2, 291.6, 888, 1818. Reference interval of fasting insulin (manufacturer's) was 11.4 to 138 pmol/l (Procedure manual, Beckman Coulter, 2005).

Daily continuous internal quality control data were analysed according to Westgard rules for acceptance or rejection of analyte data (Klee and Westgard, 2012). If there is a rejection, appropriate measures were taken to set right errors in machine functioning, reagents or calibration levels.

6.4.5. Diagnostic Criteria and Reference Intervals

Please refer Chapter 2**.** Section 2.26. for Diagnostic criteria and Reference Intervals.

6.4.6. Inter conversion of Units of Variables

Please refer Chapter 2, Methodology. **Table 2.2**. Conversion factors for interconversion between conventionl unit and SI unit.

6.4.7. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer 2.27. Statistical analysis section in Chapter 2.

6.5. Results

Samples were selected as before in different stages. In the initial stages clinical and subclinical disease conditions were excluded (Stage I and II). The insulin sample selected at Stage III were aged 18 to 25 years (n = 188) and partitioned into males (n = 63) and females (n = 125). After excluding high levels of EPO and PTH related analytes to rule out subclinical disease states, the insulin sample number decreased to n = 146. After excluding hemoglobin, iron and ferritin deficiencies, sample number decreased to n = 63. The final sample selected without any disease state or deficiencies were, male n = 33 and females n = 30. The decrease in sample number was very high for females (71.15%) and was due to deficiency of hemoglobin, iron and ferritin. In the earliest stages the decrease in male sample was more than that of females (Table 6.1).

6.5.1. Visual Evaluation of Distribution of Insulin

Distribution of fasting insulin in the insulin sample (n = 146) was found to be positively skewed by histogram and by Q-Q plot (Fig. 1A and B). Box-Whisker plot showed one far outlier and five near outliers Fig. 1C).

In the partitioned male insulin sample, distribution of fasting insulin (n = 42) was found to be positively skewed but was nearer to Gaussian distribution by histogram and by Q-Q plot (Fig. 2A and B). Box-Whisker plot showed one near outlier Fig. 2C).

After $\log_{10}$ transformation of the male insulin sample, distribution of fasting insulin (n = 42) was near to Gaussian distribution by histogram and by Q-Q plot (Fig. 3A and B). Box-Whisker plot showed one near and negative outlier Fig. 3C).

In the partitioned female insulin sample, distribution of fasting insulin (n = 104) was found to be positively skewed by histogram and by Q-Q plot (Fig. 4A and B). Box-Whisker plot showed one far outlier and three near outlier (Fig. 4C).

After $\log_{10}$ transformation of the female insulin sample, distribution of fasting insulin (n = 104) was almost Gaussian distribution by histogram and by Q-Q plot (Fig. 5A and B). Box-Whisker plot showed one near and positive outlier (Fig. 5C).

6.5.2. Gender Differences in the Concentration of Analytes

The gender differences in the analytes used for sample selection and those related to EPO and insulin were analysed for gender difference (Table 6.2). The increased concentration of ALT and triglycerides in males may be due to insulin resistance. The increased EPO in females may be due to hemoglobin and iron deficiency in females. There was no gender difference in fasting insulin but there was a borderline increase of fasting C peptide in males (Table 6.2). Increased PTH in the female sample may be due to increased EPO in females.

Comparison of gender differences in the concentration of fasting insulin and analytes related to fasting insulin were done after excluding hemoglobin, Iron and Ferritin deficiencies (Table 6.3). There were no gender differences seen for fasting insulin and HOMA-IR, indicating that in young adult males and females, there were no

gender differences in fasting insulin resistance. But there was a borderline gender difference in fasting C peptide, indicating increase in insulin secretion in the male sample by EPO.

6.5.3. Correlations of Insulin

As expected, insulin correlated well with C peptide in males and females (Table 6.4). Insulin correlated with EPO in females but not in males. Insulin also correlated with waist circumference, ALT and ferritin in males. But in females, insulin correlated with BMI and serum iron. In the presence of deficiencies, interpretations of correlation and their gender differences were become complex.

After exclusion of deficiencies of hemoglobin iron and ferritin, correlation of insulin with various analytes related to EPO and insulin were estimated (Table 6.5). After exclusion of deficiencies the correlation of insulin with EPO was lost but the correlation with C peptide was retained in both males and females. In addition to waist circumference and ALT, EPO correlated with ferritin in males. This may be due to increased insulin resistance, increasing waist circumference and fatty liver (ALT) resulting in increased cytokine, IL-1, IL-6 and TNFα. These cytokines inhibit the release of iron from ferritin. The loss of correlation of insulin with EPO may be due to interference from increased insulin resistance in males and gender differences in EPO, PTH and hemoglobin concentration. X-Y scatter was done to confirm the correlation of insulin before (Fig. 6.6) and after exclusion (Fig. 6.7) of hemoglobin, iron and ferritin. There were correlations seen with EPO and insulin in the male sample before exclusion (Fig. 6.6A). There was positive correlation of insulin with fasting C peptide in the male and female sample (Fig. 6.6B and E). In the male sample, after exclusion insulin correlated with ferritin (Fig. 6.6C).

6.6. Discussion

Increase in fasting insulin resulting in hyperinsulinaemia and is a marker for predisposition to type 2 diabetes mellitus. One of the causes of hyperinsulinaemia is insulin resistance. Insulin resistance leads to increase in insulin secretion. Increase in insulin secretion is a compensatory mechanism to overcome insulin resistance. EPO has

been reported to have beneficial effects in type 2 diabetes mellitus (McGill and Bell, 2006; Thomas, 2006), probably due to decrease in insulin resistance (Mak 1996; Pan et al, 2013). Insulin resistance may increase fasting glucose levels and to compensate this more insulin is secreted to being down glucose level.

6.6.1. Gender Differences in the Concentration of EPO, Fasting Insulin and Related Analytes

Fasting triglycerides, ALT and fasting C peptide were elevated in the male sample (Table 6.2). Though these increased levels of analytes are indicative of insulin resistance, there was no difference in the level of fasting insulin in the males and females. This may be due to deficiencies in the sample population. Hemoglobin, iron and ferritin were lowered in the female sample resulting in increased EPO and PTH in the female sample (Table 6.2).

After exclusion of deficiencies in hemoglobin, iron and ferritin there was border line gender difference in C peptide with increased concentration in males. The increased C peptide in the male sample may be due to increased insulin secretion resulting from increased insulin resistance in males. HOMA-IR, which is an indicator of fasting insulin resistance also, did not show any gender difference, indicating that increased insulin secretion and insulin resistance did not lead to fasting hyperinsulinemia in males after exclusion of deficiencies.

6.6.2. Correlations of Fasting Insulin with EPO and Analytes Related to EPO and Fasting Glucose

Before exclusion of deficiencies, fasting insulin correlated with fasting C peptide and HOMA-IR in both males and females. Fasting insulin in males correlated with waist circumference and ALT, and in females fasting insulin correlated with BMI and EPO. These results indicate gender differences in the correlation of insulin. Insulin correlated with EPO in the female sample but not in the male sample. In the male sample insulin resistance increases insulin and increase in EPO decreases insulin. This confounding effect of insulin secretion and insulin resistance may be the reason for absence of correlation between insulin and EPO in males (Table 6.4).

After exclusion of deficiencies of hemoglobin, iron and ferritin the correlation of fasting insulin with EPO was lost in the female sample. But there were correlations of insulin with C peptide in both male and female samples, indicating the concentration of insulin in circulation is directly related to insulin secretion.

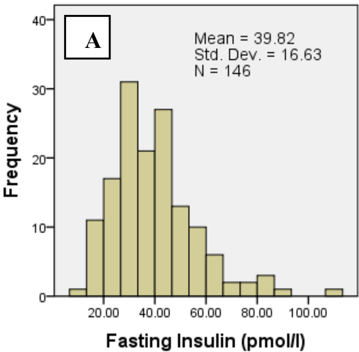
6.7. Conclusion

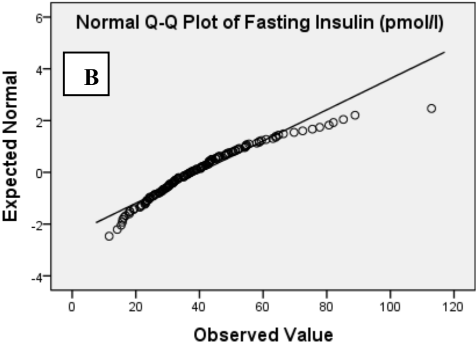
One of the causes of increase in fasting insulin or hyperinsulinaemia is insulin resistance. Insulin resistance increases insulin secretion as a compensatory mechanism. In males insulin resistance is higher than in females resulting in increased fasting insulin in males. EPO has been shown to be beneficial for type 2 diabetes, probably due to decrease in insulin resistance. In this study we have shown that fasting insulin correlates with EPO in the female sample and not in the male sample. This gender difference may be due to EPO decreases insulin resistance in males, resulting in decrease of insulin secretion. But increase in insulin secretion directly by EPO may be confounding the correlation between insulin, C peptide and EPO in the male sample. This confounding was less in the female sample.

Table 6.1. Number of participants (n) at various stages (III to V) and steps (4 to 6) of implementation of exclusion criteria for selection of the insulin sample population (n = 146; at Stage IV) and partitioning of samples according to age (18 – 25 years) and gender (males, M & females, F).

Stages	Steps	Exclusion at various stages for selection of reference fasting insulin sample population	Sample number at various phases of clinical exclusion		
			Total (M+F), n	Male, n (% males excluded at each stage)	Female, n (% females excluded at each stage)
III (3 to 4)	**4c.**	Participants after exclusion of growth phase <18 years and the influence of age >25 years (aged 18 to 25 years).	**188**	**63**	**125**
IV (4 to 5)	**5.**	Fasting insulin samples selected for this study after excluding ferritin >250, osteocalcin>35, ostase>30, NTx>200. Age 18 -25 years.	146	42 (21/63 = 33.33%)	104 (21/125 = 16.8)
V (5 to 6)	**6.**	Fasting insulin sample after excluding hemoglobin <125 g/l iron <9.85 µmol/l and ferritin <20 ng/ml.	63	33 (9/42 = 21.43)	30 (74/104 = 71.15)

Fig. 6.1. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of fasting insulin in the insulin sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 146).





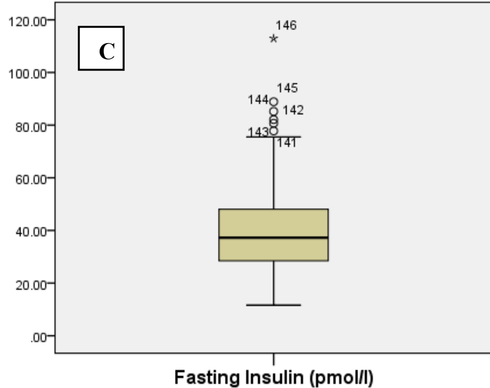


Fig. 6.2. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of fasting insulin in the **male** insulin sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 42).

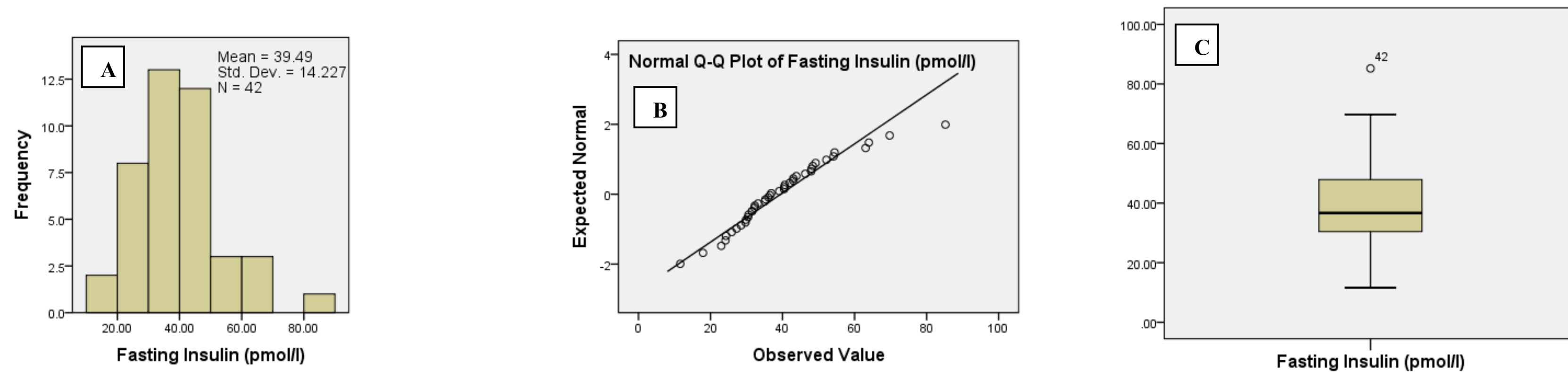


Fig. 6.3. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of $\mathbf{log_{10}}$ **transformed** fasting insulin in the **male** insulin sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 42).

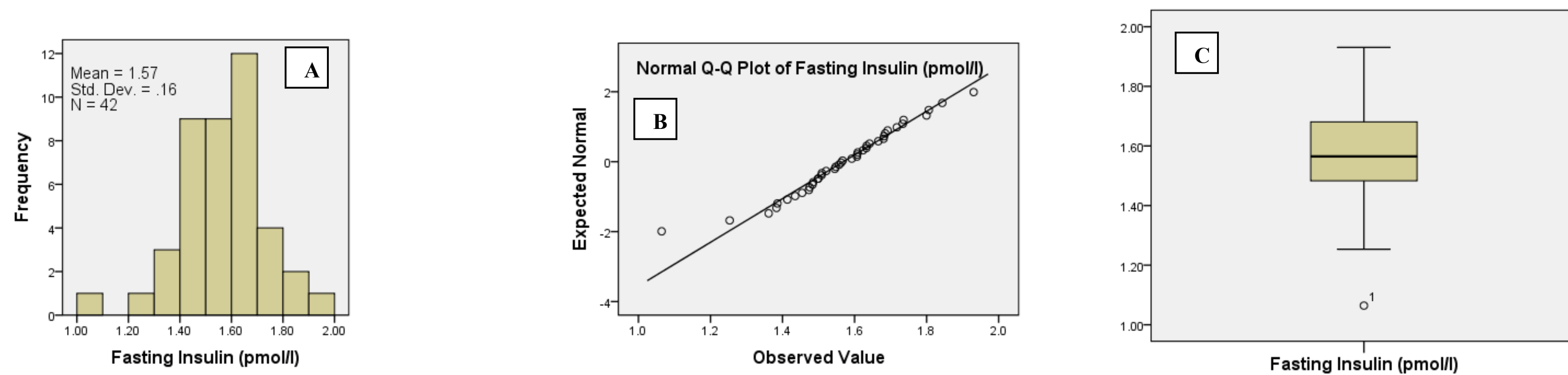


Fig. 6.4. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of fasting insulin in the **female** insulin sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 104).

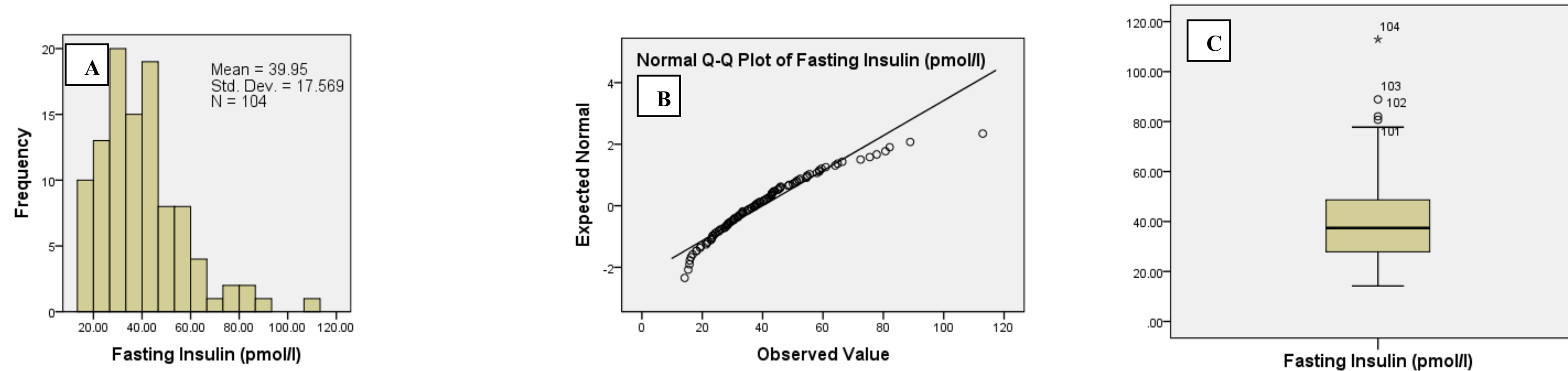


Fig. 6.5. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot(C) of **$\log_{10}$ transformed** fasting insulin in the **female** insulin sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 104).

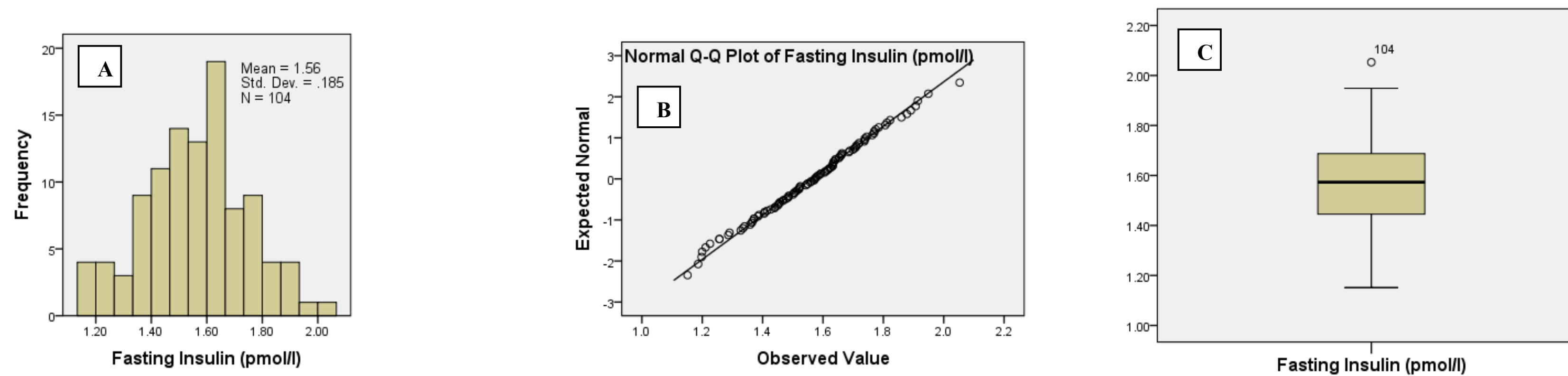


Table 6.2. Comparison of gender differences of analytes in the Insulin sample population. The Shapiro-Wilk test for normality and Levene's test for equality of variances of the male (M) and female (F) samples were analysed. The gender differences were compared by 95% CI of mean, Student t test (parametric method) or Mann Whitney U test (non parametric method).

Variables in SI units	Comparison of gender differences of analytes in the fasting insulin sample					
	M (n = 42) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 104) Mean±SD Minimum - Maximum 95% CI of mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			M	F		
Age (years)	21.57±1.84	22.22±1.72	--	--	--	--
BMI (Kg/m^2)	21.57±3.03 15.32 – 27.18 20.63 – 22.52	20.97±3.13 16.22 – 28.08 20.35 – 21.58	0.208	0.969	0.852	0.272
Waist Circum-ference (cm)	77.32±9.16 57.50 – 99.00 74.46 – 80.17	75.93±7.61 59.00 – 99.00 74.45 – 77.41	0.627	0.001	0.181	(0.286)
ALT (U/L)	29.69±11.76 16.00 – 60.00 26.02 – 33.36	23.90±8.49 10.00 – 60.00 22.25 – 25.56	0.091	0.003	0.039	**(0.006)**
Triglycerides (mmol/l)	0.981±0.314 0.37 – 1.83 0.883 – 1.08	0.855±0.338 0.42 – 2.15 0.790 – 0.921	0.122	0.012	0.930	**(0.007)**
S. EPO (U/L)	7.47±2.70 4.03 – 14.52 6.63 – 8.31	9.55±5.83 3.67 – 36.10 8.41 – 10.68	0.033	<0.001	0.090	**(0.034)**
HB (g/l)	147.53±14.52 119.9 – 181.8 143.0 – 152.1	129.60±12.43 97.0 – 172.0 127.2 – 132.0	0.656	<0.001	0.732	**(<0.001)**
S. Iron (µmol/l)	22.79±6.21 8.23 – 39.20 20.86 – 24.73	15.66±7.11 1.79 – 34.55 14.27 – 17.04	0.002	<0.001	0.004	**(<0.001)**

Ferritin (ng/ml)	52.06±30.88 10.90 – 139.7 42.44 – 61.68	21.61±19.29 2.50 – 121.90 17.87 – 25.37	0.165	0.160	0.073	**(<0.001)**
Fasting Glucose (mmol/l)	4.94±0.430 4.01 – 6.03 4.81 – 5.08	4.94±0.521 4.05 – 6.70 4.83 – 5.04	0.584	0.006	0.112	(0.616)
Fasting Insulin (pmol/l)	39.49±14.23 11.60 – 85.22 35.06 – 43.93	39.95±17.57 14.17 – 112.93 36.53 – 43.36	0.469	0.907	0.183	0.845
HOMA-IR	1.25±0.487 0.45 – 3.13 1.10 – 1.40	1.27±0.61 0.43 – 4.05 1.15 – 1.39	0.504	0.705	0.185	0.820
Fasting C peptide (nmol/l)	0.642±0.189 0.36 – 1.16 0.567 – 0.716 (n = 27)	0.570±0.155 0.30 – 0.93 0.534 – 0.606 (n = 75)	0.770	0.147	0.754	**0.070**
Intact PTH (ng/l)	26.33±10.39 10.80 – 59.90 22.30 – 30.36 (n = 28)	34.80±16.49 10.40 – 80.50 31.37 – 38.24 (n = 91)	0.276	0.290	0.133	**0.015**

Table 6.3. Comparison of gender differences of fasting insulin and analytes related to fasting insulin after excluding hemoglobin<125 g/l, Iron <9.85 µmol/l and Ferritin <20 ng/ml. Shapiro-Wilk test for normality and Levene's test for equality of variances of the male (M) and female (F) samples were analysed. 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	Comparison of gender differences of analytes after excluding hemoglobin, iron and ferritin					
	M (n = 33) Mean±SD Minimum - Maximum 95% CI of mean	F (n = 30) Mean±SD Minimum - Maximum 95% CI of mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			M	F		
Fasting Glucose (mmol/l)	4.89±0.408 4.01 – 6.03 4.74 – 5.03	4.92±0.450 4.27 – 5.92 4.75 – 5.09	0.378	0.122	0.362	0.795
Fasting Insulin (pmol/l)	40.45±15.51 11.60 – 85.22 34.94 – 45.95	37.18±19.04 15.83 – 112.93 30.07– 44.30	0.562	0.576	0.653	0.305
HOMA-IR	1.27±0.536 0.45 – 3.13 1.08 – 1.46	1.18±0.672 0.50 – 4.05 0.93 – 1.44	0.724	0.173	0.619	0.358
Fasting C peptide (nmol/l)	0.645±0.198 0.36 – 1.16 0.561 – 0.728 (n = 24)	0.546±0.163 0.30 – 0.93 0.476 – 0.616 (n = 23)	0.862	0.288	0.502	**0.068**

Table 6.4. Correlation (r) and significance of correlation (P) of fasting insulin with EPO and related analytes in total males and females samples Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Correlation coefficient, r	P	Correlation coefficient, r	P
Correlations of Insulin (before exclusion of deficiencies)				
	Male (n = 42)		**Female (n = 104)**	
BMI	0.148	0.349	**0.255**	**0.009**
WC	**0.382**	**0.013**	0.177	0.072
ALT	**0.305**	**0.050**	0.076	0.443
Triglycerides	0.253	0.106	0.172	0.081
EPO	-0.129	0.414	**0.193**	**0.050**
Fasting Glucose	0.027	0.866	0.167	0.091
Fasting C peptide	0.673 (n = 27)	**<0.001**	0.615 (n = 75)	**<0.001**
Intact PTH	0.258 (n = 28)	0.185	0.145 (n = 91)	0.171
Hemoglobin	-0.036	0.822	0.077	0.439
S. Iron	0.028	0.861	**-0.212**	**0.031**
Ferritin	**0.278**	**0.074**	-0.144	0.145
HOMA-IR	**0.974**	**<0.001**	**0.975**	**<0.001**
Correlations of EPO in the male and female insulin sample				
HOMA-IR	-0.115	0.469	**0.224**	**0.022**

Table 6.5. Correlation (r) and significance of correlation (P) of fasting insulin with EPO and related analytes in males and in females, after exclusion of samples with hemoglobin <125 g/l iron <9.85 µmol/l and ferritin <20 ng/ml. Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after $\log_{10}$ transformation.

Variables	Correlation coefficient, r	P	Correlation coefficient, r	P
Correlations of Insulin after exclusion of anemia, iron and ferritin deficiencies				
	Male (n = 33)		**Female (n = 30)**	
BMI	0.155	0.388	0.229	0.223
WC	0.395	**0.023**	0.207	0.273
ALT	0.340	**0.053**	0.050	0.794
Triglycerides	0.194	0.279	0.127	0.502
EPO	-0.184	0.305	-0.012	0.949
Fasting Glucose	0.109	0.547	0.274	0.142
Fasting C peptide	**0.667 (n = 24)**	**<0.001**	**0.464 (n = 23)**	**0.026**
Intact PTH	0.274 (n = 25)	0.184	0.225 (n = 26)	0.270
Hemoglobin	-0.116	0.522	-0.071	0.709
S. Iron	-0.085	0.639	-0.151	0.427
Ferritin	**0.374**	**0.032**	-0.200	0.289
HOMA-IR	**0.981**	**<0.003**	**0.983**	**<0.001**
Correlations of EPO with HOMA-IR after exclusion of deficiencies				
HOMA-IR	-0.151	0.403	0.006	0.977

Fig. 6.6. X-Y scatter diagram, of fating insulin with erythropoeitin (A, D), fasting C peptide (B, E) and ferritin (C, F) in **male (n = 42, A – C) and in female (n = 104, D – F)** insulin sample population.

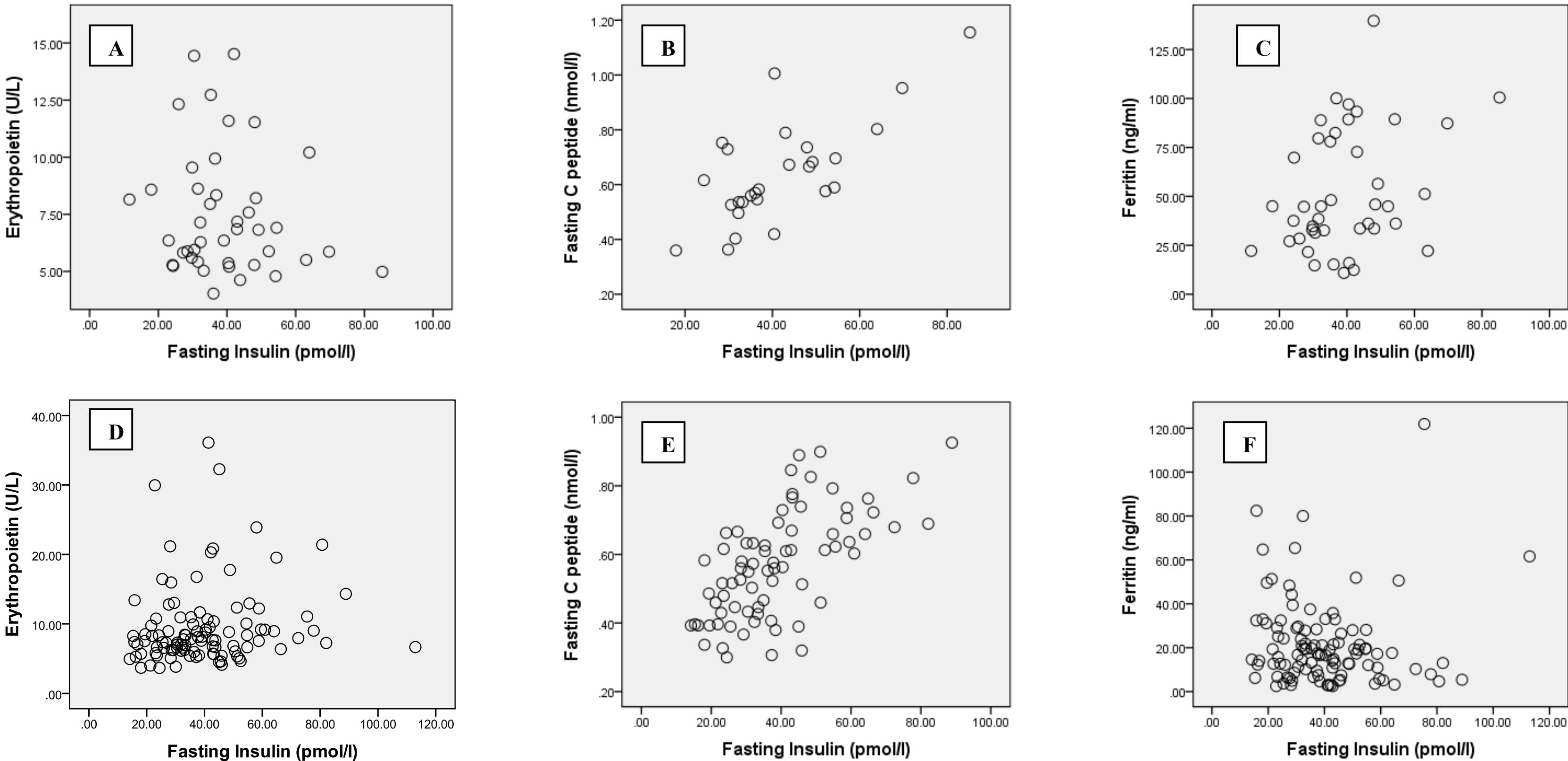
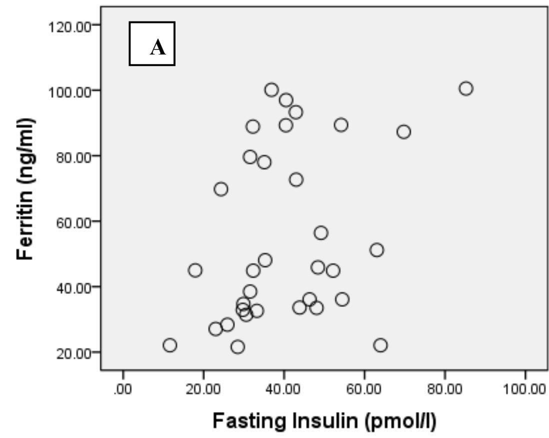
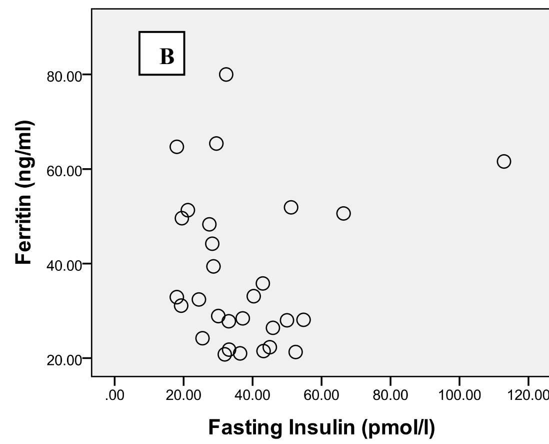


Fig. 6.7. X-Y scatter diagram, of fating insulin with ferritin in **males (A) (n = 33) and in females (B) (n = 30)** after exclusion of samples deficient in hemoglobin <125 g/l iron <9.85 µmol/l and ferritin <20 ng/ml.





NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 7.

Baseline Reference Interval of Erythropoietin Sample Population, and Partitioned Male and Female Groups

7.1. Abstract

In calculating the reference intervals, it was assumed that α error was 2.5.to 5% of area on either side of the distribution plot by parametric methods or 2.5 to 5% of the sample number in the non parametric percentile method was abnormal and were excluded. But we observed that the number of α and β errors were much more than 5% each. Clinical exclusion, Clinical Biochemistry exclusion and partitioning of samples were used to exclude samples with disease states and deficiencies to obtain the baseline reference sample. The three reference levels used were 95% parametric, 95% non parametric and the minimum-maximum levels. The minimum-maximum levels of reference intervals were used as errors were removed to a maximum after all three exclusions. When the sample number was 120 or above, the 95% non parametric method was used without evaluation of the distribution characteristics. When the sample number was below 120, but above 30, parametric method with robust calculation and bootstrap sampling were used to calculate 95% reference interval. When the sample number was below 30, the only method used was the non parametric minimum-maximum levels as the reference interval. Reference Interval of fasting sample population (n = 411) calculated by non parametric percentile method with bootstrap sampling: 3.84 – 26.7 U/L. Baseline Reference Interval of fasting EPO sample after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin calculated by non parametric minimum-maximum levels. Male sample: 4.11 – 12.73 U/L; Female sample: 3.67 – 13.41 U/L. When values were compared with reported reference interval, it was seen that the lower limits were similar but the upper limits of the baseline reference intervals were much lower.

7.2. Introduction

Methods of calculating reference intervals of Clinical Biochemistry analytes assumed that after converting a sample to Gaussian distribution, the samples falling in an area of less than 5% at the two extremes of the distribution plot by parametric methods, or less than 5% of the sample number in the non parametric percentile methods on either were abnormal levels and were excluded. We observed that the number of samples that

were alpha (α) or beta (β) errors were much more than this. It was not easily possible to define α errors and even more difficult to define β errors, statistically. Clinical exclusion criteria were required. Detection and partitioning of groups by clinical and statistical methods can remove α and β errors.

Quantitative data of analytes from the laboratory, along with other clinical data collected from a patient can be interpreted by comparison with the reference data to arrive at a diagnosis of a disease. The interpretation of quantitative biochemistry laboratory data is done with reference to comparison by a healthy sample. Healthy reference values are required for all clinical laboratory tests, to differentiate healthy individuals from patients with a clinically suspected disease.

Recommended requirements are satisfied to make the reference values for comparison of patient's laboratory results possible and valid (IFCC 1987-1991; Mardia 1980; Solberg & Grasbeck, 1989). The population from which reference data is collected should be defined. All the groups of reference individuals should be clearly defined with inclusion and exclusion criteria. Conditions in which the sample were obtained and processed for analysis should be known. All quantities compared should be of same type. All laboratory results should be produced with the use of adequately standardized methods under sufficient analytical quality control. The diagnostic sensitivity and specificity, prevalence and clinical costs of misclassification should be known for all lab tests used. From the parent population, who fulfill the selection criteria, random sampling is the best method for selecting reference individuals. But strict random sampling is not possible due to practical reasons. Therefore, using the best reference sample that can possibly be obtained after all practical considerations have been taken into account is necessary (Horowitz, 2012).

7.2.1. Partitioning the Sample

The heterogeneity in the sample should be partitioned to separate reference values according to sex and age groups, and other criteria if required. The number of criteria for partitioning the reference interval should be kept small and significant, so that sufficient sample sizes for valid statistical estimates must be available.

Several analytes vary significantly among different age and gender groups, so age and sex are most frequently used criteria for sub grouping. Other examples of partitioning criteria to be used for possible sub grouping are the genetic factors; the race, ethnic, blood and HLA groups; physiological factors such as stage of menstrual cycle, pregnancy, physical condition, and factors such as socio economic, environmental and chronobiological states (Horowitz, 2012). In this study, partitioning based on age and sex was done.

7.2.2. Sample Collection and Data Generation

The step of sample collection was essential to minimize bias and variation, standardization of pre analytical preparation of individuals before sample collection, sample collection itself and handling of sample before analysis, etc. were necessary. Venipuncture and skin puncture are standardised procedures for blood sample collection (Grasbeck & Alstrom, 1981). After sample collection its analysis is very important. Same analytical method should be used to ensure comparability between reference and observed value. In the analytical procedures the essential components required specification are: Analysis method, including information on equipment, reagents, calibration type of raw data and calculation methods; quality control; reliability and validity criteria. The specification and validity of the tests should be so clear that another investigator can reproduce the study.

7.2.3. Statistical Treatment of Reference Interval

Statistical analysis for reference interval calculations was done for partitioning the sample population for forming the minimum valid groups, analysis of sample distribution and calculation of reference intervals (Harris & Boyd, 1995; IFCC, 1987; Solberg & Grasbeck, 1989). Baseline reference interval is the reference interval obtained after exclusions by clinical and clinical biochemistry evaluations to remove all clinical and subclinical disease states and deficiencies in the sample population.

7.2.3.1. Partitioning of the reference data of fasting erythropoietin sample

Various influences and correlation of the parameters for reference interval calculation are analyzed for partitioning and to understand the relationship of the

parameter. In this study, the major analytes that influenced fasting EPO were anemia, iron and ferritin deficiencies, vitamin D deficiency and parathyroid hormones levels. Partitioning by gender differences and age were also required. The influences of age and growth phase were restricted in the study by selecting the sample population of male and female between 18 - 25 years of age. Biochemical cut off values of laboratory data are also used to exclude individuals with family history of obesity, liver dysfunction, abnormal lipid profile and other clinical secondary influences.

Significant differences in the partitioned group were analysed by parametric and non parametric statistical methods. Before choosing parametric methods, it was required to verify sample distribution by a number of methods, such as Shapiro-Wilk, D'Agostino and Anderson-Darling methods. The parametric statistical methods used in this study were Student t test. The non parametric method was Mann-Whitney U test (IFCC, Mardia 1980; Solberg 1986).

7.2.3.2. Analysis of sample distribution

Sample distribution can be analysed visually and by calculating distribution statistic. The visual method of data analysis was done by Anderson–Darling probability plot. The distribution statistic was calculated by Shapiro-Wilk, D'Agostino and Anderson-Darling methods. While Anderson-Darling methods were based on the distribution of the data in relation to Gaussian distribution, D'Agostino methods rely on the skewness and kurtosis character of the sample distribution (IFCC, 1987; Horowitz, 2012).

When sample distribution was not Gaussian or when the statistic was too low, data transformations were done by $\log_{10}$ and Box-Cox methods.

7.3. Objectives

7.3.1. Aspects of the Original Objectives Addressed in this Chapter

The objectives and aim of this chapter was to define methods and calculate the reference intervals of the EPO sample population and the baseline reference interval of

partitioned male and female samples. This is done to identify erythropoietin in healthy individuals and significant variations in disease conditions.

The above aim of this chapter was to fulfill the following aspects of the original objective.

7.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine

1. Correlation and significant variations of **erythropoietin** with other analytes **in healthy individuals,**

2. Identification of such correlations and **significant variations in disease conditions** and the changes in these analytes during treatment.

7.4. Materials and Methods

7.4.1. Study Setting and Research Design

Please refer 2.1. Study setting and Research design section in chapter 2

7.4.2. Selection of Participants, Inclusion and Exclusion Criteria

Exclusion of subclinical disease states and deficiencies by Clinical Biochemistry assays for EPO sample selection. Please refer 2.2. Selection of participants, inclusion and exclusion criteria in chapter 2.

7.4.3. Sample collection and sample preparation

Please refer 2.3. Sample collection section in chapter 2

7.4.4. Analytical control and assays

EPO assay was done with Access 2 machine and their reagents, using immunometric assay with magnetic bead coated anti EPO (Beckman Coulter, 2005). **Please refer detailed description of EPO assay in chapter 2.**

7.4.5. Diagnostic Criteria and Reference Interval

Please refer 2.26. Diagnostic criteria and reference interval section in chapter 2.

7.4.6. Statistical Analysis

Statistical softwares used for this study were SPSS version 23.0, Minitab and MEDCALC (trial versions).

Comparisons of gender differences in EPO were done by 95% confidence interval of mean, two-tailed Student t test and Mann Whitney U test. (Altman, 1991).

7.4.6.1. Baseline reference interval calculation

Baseline reference intervals of young adult population between the ages of 18 - 25 years excluded the influence of growth phase upto 18 years and the environmental effects after 25 years. Strict clinical criteria and cutoff levels of a number of clinical biochemistry parameters were used for inclusion and exclusion criteria to reduce secondary influences on fasting erythropoietin (Refer Chapter 2, General Methods).

The following methods for calculation of total sample population baseline reference intervals and partitioned sample reference interval were from the recommendations given by IFCC and from the articles on Reference intervals in Tietz Textbook of Clinical Chemistry (IFCC, 1987; Horowitz, 2012; Solberg & Grasbeck, 1989; Harris & Boyd, 1995).

When the sample number was above 120, the non parametric method of 95% reference interval calculation was done without any transformation. This was done by calculating the 95% middle percentile values, leaving 2.5% at both ends.

When the sample number was below 120, but above 30, parametric method with robust calculation was used to calculate 95% reference interval. The parametric methods

require Gaussian distribution of the sample, different methods of transformation were used to convert a non-Gaussian distribution to Gaussian distribution preferably with high statistic by Shapiro-Wilk, D'Agostino and Anderson-Darling methods. Anderson-Darling method works well for analysis of the low sample number distribution. For 95% reference interval calculations, Box-Cox transformations were preferred, as the transforming criteria was variable and when $\log_{10}$ transformations were not effective.

When the sample number was below 30, non parametric range was used to calculate 95% reference interval. This method requires careful analysis of two or three data points near lowest and highest range points. This was to reduce errors at critical lower and upper limits of reference interval.

When the sample number was above 120 and when non parametric method was used, bootstrap sampling was done to make the reference interval close to population reference interval. The 90% confidence interval of the upper and lower limits of the reference interval was also calculated. When parametric methods were used to calculate the 95% reference interval, Robust method of calculations were used to get better values for the reference interval.

7.5. Results and Discussion

7.5.1. Reference Interval of EPO after Clinical Exclusion of Samples

(EPO sample n = 411; female n = 224, male n = 187)

The sample distribution was positively skewed and Anderson-Darling probability plot, and D'Agostino-Pearson showed a P value <0.005 (Fig. 7.1; Table 7.1A). As the sample number was above 120 non-parametric percentile methods was used to estimate reference interval. The reference interval was from 3.84 - 26.70U/L (Table 7.2A) for the sample population (n = 411); the male sample reference interval was 3.61 - 19.91 U/L; and for the female sample was 3.84 - 32.28 U/L. The lower limit of the reference interval was close together for males and females. But the upper limit was much higher for the female sample and may be due to the lower level of hemoglobin, iron and ferritin in females. The gender difference of lower EPO in males can also been seen by 95% CI of

mean and student t test (Table 7.3A). The above reference intervals for males and females were very close to the reference interval reported (Table 7.4).

7.5.1.1. Discussion

The above reference interval was calculated by the traditional method of estimation of reference interval. In this sample the reference interval is influenced by the deficiencies in vitamin D, hemoglobin, iron and ferritin. The deficiencies of all these analytes were much more in female sample than male sample. All these analytes are related to EPO and deficiencies increase EPO and, therefore, the upper limit of EPO reference interval was higher, especially for the female sample.

7.5.2. Reference Interval of the EPO Sample Population, Males and Female after Exclusion by Clinical Examination, Clinical Biochemistry Evaluation and After Partitioning

The total number of EPO sample in this study was 159; there were 49 males and 110 females. As the sample number was above 120 non-parametric percentile methods was used to estimate reference interval. As the test number is below 120 in male and female, parametric method of reference interval calculation was taken. The method used was parametric Robust calculation with Bootstrap repeated sample selection. The sample was Box-Cox transformed and visual analysis of data showed a Gaussian distribution (Fig. 7.2). Estimation of the distribution of Box-Cox transformed sample by various methods showed a Gaussian distribution (Table 7.1B).

The reference interval calculated for the EPO sample was 4.01 – 23.89U/L, for the male sample it was 4.03 - 16.98 U/L and for the female sample it was 4.04 - 26.06 U/L (Table 7.2B). The lower limits of the reference interval were same in male and female. The upper limit was lower than that estimated for sample at step I (Table 7.2A). But the upper limit of the EPO reference interval was lower in males than female. The gender differences also showed lower EPO value for males by both student t test and 95% CI of mean (Table. 7.3B).

7.5.2.1. Discussion

After exclusion of subclinical disease state and after partition of age and gender, the upper limit of the reference interval was markedly lower than that by the previous method. This was due to the lower value of EPO resulting from exclusion of EPO related subclinical diseases and deficiencies.

7.5.3. Reference Interval of EPO after Exclusion of Subclinical Deficiencies in Vitamin D, Hemoglobin, Iron and Ferritin

The distribution of the EPO sample after exclusion of deficiencies and after $\log_{10}$ transformation was estimated by various methods and was found to be Gaussian (Table 7.1C). As the sample was less than 30 in number, minimum-maximum (range) was used to calculate the reference interval. The non parametric minimum-maximum levels gave a reference interval of 4.11 -12.73 in males and 3.67 - 13.41 in the female sample (Table 7.2C and Table 7.4). In this sample the upper reference interval limits were even lower than that obtained after exclusion of subclinical deficiencies. Also, the upper limit of the reference interval in the males and females were nearly the same. There was no gender difference between male and female EPO sample by 95% CI of mean and student t test (Table 7.3C).

7.5.3.1. Discussion

Deficiencies of vitamin D, hemoglobin, iron and ferritin increase EPO levels. Therefore, exclusion of these deficiencies decreases the upper limit of EPO reference interval. The male and female sample showed no gender difference in EPO as all the subclinical disease states and deficiencies were removed (Table 7.4).

7.5.4. Reference Interval Chosen for Fasting Erythropoietin

1. Reference Interval of fasting sample population (n = 411): selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.84 – 26.7 U/L.
2. Reference Interval of fasting EPO **male** sample: selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.61 – 19.91 U/L.
3. Reference Interval of fasting EPO **female** sample: selected method non parametric percentile with bootstrap sampling; calculated reference interval: 3.84 – 32.28 U/L.

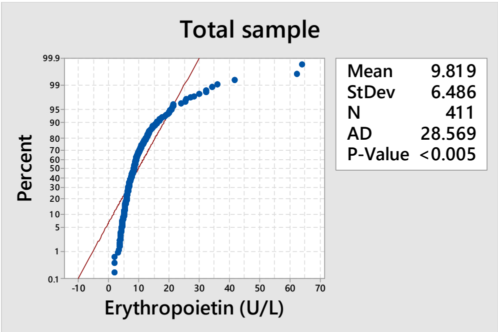
4. Reference Interval of fasting EPO **samples with age 18 -25 years** (n = 159) **after exclusion by clinical examination and clinical biochemistry assays** selected method non parametric percentile with bootstrap sampling; calculated reference interval: 4.01 – 23.89 U/L.
5. Baseline Reference Interval of fasting EPO in male sample **with age 18 - 25 years and after exclusion by clinical examination and clinical biochemistry assays**: selected method parametric robust method; calculated reference interval: 4.03 – 16.98 U/L.
6. Baseline Reference Interval of fasting EPO in female sample **with age 18 -25 years and after exclusion by clinical examination and clinical biochemistry assays**: selected method parametric robust method; calculated reference interval: 4.04 – 26.06 U/L.
7. Baseline Reference Interval of fasting EPO sample (n = 45) **after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin:** selected method parametric robust method; calculated reference interval: 3.16 – 12.55 U/L.
8. Baseline Reference Interval of fasting EPO in **male** sample **after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin:** selected method non parametric range (minimum-maximum levels); calculated reference interval: 4.11 – 12.73 U/L.
9. Baseline Reference Interval of fasting EPO in **female** sample **after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin:** selected method non parametric range (minimum-maximum levels); calculated reference interval: 3.67 – 13.41 U/L.

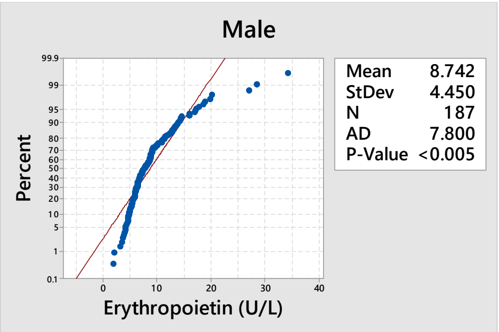
7.6. Conclusions

Clinical exclusion, Clinical Biochemistry exclusion and partitioning of samples were used to exclude samples with disease states and deficiencies to obtain the baseline reference sample. The three reference levels used were 95% parametric, 95% non parametric and the minimum-maximum levels. The minimum-maximum levels of reference intervals were used as errors were removed to a maximum after all three exclusions. Reference Interval of fasting sample population (n = 411) calculated by non

parametric percentile method with bootstrap sampling was 3.84 – 26.7 U/L. This reference interval was very similar to the reported reference interval of 4 – 27 U/L (Kratz et al, 2015**).** Baseline Reference Interval of fasting EPO sample, after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin, was calculated by non parametric minimum-maximum levels. In the male sample it was 4.11 – 12.73 U/L. In the female sample it was 3.67 – 13.41 U/L. When values were compared with reported reference interval, it was seen that the lower limits were similar but the upper limits of the baseline reference intervals were much lower.

Fig.7.1. Distribution characteristics of EPO in the sample population (n = 411), male (n = 187) and female (n = 224) by Anderson – Darling probability plot.





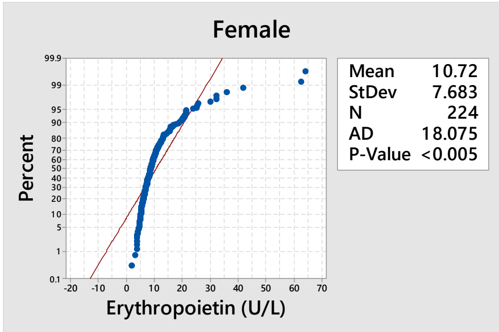


Fig.7.2. Anderson – Darling probability plot of EPO after Box-Cox transformation (male $\lambda = -0.92$, female $\lambda = -0.6$) in the EPO sample population aged 18 to 25 years after exclusion by Clinical examination and Clinical Biochemistry assays (male, n = 49 and female, n = 110).

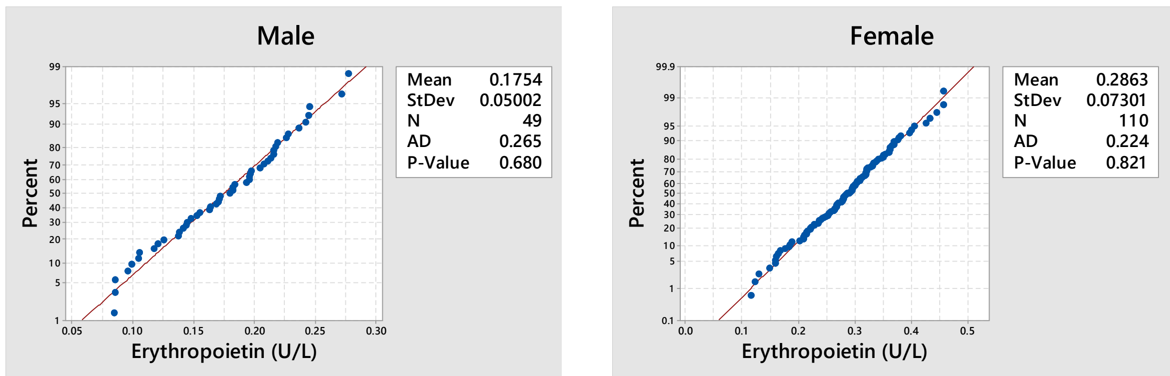


Fig.7.3. Anderson – Darling probability plot of log_{10}transformed EPO in sample population aged 18 to 25 years after excluding vitamin D< 50 nmol/l, hemoglobin <125 g/l, iron <9.85 µmol/l and ferritin <20 ng/ml (male, n = 25 and female, n = 20).

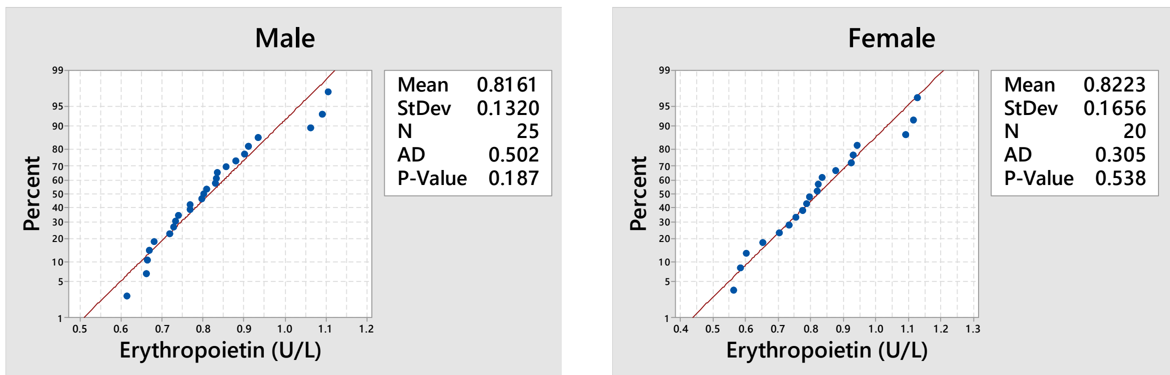


Table 7.1. Distribution characteristics of EPO at various stages of sample selection.

EPO	Coefficient of Skewness (P)	Coefficient of Kurtosis (P)	Shapiro - Wilk test P	D'Agostino-Pearson test P	Anderson Darling test P
A. Samples selected after clinical history and examination (Stage I, step 2)					
sample population (n = 411)	3.94 (<0.0001)	24.45 (<0.0001)	<0.0001	<0.0001	<0.005
Male (n = 187)	2.28 (<0.0001)	8.12 (<0.0001)	<0.0001	<0.0001	<0.005
Female (n = 224)	3.68 (<0.0001)	20.17 (<0.0001)	<0.0001	<0.0001	<0.005
B. Samples with age 18 -25 years (n = 159) after exclusion by Clinical examination and Clinical Biochemistry assays					
Samples with age 18 -25 years (n = 159)	2.60 (<0.0001)	8.84 (<0.0001)	<0.0001	<0.0001	<0.005
Male (n = 49) (Box-Cox transformed λ = -0.92)	-0.091 (0.779)	-0.694 (0.201)	0.438	0.424	0.680
Female (n = 110) (Box-Cox transformed λ = -0.6)	-0.025 (0.912)	-0.064 (0.975)	0.729	0.993	0.821
C. After Excluding hemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50nmol/l (n = 45)					
Total sample (n = 45) (log_{10} transformed)	0.523 (0.129)	-0.176 (0.945)	0.063	0.316	0.094
Male (n = 25) (log_{10} transformed)	0.772 (0.094)	0.188 (0.653)	0.115	0.222	0.187
Female (n = 20) (log_{10} transformed)	0.365 (0.454)	-0.391 (0.807)	0.399	0.733	0.538

Table 7.2. 95% Reference interval of erythropoietin (U/L) done by non-parametric (percentile and minimum-maximum levels) and parametric (Robust) methods.

EPO	Parametric method		Non-parametric method	
	Robust method		percentile method by bootstrap sampling	
	Lower limit (90% CI)	Upper limit (90% CI)	Lower limit (90% CI)	Upper limit (90% CI)
A. Samples selected after clinical history and examination (Stage I, step 2)				
EPO sample (n = 411)	--		3.84 (3.21 - 4.11)	26.70 (21.30 - 34.22)
Male (n = 187)	--		3.61 (1.94 – 4.11)	19.91 (17.32 – 34.22)
Female (n = 224)	--		3.84 (3.07 – 4.50)	32.28 (23.89 – 62.45)
B. Samples with age 18 -25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 159)				
EPO samples with age 18 -25 years (n = 159)	--		4.01 (3.67 – 4.50)	23.89 (20.30 – 36.10)
Male (n = 49) (Box-Cox transformed λ = -0.92)	4.03 (3.74 – 4.34)	16.98 (12.85 – 23.05)	--	
Female (n = 110) (Box-Cox transformed λ = -0.6)	4.04 (3.77 – 4.37)	26.06 (20.56 – 33.46)	--	
C. After Excluding hemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50 nmol/l				
EPO sample (n = 45) (log_{10} transformed)	3.16 (2.82 – 3.64)	12.55 (10.66 – 14.84)	--	
Male (n = 25) (log_{10} transformed)	3.27 (2.81 – 3.93)	11.83 (9.61 – 14.73)	(4.11 – 12.73 Min – Max)	
Female (n = 20) (log_{10} transformed)	2.85 (2.36 – 3.62)	14.80 (11.07 – 19.15)	(3.67 – 13.41 Min – Max)	

Table 7.3. Comparison of gender difference in the concentration of EPO (U/L) by 95% CI of mean and Student t test at various stages of sample selection.

EPO	Mean±SD	95% CI of mean	Range	Median	Mode	Student t test (or Mann-Whitney U test), P
A. Samples selected after clinical history and examination (Stage I, step 2) (n = 411)						
Male (n = 187)	8.74±4.45	8.10 – 9.38	1.94 – 34.22	7.68	4.59	(0.002)
Female (n = 224)	10.72±7.68	9.71 – 11.73	2.03 – 64.11	8.77	6.65	
B. Samples with age 18 -25 years after exclusion by Clinical examination and Clinical Biochemistry assays (n = 159)						
Male (n = 49)	7.40±2.80	6.60 – 8.20	4.03 – 14.52	6.45	5.28	(0.006)
Female (n = 110)	9.64±5.70	8.56 – 10.72	3.67 – 36.10	7.88	6.60	
C. **After Excluding hemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50 nmol/l (n = 45)**						
Male (n = 25)	6.87±2.34	5.90 – 7.83	4.11 – 12.73	6.35	5.88	0.889
Female (n = 200)	7.13±2.90	5.77 – 8.49	3.67 – 13.41	6.43	3.67	

Table 7.4. Reference intervals (RI) chosen for fasting EPO.

EPO U/L	Earlier reported reference interval (Kratz et al, 2015)	RI of samples after exclusion by clinical history and examination	RI of samples after exclusion by Clinical Biochemistry evaluation and partitioning (18 - 25 years)	RI of samples after excluding hemoglobin <125 g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and vitamin D <50nmol/l
Total sample	4 – 27	3.84 - 26.7 **(n = 411)**	4.01 – 23.89 **(n = 159)**	3.16 - 12.55 (n = 45)
Male	--	3.61 – 19.91 **(n = 187)**	4.03 – 16.98 **(n = 49)**	(4.11 – 12.73 Min – Max) **(n = 25)**
Female	--	3.84 – 32.28 **(n = 224)**	4.04 – 26.06 **(n = 110)**	(3.67 – 13.41 Min – Max) **(n = 20)**

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Chapter 8.

Deficiencies of Hemoglobin, Iron and Ferritin, and their Influence on Erythropoietin and the Related Analytes

8.1. Abstract

In the healthy state, a number of analytes are related to other analytes. In the disease state, when one analyte varies, the related analytes may or may not vary. Clinical interpretations of the concentrations and correlations of analytes are influenced by subclinical disease states and deficiencies. In this study, the influence of deficiencies of hemoglobin, iron and ferritin were analysed on EPO and its related analytes in males and females. These deficiencies are much more prevalent in females than in males. These deficiencies were found to influence concentrations of EPO, related analytes and their correlations with EPO. The relative prevalence and biochemical functions of analytes were used to group the deficiencies according to their presence and absence, in isolation or in combination. Differences in concentrations of analytes and in their correlations with EPO were observed with and without deficiencies. Differences in the correlations influenced the regulation of analytes and caused alterations in the biochemical functions. In samples with anemia and iron deficiency, there were major differences in the correlations of EPO with C peptide. In the female sample, correlation of EPO with hemoglobin was better in the absence of deficiency. In the male sample, EPO did not correlate with hemoglobin in presence or absence of deficiencies. These results indicated that in the absence of deficiencies clinical interpretations of concentrations and correlations of analytes are complex and difficult.

8.2. Introduction

8.2.1. Iron Absorption and Transport

Iron released as ferrous iron (Fe^{2+}) is absorbed by the intestinal proton-coupled divalent metal transporter 1 or DMT-1. Ferric iron is (Fe^{3+}) is reduced to Fe^{2+} by duodenal Cytochrome b (DcytB) reductase, and is transported into the intestinal mucosal cell by the proton-coupled divalent metal transporter 1 (DMT-1). The basolateral export of iron from the enterocyte to the circulation is mediated through ferroportin in association with hephaestin. In the circulatory system iron is associated with the plasma transferrin (Yeh et al, 2009). Recent studies showed that the transferrin iron pool is not

homogeneous but is composed of apo-transferrin (apo-Tf), when no iron is bound; monoferric transferrin (bounded to a single iron atom); and diferric transferrin (bounded to two iron atoms) (Young and Bomford, 1984).

Dietary ferritin is absorbed through an adaptor-related 2 protein complex (AP2)-dependent endocytosis mechanism by the enterocytes (San Martin et al, 2008). In enterocyte, heme is broken by heme oxygenase 1 and iron is released as ferric iron.

The low pH of the stomach coupled to proteolytic enzymes is responsible for the release of heme from hemoproteins. The mechanism responsible for heme uptake is not well understood (Silva and Faustino, 2015). It seems to be occurring by receptor-mediated endocytosis. The HCP1 (heme-carrier protein 1), has been identified as the receptor involved in this process. It is identical to the proton coupled folate transporter (PCFT) and has low-affinity to heme and is more involved in folate absorption (Laftah et al, 2009).

In the cytoplasm of enterocyte iron is either driven to the basolateral membrane or stored as ferritin. Here, the 'mucosal block' mediated by ferritin plays a critical role in the effective absorption of iron (Beutler, 2010).

8.2.2. Cellular Metabolism of Iron

The cellular uptake of transferin-bound iron (TBI) is mainly mediated by the transferrin receptor 1 (TfR1), located at the cell membrane. Upon binding, this TBI–TfR1 complex undergoes a clathrin-dependent endocytosis. The endosome pH is decreased by the entry of H^+ and Fe^{3+} is released from transferrin. The apotransferrin remains bound to TfR1 and this complex then returns to the cell membrane and is released to the plasma. In the erythroblast, free iron is reduced by Steap 3 and transported to the cytoplasm by DMT-1, which induces the release of Fe^{2+} in to the cytosol, where it is taken up by mitochondria for heme synthesis (Beutler, 2010; Silva and Faustino, 2015).

Mitochondria have a high requirement for iron, as majority of iron is used by the erythroblasts for hemoglobin synthesis. Circulating erythrocytes consist mainly of hemoglobin which constitutes about 70% of iron in the body. Mitochondrion is the main

cell organelle responsible for maintaining the iron homeostasis. Mitochondria also require iron for components of the electron transport chain.

Iron is stored as ferritin and storage prevents the presence of free iron whenever body iron levels increase. Stored iron also ensures its immediate availability during iron deficiency. The release of iron from ferritin is driven by both lysosome- and proteasome-mediated degradation mechanisms (Mehlhase et al, 2005).

8.2.3. Recycling of Iron

During senescent, the erythrocytes are scavenged by splenic and hepatic macrophages and cause the release of iron from hemoglobin and rendering it available for another hemoglobin cycle. Recycling macrophages release approximately 20 percent of hemoglobin iron form the cell to the plasma, from these 80 percent of iron is reincorporated into hemoglobin. The remaining iron is enters the storage pool as ferritin or hemosederin. When there is an increased demand of iron for hemoglobin synthesis, stored iron is released more rapidly. Where as in inflammatory condition or infection or malignancy the usage of iron for hemoglobin synthesis is reduced and stored iron remains high (Beutler, 2010; Silva and Faustino, 2015).

8.2.4. Regulation of Iron Metabolism

Human beings have several mechanisms through which iron levels are kept in homeostasis. Iron homeostasis is regulated at the level of dietary iron absorption by the duodenum, transport of iron in the circulation, cellular uptake and consumption, recycling by macrophages and storage in the liver (Charlton and Bothwell, 1983).

Iron homeostasis is controlled by iron absorption, recycling and storage. There are no active ways for iron excretion. The daily requirement of a human is approximately 1 mg of dietary iron. This iron overcomes the non-specific iron losses through bleeding, sweat and sloughing of epithelial cells. The organism increases its duodenal absorption whenever more iron is required, and release from macrophages and from storing cells. On the other hand, when there is a condition of iron overload the absorption is inhibited and storage form of iron is increased, in order to prevent the toxic effects of free iron excess (Finch, 1994).

Commonly, iron homeostasis is controlled by hepcidin, a liver-derived hormone. It plays a central role in regulating iron absorption from intestine mucosal cells and its release from macrophages. The regulation of hepcidin production is entirely transcriptional. Hepcidin gene (HAMP) transcription is increased by high iron levels, infection and inflammatory stimuli, and is decreased by anemia, hypoxia, HIF-1 and erythropoiesis. Hepcidin acts by binding to the cell surface ferroportin-1, a transmembrane iron-transport protein expressed both on intestinal mucosal cells and macrophages. Binding of hepcidin with ferroportin induces its internalization and degradation. Depletion of membrane ferroportin prevents iron release from enterocytes, macrophages and hepatocytes and results in a fall in iron level (Ganz, 2005). Alterations in hepcidin production may also lead to changes in iron absorption and recycling. Multiple factors including iron, erythropoiesis, inflammation, and growth factors regulate hepcidin expression.

8.2.5. Disorders of Iron Metabolism

Low hepcidin levels are seen in iron overload, while elevated hepcidin levels in anemia. Alteration in systemic iron homeostasis results in two major classes of disease, anemia and hemochromatosis.These iron-related diseases are caused mainly by disturbances in hepcidin regulation (Zhang and Rovin, 2013).

8.2.5.1. Anemia

World Health Organization reported that anemia is an indicator of poor nutrition and low health status. Approximately 126 million peoples were suffering from anemia worldwide (McLean et al, 2009). The World Health Organization defines that anemia occurs if the hemoglobin level drops below 120 g/l for women and below 130 g/l for men at sea level (World Health Organization, 1968). Generally, in anemic patients the quality of life is impaired due to fatigue, dizziness, and impaired cognitive functions. The symptoms are due to iron deficiency in red blood cells which diminishes the oxygen supply for the body.Iron deficiency can occur either with or without anemia. The prevalence of different anemia subtypes is: about 50% of anemias arise from nutritional iron deficiency, 42% are caused by inflammation and infection and 8% of anemias

develop due to nutritional deficiencies (such as vitamin A, vitamin B12, folate, riboflavin, or copper), or are genetically caused (Denic and Agarwal, 2007).

8.2.5.2. Iron deficiency anemia

Iron deficiency anemia (IDA) is the most frequent form of anemia caused by a relative or absolute deficiency of dietary iron. In IDA, iron is insufficient to satisfy the iron demand for erythropoiesis. In earlier stage, iron deficiency occurs without anemia, but later anemia develops as a result of depleted iron stores. In this case, treatment is by oral or intravenous iron supplementation (Clark, 2008).

Pathogenic factors in anemia of iron deficiency are impaired hemoglobin synthesis as a consequence of reduced iron supply, defect in cellular proliferation and reduced survival of erythroid precursors and erythrocytes, when the anemia is severe.

8.2.5.3. Anemia in chronic kidney disease (CKD)

The main etiology of anemia in CKD is the failure in EPO production by the kidney which leads to decrease in the level of hemoglobin (Delaney et al, 2012). In patients with CKD and low endogeneous serum erythropoietin level **rhEPO-epoitin alfa and beta** was used to correct anemia. 90-95% of patients treated with rhEPO are effective in correcting anemia of CKD (Winearl et al, 1986; Eschbach et al, 1987).

8.2.6. Ferritin

Ferritin is localized in cell cytosol, nucleus and mitochondria, but is also present in the serum. Iron is incorporated into ferritin as Fe^{2+}, but oxidized quickly to Fe^{3+} within its spherical shell by H ferritin ferroxidase (Wang et al, 2010). The major function of ferritin is to provide a storage site for iron which may be used for heam synthesis whenever required.

Serum ferritin concentrations are normally within the range 15–200 ng/ml and are lower in children than adults. Ferritin levels are lower in women before the menopause than in men. It is mainly due to blood losses during menstruation and childbirth. But after menopause the ferritin concentration increases but remains lower than in men. Low serum ferritin was observed in patients with iron deficiency anemia and is typically less than 12–15 ng/ml (Worwood, 1982).

8.3. Objectives

8.3.1. Aspects of the Original Objectives Addressed in this Chapter

Common disease conditions associated with EPO and iron metabolism are deficiencies in hemoglobin (anemia), iron and ferritin. These disease states can occur in isolation or in combination. Identification of significant variations and correlations of analytes in disease conditions:

1. Samples were grouped into those without and with deficiencies in hemoglobin (anemia) and/or iron and/or ferritin in isolation or in combination.
2. Significant differences in concentrations of analytes related to EPO in samples without and with deficiencies were evaluated.
3. Differences in the correlations of EPO with these analytes in samples without and with deficiencies were evaluated.

8.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. **These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine**

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment

8.4. Materials and Methods

8.4.1. Study Setting and Research Design

Please refer 2.1. Study setting and Research design section in Chapter 2

8.4.2. Selection of Participants, Inclusion and Exclusion Criteria

Healthy cross section of participants 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Chapter 2; Table 2.1; Stage I).

8.4.2.1. Exclusion of disease states by Clinical Biochemistry assays for sample selection

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of individuals with diseases.

Samples with or without hemoglobin, iron and ferritin deficiencies were selected. Sample in which fasting EPO, PTH, fasting Insulin, fasting C peptide and analytes related to iron metabolism have been estimated, were selected for the study. The exclusion criteria reduced the study sample number from over 600 to 211. After selection of study samples, they were subjected to groupings according to deficiencies in hemoglobin <125 g/l, iron <9.85 µmol/l (55 µg/dl) and ferritin <20 ng/ml (Table 8.1). The above cut off levels was designed for this study to include individuals with increased concentrations of EPO, intact PTH, and deficiencies hemoglobin, iron and ferritin.

8.4.3. Sample collection and sample preparation

Please refer 2.3. Sample collection section in chapter 2

8.4.4. Analytical Control and Assays

Analytical measurements of **ALT, triglycerides, fasting glucose, intact PTH, EPO, vitamin D, hemoglobin, iron, ferritin fasting insulin and fasting C peptide** assays were done. **Please refer detailed description of these assays in chapter 2.**

8.4.5. Diagnostic criteria and Reference Interval

Please refer 2.26. Diagnostic criteria and reference interval section in chapter 2.

8.4.6. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer section 2.27. Statistical analysis in chapter 2.

8. 5. Result

8.5.1. Subgroups formed from Isolated Deficiencies of Hemoglobin, Iron or Ferritin and In Combination

Deficiency of one analyte may occur in isolation or in combination with other analytes. There were also gender differences in the deficiencies. In females, hemoglobin, iron and ferritin deficiencies were very much more (Table 8.1; Column A and H). Ferritin deficiency was found to occur in isolation in females and in significant numbers (Table 8.1; Column E). In the female sample population, hemoglobin deficiency occurred more in combination with iron and/or ferritin deficiencies (Table 8.1; Column B and F) and less in isolation (Table 8.1; Column D). Iron and ferritin deficiency combination was also found to occur in significant numbers in females among the sample population (Table 1; F).

In the male sample, hemoglobin, iron and ferritin deficiencies were uncommon and occurred in isolation (Table 8.1; Column C, D and E). Deficiencies in combination were rare.

These observations made us group the deficiencies according to the presence and absence of deficiencies, and deficiency in isolation or in groups. The most frequent

deficiency in isolation was ferritin deficiency in females (Table 8.1; D). In females, the most frequent deficiencies that occurred in isolation and in combination were that of hemoglobin (Table 8.1; Columns B, D and F) and ferritin (Table 8.1; Columns B, E, F). This was followed by iron in isolation and in combination.

8.5.2. Distribution of EPO in the Deficient Samples

The concentration of EPO increased during deficiencies in hemoglobin and iron. The distribution of EPO in the female sample population with deficiencies was found to be positively skewed, with mode of EPO histogram at <10 and mean at 11.4 (Fig.8.1). These results indicate that even in the presence of deficiencies, large number of EPO samples were within the reference interval.

8.5.3. Analytes in Males and Females Without and With Deficiencies in Hemoglobin, Iron and/or Ferritin

In the male sample, deficiencies of hemoglobin, iron and/or ferritin did not affect the concentrations of EPO (Table 8.2). Fasting C peptide and vitamin D were higher in the group with deficiency. The absence of differences in the concentration of EPO in the presence or absence of deficiencies may be due to the higher hemoglobin in males resulting from androgens restricting general hypoxia. Increased C peptide in the deficient sample may be contributed by the action of EPO and insulin resistance.

In the female samples, the deficiencies increased the concentrations of EPO (Table 8.3). As expected, hemoglobin, iron and ferritin were lower in the group with deficiencies. There were no differences in the concentration of C peptide in the samples with and without deficiencies. In the female sample, there was increased EPO in the deficient sample, but C peptide is not increased in the deficient sample. Vitamin D was higher in the non deficient samples.

The concentration of EPO was found to be highest (mean 18.6) in the samples with all three deficiencies (Table 8.4). Closely following this was iron and ferritin deficient samples with mean EPO of 17.55. In the samples with hemoglobin and ferritin deficiencies the mean EPO was 10.11, within the reference interval showing that this combination was not increasing the concentration of EPO. Similarly, ferritin deficient

samples also did not increase the concentration of EPO. EPO was highest when all the samples in a group had iron deficiency.

In the male sample without deficiencies, EPO did not correlate with hemoglobin, iron and ferritin. In the sample with deficiencies, EPO correlated with fasting C peptide (Table 8.5). In the deficient sample, the fasting EPO was higher which increased insulin secretion and correlated with C peptide. X-Y scatter diagram confirmed the positive correlation of EPO with fasting glucose and C peptide (Fig.8.2).

In female samples, EPO correlated negatively with hemoglobin, iron and ferritin, and positively with intact PTH much better in the presence of deficiencies than in its absence. This may be due to resistance to EPO action was by the deficiencies of iron and ferritin. EPO correlated positively with C peptide in the absence of deficiencies (Table 8.6). X-Y scatter confirm the positive correlation of EPO with fasting C peptide and intact PTH (Fig.3).

8.6. Discussions

Clinical interpretations of analytes and their relationships were affected by subclinical deficiencies. In this section, the influence of anemia and deficiencies in iron and ferritin were analysed. Our observations made us group the deficiencies according to the presence and absence of deficiencies, and deficiency in isolation or in groups. The most frequent deficiency was that of hemoglobin in females. This could be explained by the monthly blood loss in females. Hemoglobin deficiency occurred in isolation and in combination with iron and ferritin (Table 8.1; B, D and F). Ferritin in the reticulo endothelial cells was the immediate source of iron for hemoglobin synthesis. Iron and hemoglobin deficiency increased EPO and PTH which increased erythropoiesis. Therefore, the most frequent deficiency in isolation was that of ferritin deficiency in females (Table 8.1; D).

In the female sample, the major subgroups of deficiencies were 1) Hemoglobin with ferritin, 2) Hemoglobin with iron, 3) Hemoglobin with ferritin and iron, 4) Isolated ferritin, 5) Isolated hemoglobin, 6) Sample without deficiencies.

In the male sample, the number of deficiency was few. Only three sub groups of deficiencies could be defined: 1) Hemoglobin, 2) Iron, 3) Ferritin, 4) Sample without deficiency.

In a resting fasting sample of serum, iron was found to be the most important factor that increased EPO concentration. Hemoglobin and ferritin deficiencies resulted in small increase of EPO (Table 8.4). This may be reason for the large number of EPO samples to be within the reference interval even with deficiencies (Fig. 8.1).

The higher hemoglobin in male sample without deficiency was due to availability of iron, and androgens increasing hemoglobin in males. The lower iron and ferritin decreased hemoglobin in the deficient sample (Table 8.2). EPO was higher in the deficient sample but was not significant (Table 8.2; Mean EPO in males = 7.19 and in females = 8.91). The higher level of EPO may be the cause for the higher concentration of C peptide in females.

In the male non deficient sample, EPO correlated with glucose and weakly correlated with C peptide. In the deficient male sample EPO correlated positively with C peptide. Again gender differences in insulin secretion and influence of concentrations of hemoglobin, iron and ferritin causing differences in insulin secretion were observed.

In the female non deficient sample, EPO, as expected and observed earlier, was correlating with C peptide (Table 8.6). EPO was higher in the female deficient sample due to the deficiencies. But EPO did not increase C peptide in females. This may be due to the gender differences in relationships of EPO with PTH and hemoglobin, and the deficiencies may be confounding with the relationships of EPO and C peptide.

8.7. Conclusions

Deficiencies were found to influence concentrations in analytes and their relationships. Differences in concentrations of analytes and in their correlations with EPO were observed with and without deficiencies. Differences in the correlations influenced the regulation of analytes and caused alterations in the biochemical functions. In samples with anemia and iron deficiency, there were major differences in the correlations of EPO

with C peptide. In the female sample, correlation of EPO with hemoglobin was better in the absence of deficiency. In the male sample, EPO did not correlate with hemoglobin in presence or absence of deficiencies.

Table 8.1. The study population (n = 211) was grouped according to the absence (n = 89) or presence of deficiencies (n = 122) in hemoglobin (<125 g/l) and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml). Sample without deficiencies was the control sample.

Gender	(A) Sample Without Deficiencies (n = 89)	Samples (n) grouped according to deficiencies in following analytes						
		(B) Hemoglobin, Iron and Ferritin	(C) Iron	(D) Hemoglobin	(E) Ferritin	(F) Hemoglobin and Ferritin	(G) Iron and Ferritin	(H) Total with deficiencies (122)
Male (n)	53	0	5	5	5	0	1	16
Female (n)	36	18	2	16	39	21	10	106

Fig. 8.1. Histogram (A), Normal probability Quantile-Quantile (Q-Q) Plot (B) and Box-Whisker plot (C) of EPO in **female sample population with hemoglobin (<125 g/l), and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml) deficiency** (n = 106). Number of deficient samples in males were low (n = 16). Distribution of sample without these deficiencies is shown in Fig.3.11, Chapter 3.

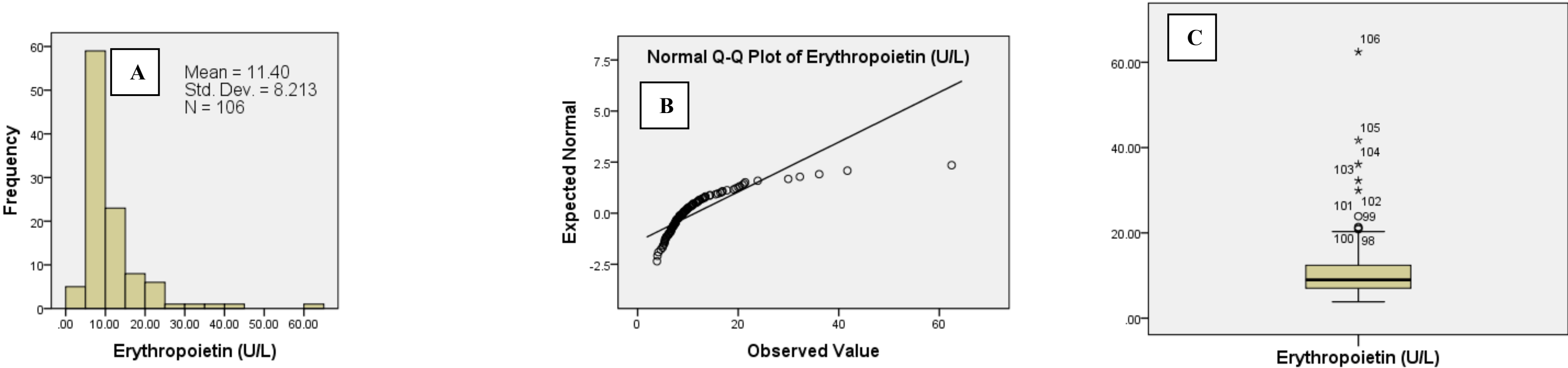


Table 8.2. Comparison of analytes in **male sample population** in groups **without** and **with deficiencies** in hemoglobin (<125 g/l) and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml) by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	Without deficiencies (n = 53) Mean±SD Minimum - maximum 95% CI mean	With deficiencies (n = 16) Mean±SD Minimum - maximum 95% CI mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			Without deficiencies	With deficiencies		
Age (>18 years)	26.06±10.39 18.00 – 59.00 23.19 – 28.92	25.00±14.18 18.00 – 80.00 17.95 – 32.05	--	--	--	--
BMI (kg/m^2)	22.13±3.02 15.32 – 27.18 21.30 – 22.96	23.211±4.21 16.54 – 29.09 21.11 – 25.31	0.075	0.054	**0.036**	(0.221)
Waist circum-ference (cm)	80.61±9.31 63.00 – 99.00 78.04 – 83.17	81.50±12.96 57.50 – 99.00 75.06 – 87.94	0.455	0.135	**0.044**	(0.539)
ALT (U/L)	29.26±10.01 16.00 – 60.00 26.51– 32.02	34.50±16.36 16.00 – 72.00 26.36 – 42.64	0.281	0.300	0.074	0.247
Triglycerides (mmol/l)	1.04±0.400 0.37 – 2.19 0.925 – 1.15	1.20±0.495 0.57 – 2.40 0.955 – 1.45	0.511	0.672	0.951	0.173
S. EPO (U/L)	7.19±2.39 4.11 – 13.91 6.53 – 7.85	8.91±4.93 2.01 – 19.83 6.46 – 11.37	**0.050**	0.362	**<0.001**	(0.447)
Hemoglobin (g/l)	146.92±11.73 125.0 – 172.0 143.7 – 150.2	133.51±16.55 110.8 – 181.0 125.3 – 141.8	0.410	0.137	**0.042**	**(<0.001)**
Ferritin (ng/ml)	56.90±26.83 21.60 – 113.0 49.51 – 64.30	50.93±42.11 10.90 – 139.7 29.98 – 71.87	**0.019**	0.084	**<0.001**	(0.128)
S. Iron (µmol/l)	23.14±5.99 11.10 – 39.20 21.49 – 24.79	17.81±7.62 1.79 – 30.61 14.02 – 21.60	0.689	**0.001**	**<0.001**	**(0.016)**
Fasting	5.03±0.541	5.18±0.425	**0.041**	0.881	0.391	(0.119)

Glucose (mmol/l)	4.01 – 6.80 4.89 – 5.19	4.73 – 5.94 4.97 – 5.39				
Fasting Insulin (pmol/l)	40.99±15.74 11.60 – 85.22 36.65 – 45.33	49.62±26.34 24.17 – 122.9 36.52 – 62.72	0.535	0.132	0.708	0.156
Fasting C peptide (nmol/l)	0.652±0.185 0.35 – 1.16 0.596 – 709	0.778±0.227 0.55 – 1.19 0.634 – 0.922	0.581 (n = 44)	0.085 (n = 8)	0.605	**0.059**
Intact PTH (ng/l)	29.28±15.62 8.40 – 86.40 24.59 – 33.98	33.02±14.47 12.60 – 55.50 23.83 – 42.21	0.751 (n = 45)	0.258 (n = 12)	0.776	0.342
Vitamin D (nmol/l)	63.35±32.43 12.46 – 150.0 53.72 – 72.98	93.55±31.32 53.40 – 133.0 73.65 – 113.5	**0.048** **(n = 46)**	**0.049** **(n = 8)**	0.286	**(0.010)**

Table 8.3. Comparison of analytes in **female sample population** in groups **without** and **with deficiencies** in hemoglobin (<125 g/l) and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml) by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	Without deficiencies (n = 36) Mean±SD Minimum - maximum 95% CI of mean	With deficiencies (n = 106) Mean±SD Minimum - maximum 95% CI of mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			Without deficiencies	With deficiencies		
Age (18-25 years)	25.36±7.62 18.00 – 47.00 22.78 – 27.94	24.49±7.74 18.00 – 70.00 23.00 – 25.98	--	--	--	--
BMI (kg/m^2)	21.62±3.32 17.29 – 29.21 20.50 – 22.74	21.78±3.47 16.22 – 30.49 21.11 – 22.45	0.066	0.062	0.958	0.835
Waist circum-ference (cm)	78.65±9.38 63.00 – 98.00 75.48 – 81.82	77.89±8.93 59.00 – 110.0 76.17 – 79.61	0.574	0.314	0.425	0.674
ALT (U/L)	23.08±8.06 10.00 – 48.00 20.36 – 25.81	24.73±10.26 9.00 – 71.00 22.75 – 26.70	0.106	**0.044**	0.325	(0.386)
Triglycerides (mmol/l)	0.870±0.303 0.52 – 1.74 0.767 – 0.972	0.925±0.384 0.42 – 2.19 0.851 – 0.999	0.061	0.057	0.108	0.588
S. EPO (U/L)	6.72±2.57 2.03 – 13.41 5.85 – 7.60	11.40±8.21 3.85 – 62.45 9.82 – 12.99	0.261	**<0.001**	0.063	**(<0.001)**
Hemoglobin (g/l)	136.30±8.47 125.0 – 156.2 133.4 – 139.2	123.73±13.37 70.6 – 160.6 121.2 – 126.3	**0.029**	**<0.001**	**0.020**	**(<0.001)**
Ferritin (ng/ml)	40.67±23.64 20.80 – 138.6 32.69 – 48.68	14.88±13.73 1.40 – 121.90 12.23 – 17.52	**0.010**	**0.018**	**0.012**	**(<0.001)**
S. Iron (µmol/l)	19.38±5.60 10.20 – 34.55 17.48 -21.27	13.87±6.61 1.79 – 30.97 12.59 – 15.14	0.888	**<0.001**	**0.001**	**(<0.001)**
Fasting	4.96±0.536	5.03±0.811	0.311	**<0.001**	0.255	(0.976)

Glucose (mmol/l)	4.05 – 6.73 4.78 – 5.14	3.86 – 10.77 4.87 – 5.18				
Fasting Insulin (pmol/l)	38.96±19.12 15.83 – 112.9 32.49 – 45.43	47.73±33.36 9.79 – 233.98 41.31 – 54.16	0.685	**0.027**	0.711	(0.159)
Fasting C peptide (nmol/l)	0.60±0.20 0.30 – 0.99 0.519 – 0.671	0.614±0.192 0.19 – 1.56 0.572 – 0.656	0.209 (n = 29)	**0.024** (n = 83)	0.167	(0.673)
Intact PTH (ng/l)	36.32±19.05 8.00 – 82.50 29.45 – 43.19	37.43±20.91 2.20 – 139.40 33.22 – 41.65	0.350 (n = 32)	**<0.001** (n = 97)	0.924	(0.939)
Vitamin D (nmol/l)	58.19±28.64 13.20 – 127.8 47.86 – 68.51	49.33±31.68 7.74 – 152.01 42.91 – 55.75	0.195 (n = 32)	**0.018** (n = 96)	0.443	**(0.030)**

Table 8.4. Comparison of EPO in **female sample population** in partitioned groups as in Table 8.1, **without** and **with deficiencies** in hemoglobin (Hb <125 g/l) and/or iron (<9.85 μmol/l) and/or ferritin (<20 ng/ml) by 95% CI of mean and Student t test (Mann Whitney U test).

Groups		Mean±SD Range 95% CI of mean	Student t test (Mann-Whitney U test), P
A.	**Without deficiencies (n = 36)**	6.72±2.57 2.03 – 13.41 5.85 – 7.60	--
B.	**Hb, iron and ferritin deficient (n = 18)**	18.60±13.62 4.12 – 62.52 11.82 – 25.37	**(<0.001)**
C.	**Hb or iron deficient (n = 18)**	8.89±2.88 5.39 – 13.36 7.45 – 10.32	**0.007**
D.	**Ferritin deficient (n = 39)**	8.37±2.92 3.85 – 19.10 7.42 – 9.31	**0.006**
E.	**Hb and ferritin deficient (n = 21)**	10.11±3.87 5.34 – 17.77 8.35 – 11.87	**<0.001**
F.	**Iron and ferritin deficient (n = 10)**	17.55±12.24 6.05 – 41.72 8.79 – 26.30	**(0.001)**

Table 8.5. Correlation (r) and significance of correlation (P) of EPO with related analytes in **males** of groups **without** and **with deficiencies** in hemoglobin (<125 g/l) and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml). Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after $\log_{10}$ transformation.

Variables related to EPO evaluated for correlation	**Pearson's, r**	**P**	**Pearson's, r**	**P**
	Without deficiencies (n = 53)		**With deficiencies (n = 16)**	
Blood Hemoglobin	0.059	0.675	-0.217	0.386
S. Iron	-0.138	0.324	**-0.252**	0.314
S. Ferritin	-0.011	0.936	0.181	0.473
Intact PTH	-0.054 (n = 45)	0.723	**0.274** (n = 12)	0.388
Vitamin D	-0.045 (n = 46)	0.766	**0.251** (n = 12)	0.432
Fasting Glucose	**0.295**	**0.032**	0.089	0.724
Fasting Insulin	0.007	0.961	0.149	0.724
Fasting C peptide	0.200 (n = 44)	0.193	**0.573** (n = 8)	**0.051**

Fig. 8.2. X-Y scatter diagram of analytes in **males** significantly **correlating with EPO** as in Table 8.5. (A) Correlating with **fating glucose** in without deficiencies (n = 53) and (B) with **fating C peptide** in groups with hemoglobin <125 g/l and/or iron <9.85 µmol/l and/or ferritin <20 ng/ml deficient (n = 8).

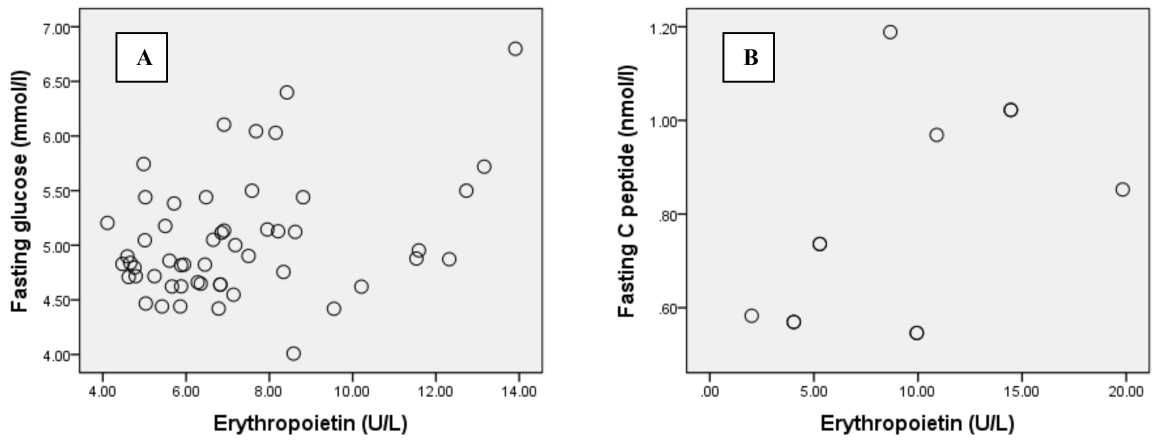
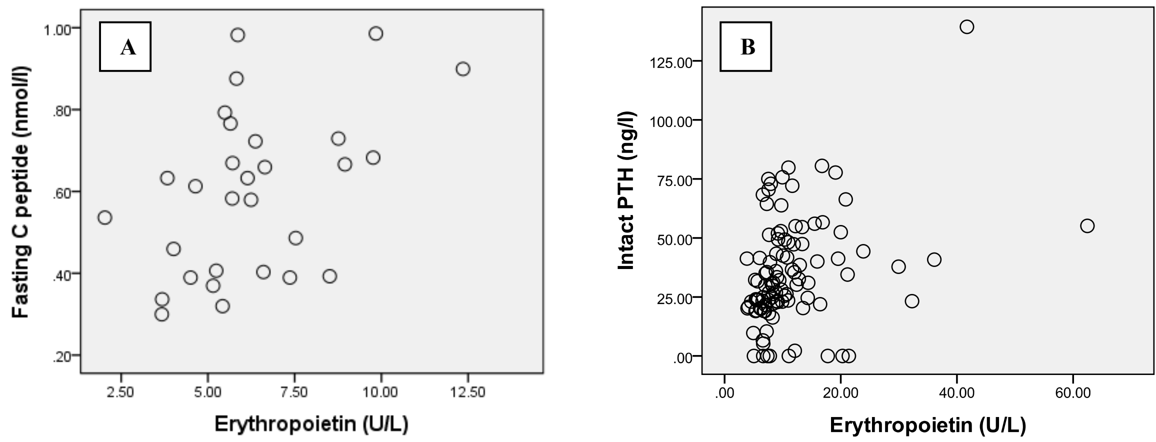


Table 8.6. Correlation (r) and significance of correlation (P) of EPO with related analytes in the **female sample** groups **without** and **with deficiencies** in hemoglobin (<125 g/l) and/or iron (<9.85 µmol/l) and/or ferritin (<20 ng/ml) by parametric (Pearson's, r) and non parametric (Spearman's rho ρ) methods.

Variables related to EPO evaluated for correlation	Pearson's, r	P	Spearman's rho ρ	P
	Without deficiencies (n = 36)		With deficiencies (n = 106)	
Blood Hemoglobin	**-0.563**	**<0.001**	**-0.199**	**0.041**
S. Iron	**-0.270**	0.111	**-0.409**	**<0.001**
S. Ferritin	0.239	0.160	**-0.407**	**<0.001**
Intact PTH	**0.341** (n = 32)	**0.056**	**0.476** (n = 97)	**<0.001**
Vitamin D	-0.158 (n = 32)	0.389	-0.034 (n = 96)	0.741
Fasting Glucose	0.134	0.437	0.158	0.107
Fasting Insulin	0.053	0.759	0.148	0.131
Fasting C peptide	**0.406** (n = 29)	**0.029**	0.173 (n = 83)	0.117

Fig. 8.3. X-Y scatter diagram in the **female sample**, of **EPO** with (A) **fasting C peptide** in groups without deficiencies (n = 29) and (B) **with intact PTH** in groups with hemoglobin <125 g/l and/or iron <9.85 µmol/l and/or ferritin <20 ng/ml deficient (n = 97).



NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 9.

Influence of Vitamin D Deficiency in Combination with Other Deficiencies, on Erythropoietin and the Related Analytes

9.1 Abstract

Deficiencies in vitamin D in isolation or in combination with hemoglobin (anemia), iron and ferritin were found to influence concentrations of related analytes resulting in changes in their correlations with erythropoietin. In the vitamin D sample (n = 148), the most abundant deficiency group was vitamin D deficiency in isolation (n = 33) followed by vitamin D deficiency in combination with ferritin (n = 24) and hemoglobin (n = 24). In the groups formed by the presence and absence of vitamin D deficiency, there were no major differences in concentrations of related analytes and in their correlations with EPO, both in the male and in the female samples. But in the samples with deficiency of vitamin D, there were significant differences both in the concentrations of analytes and in their correlations with EPO and PTH. Thought interpretations of the influences of few of the deficiencies could be made, in most cases the interpretations were complex. After correction of vitamin D in the participants, there were no major changes in the concentrations of analytes. But there were major change in the correlations of PTH with calcium in the male sample. In the female sample, there were major changes in the correlations of EPO and PTH with hemoglobin, ferritin and iron in the female sample in which the deficiencies were more.

9.2. Introduction

Ergosterol (D_2) and cholecalciferol (D_3) are metabolized to 25-hydroxyl vitamin D [25(OH)D] in the liver by the enzyme vitamin D 25-hydroxylase and cytochrome P450 (DeLuca, 2004; Lensmeye et al, 2006). The concentration of 25(OH)D in serum is 10 to 65 ng/ml or 25 to 162 nmol/l (conversion factor = 2.5). The half-life of circulating 25(OH)D is 2 to 3 weeks. At 25(OH)D concentrations near 30 ng/ml, dietary calcium absorption is maximal. Therefore, any reference interval for 25(OH)D, and the healthy range for 25(OH)D required for optimum functional activities should be discriminated. Both $25(OH)D_2$ and $25(OH)D_3$ are metabolized to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the biologically active hormone, by 25(OH)D 1α-hydroxylase, a cytochrome P450 enzyme, in kidney and placenta. Normal circulating concentrations of

$1,25(OH)_2D$ are approximately 15 to 60 pg/ml or 36 to 144 pmol/l. The half-life of $1,25(OH)_2D$ is 4 to 6 hours.

Circulating concentrations of $1,25(OH)_2D$ are regulated by PTH, phosphate, calcium, and $1,25(OH)_2D$ (Carmeliet et al, 2006; DeLuca, 2004). PTH and hypophosphatemia increase the synthesis of $1,25(OH)_2D$ by increasing the activity of 25(OH)D-1α-hydroxylase, whereas hypocalcemia acts indirectly by stimulating the secretion of PTH. Hypercalcemia, hyperphosphatemia and $1,25(OH)_2D$ reduce 25(OH)D 1α-hydroxylase activity and $1,25(OH)_2D$. $1,25(OH)_2D$ induces 25(OH)D 24-hydroxylase enzyme activity that produce 24,25- dihydroxyvitamin D (24,25(OH)2D), the most prevalent dihydroxylated vitamin D form in serum.

The major functions of vitamin D are related to absorption of calcium and bone metabolism. In bone metabolism vitamin D is involved in bone formation, bone turnover and may also be in erythropoiesis.

Biological actions of $1,25(OH)_2D$ maintains calcium and phosphate in blood through its actions on intestine, bone, kidney, and parathyroids glands. In the small intestine, $1,25(OH)_2D$ stimulates calcium absorption from the duodenum and phosphate absorption by the jejunum and ileum (Bikle et al, 2008; Carmeliet et al, 2006; DeLuca, 2004). The events which cause the absorption of calcium from the diet are: (1) calcium entry into the brush border cytoplasm of the intestinal epithelium, mediated by an epithelial Ca^{2+} transporter or channel (CaT1); (2) diffusion of calcium within the cell by calbindin-D9k which is a cytosolic calcium-binding protein and (3) exit of calcium from the cell across its basolateral membrane by the action of a CaATPase (e.g., a Na+/Ca2+ exchanger). Most of CaT1 and whole of calbindin D synthesis are vitamin D-dependent. High calcium diet down regulates CaT1 and calbindin D expression by down regulating the production of $1,25(OH)_2D$.

High concentrations of $1,25(OH)_2D$ increases bone resorption by inducing the differentiation of monocytic stem cells in bone marrow into osteoclasts and by stimulating osteoblasts to produce cytokines and other factors that influence osteoclast activity. By stimulating osteoblasts, $1,25(OH)_2D$ also increases alkaline phosphatase and osteocalcin concentration in circulation. In the kidneys, $1,25(OH)_2D$ exhibits a short

negative feedback loop mechanism inhibiting its own synthesis and stimulates its own metabolism to 24,25(OH)2D. 1,25$(OH)_2$D also acts directly on the parathyroid gland to inhibit the synthesis and secretion of PTH. 1,25$(OH)_2$D also increases the calcium sensing receptor in the parathyroid gland, thus sensitizing the gland to calcium inhibition (Bikle et al, 2008).

In target cells 1,25$(OH)_2$D exerts its actions by associating with a specific nuclear vitamin D receptor (VDR). This receptor is analogous to the steroid receptors for androgens, estrogens, and corticosteroids and is expressed widely in tissues, and most cells respond to 1,25$(OH)_2$D (Bouillon et al, 2008). The vitamin D receptor can form heterodimers with members of the retinoid X receptor and is a member of the NR1I family. Other members in this family are the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR).

Determination of circulating 25(OH)D may be useful in the differential diagnosis of hypocalcemia, hypercalcemia, or hypercalciuria and for evaluating vitamin D status in health and in bone and mineral disorders. Only measurements of 25(OH)D and 1,25$(OH)_2$D have proven clinical value. 1,25$(OH)_2$D measurements are rarely needed in routine or even subspecialty clinical practice (Lee et al, 2007; Prentice et al, 2008).

The nutritional status of vitamin D is best determined through the measurement of 25(OH)D, rather than vitamin D, because (1) 25(OH)D is the main circulating form of vitamin D, (2) have longer half-life and is less affected by day-to-day variation, exposure to sunlight, or food intake and (3) measurement of 25(OH)D is easier compared to vitamin D which require more technically complex methods (Hollis, 2007; Hollis, 2008; Lensmeyer et al, 2006). Nutritional vitamin D deficiency is higher in breast-fed infants, strict vegetarians who abstain from eggs and milk, individuals with darker skin pigmentation, and the elderly.

Circulating 25(OH)D may be decreased by reduced availability of vitamin D, inadequate conversion of vitamin D to 25(OH)D, increased metabolism of 25(OH)D, and urinary loss of 25(OH)D with its transport protein DBP. Reduced availability of vitamin D is mainly due to the inadequate exposure to sunlight, dietary deficiency, malabsorption syndromes, and gastric or small bowel resection.

Severe hepatocellular disease has been associated with vitamin D deficiency and may be due to the inadequate conversion of vitamin D to 25(OH)D. Drugs such as phenytoin, phenobarbital and rifampin stimulate drug metabolizing enzymes which accelerate the metabolism of vitamin D and its metabolites. In nephrotic syndrome, the serum 25(OH)D concentrations may be reduced because of the urinary loss of DBP and 25(OH)D.

Vitamin D deficiency is associated with a greater risk of anemia, lower hemoglobin and higher usage of erythrocyte-stimulating agents (Sim et al, 2010). Vitamin D deficiency is also associated with a number of clinical consequences like cardiovascular disease (Zittermann, 2006; Bischoff et al, 2006), decreased bone mineralization and early mortality (Bouillon et al, 1993; DeLuca, 2004).

The main role of vitamin D is to regulate bone and mineral metabolism. In addition to this, it had many other biological actions including muscle strengthening, cellular proliferation and differentiation, immune system modulation (DeLuca, 2004), inhibition of rennin synthesis (Li, 2003), insulin production (Chiu et al, 2004) and also have a role in erythropoiesis (Sim et al, 2010).

Vitamin D supplementations in hemodialysis patients was associated with a reduction in erythrocyte-stimulating agents (ESA), increased reticulocytosis and also affect marrow function (Saab et al, 2007). Correction of 25-D deficiency appears to have a limited impact on renal osteodystrophy in patients with end stage renal disease; it may improve other clinical outcomes, such as anemia in renal disease. ERGO supplementation in hemodialysis patients may decrease the need for EPO but probably has no impact on markers of mineral metabolism (Kumar et al, 2011).

Measurement of circulating 1,25$(OH)_2$D is diagnostic in vitamin D–dependent rickets types 1 and 2, and in disease states associated with overproduction of 1,25$(OH)_2$D such as sarcoidosis, tuberculosis, Hodgkin's disease, fungal infection, Wegener's granulomatosis, and lymphoma. 1,25$(OH)_2$D test result also gives confirmatory information in the evaluation of hypercalcemia, hypercalciuria, hypocalcemia, and bone and mineral disorders. Concentrations of 1,25$(OH)_2$D are increased in type 2 vitamin D–dependent rickets, primary hyperparathyroidism, although its diagnosis does not require

measurement of $1,25(OH)_2D$ and in $1,25(OH)_2D$ intoxication. Reduced concentrations of $1,25(OH)_2D$ are seen in patients with renal failure, hypercalcemia of malignancy, hyperphosphatemia, hypoparathyroidism, pseudohypoparathyroidism, type 1 vitamin D–dependent rickets, hypomagnesemia, nephrotic syndrome and severe hepatocellular disease.

Vitamin D is one of the targets of PTH action by regulation of the formation of 1,25- dihydroxy vitamin D ($1,25(OH)_2D$) and they may be required for erythropoiesis. This chapter analysis to see if there is an influence by vitamin D deficiency on analytes related to EPO.

9.3. Objectives

8.3.1. Aspects of the Original Objectives Addressed in this Chapter

Vitamin D deficiency is a common life style disease associated with EPO and iron metabolism is deficiencies in hemoglobin (anemia), iron and ferritin. These disease states can occur in isolation or in combination with other common deficiencies such as anemia and deficiencies in iron and ferritin.

1. Samples were grouped into those without and with deficiencies in vitamin D in isolation or in combination with hemoglobin, iron and ferritin.
2. Significant differences in concentrations and correlations of the analytes related to EPO in samples without and with deficiencies were evaluated. These changes were also evaluated after correcting the deficiencies.

8.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may **cause variations in factors that are regulated by EPO**. In this study, we examine

1. **Correlation and significant variations of erythropoietin with other analytes in healthy individuals,**

2. **Identification of such correlations and significant variations in disease** conditions and the **changes in these analytes during treatment**.

9.4. Materials and Methods

9.4.1. Study setting and Research design

Please refer 2.1. Study setting and Research design section in chapter 2

9.4.2. Selection of participants, inclusion and exclusion criteria

Healthy cross section of participants 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Chapter 2; Table 2.1; Stage I).

9.4.2.1. Exclusion of subclinical diseases and deficiencies by Clinical Biochemistry assays

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of individuals with diseases.

Samples with or without vitamin D, hemoglobin, iron and ferritin deficiency, and increased EPO were selected. Sample in which fasting EPO, PTH, vitamin D, fasting insulin, fasting C peptide and analytes related to iron metabolism have been estimated, were selected for the study. The exclusion criteria reduced the study sample number from

over 600 to 148. After selection of study samples, they were subjected to groupings according to deficiencies in vitamin D (<50nmol/l), hemoglobin <125 g/l, iron <9.85 µmol/l (55 µg/dl) and ferritin <20 ng/ml (Table 9.1). The above cut off levels was designed for this study to include individuals with increased concentrations of EPO, intact PTH, and deficiencies in vitamin D, hemoglobin, iron and ferritin.

9.4.3. Sample Collection and Sample Preparation

Please refer 2.3. Sample collection section in chapter 2

9.4.4. Analytical Control and Assays

Analytical measurements of **ALT, triglycerides, creatinine, hsCRP, total calcium, fasting glucose, intact PTH, EPO, vitamin D, hemoglobin, iron, ferritin fasting insulin and fasting C peptide** assays were done. **Please refer detailed description of these assays in chapter 2.**

9.4.5. Diagnostic Criteria and Reference Interval

Please refer 2.26. Diagnostic criteria and reference interval section in chapter 2.

9.4.6. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer section 2.27. Statistical analysis in chapter 2

9.5. Result

9.5.1. Subgroups formed from Vitamin D Deficiency in Isolation and in Combination

Vitamin D deficiency may influence other deficiencies. These deficiencies in isolation or in combination may influence other analytes. Deficiency of vitamin D may occur in isolation or in combination with other analytes. There were also gender differences in the deficiencies. In females, vitamin D, hemoglobin, iron and ferritin deficiencies were very much more (Table 9.1, Column A and H). Vitamin D deficiency was found to occur in isolation in significant numbers (Table 9.1, Column B; n = 16). But

the highest relative prevalence was found in vitamin D deficiency in combination with ferritin deficiency in females (n = 24). In the female sample population, hemoglobin, iron and ferritin deficiencies were very much more than in males (Table 9.1, Column C to G). The results on the deficiencies in hemoglobin and iron in the female sample could be explained by the monthly blood loss. The higher prevalence of vitamin D deficiency in females may be due to the decreased exposure to sunlight.

In the male sample, vitamin D deficiencies were present in significant numbers, but were less than in females (Table 9.1, Column B). Understandably, hemoglobin, iron and ferritin deficiencies were absent or uncommon (Table 9.1, Column C to G).

Vitamin D deficiencies occurred in combination with hemoglobin, ferritin and iron deficiency. These observations made us group vitamin D deficiencies according to the presence and absence of deficiencies in isolation or in combination with hemoglobin, ferritin and iron deficiency. Vitamin D deficiency can occur in isolation in male groups. In females, the most frequent deficiencies occurred in combination were that of vitamin D with ferritin. This was followed by isolated vitamin D deficiency.

9.5.2. Concentration of Analytes without and with Deficiencies in Vitamin D

In the male sample, deficiencies of vitamin D did not affect the concentrations of EPO, PTH or other analytes related to them (Table 9.2). In the female samples, the deficiency of vitamin D did not show significant difference in most analytes, except ALT, creatinine which decreased in sample with vitamin D and ferritin deficiencies (Table 9.3). This probably was due to decrease in the damage to cell membranes with deficiencies in intracellular iron or ferritin.

9.5.3. Correlations of EPO and PTH in Absence and Presence of Vitamin D Deficiency

9.5.3.1. Correlations of EPO

In the male and female samples without deficiencies, EPO correlated positively with PTH, C peptide and fasting insulin (with correlation statistic, r from 0.313 to 0.575).

In these samples EPO correlated negatively with hemoglobin and iron (with correlation statistic, r from -0.355 to -0.773). The correlations were very similar in the male and female samples, except for fasting insulin in females which had the lowest correlation statistic, r (r = 0.313 and P = 0.120).

In the male sample with vitamin D deficiency, all correlations were lost (Table 9.4A). In females, most of the correlations were lost or decreased in presence of vitamin D deficiency. There was higher prevalence of vitamin D deficiency in females and, therefore, there was a negative correlation of vitamin D with EPO. In the female sample, in the presence of vitamin D and ferritin deficiency, EPO correlated with PTH. Interpretations were complex and results could not be easily explained.

9.5.3.2. Correlations of PTH

But PTH, in the male sample without deficiencies, correlated positively with EPO, fasting insulin and C peptide (Fig.9.1 A, B, C) but did not correlate with hemoglobin and iron (Table 9.4B). In the female sample without deficiencies, PTH correlated positively with EPO, but negatively with hemoglobin (Fig. 9.2 A, B) but did not correlate with fasting insulin, C peptide and iron (Table 9.5B). This was due to gender differences in the regulation of hemoglobin synthesis in males and females.

In the male sample with deficiencies, PTH did not correlate with EPO as before, but correlated negatively with vitamin D and calcium (Fig. 9.1D), and correlated weakly and negatively with hemoglobin and iron. PTH also correlated weakly and positive with fasting insulin (Table 9.4B). Again, explanation of the influence of deficiency on correlations of PTH was difficult. The negative correlation of PTH with vitamin D and calcium could be explained.

In the female sample with deficiencies of vitamin D, PTH did not correlate with EPO, but correlated negatively with vitamin D (Fig. 9.2C) and calcium, and correlated weakly and negatively with hemoglobin. PTH also correlated weakly and positively with fasting insulin and C peptide (Table 9.5B). In the female sample with deficiencies of vitamin D and ferritin, PTH correlates positively with EPO and fasting insulin (Fig 9.2D). Here also interpretations of the result are difficult.

9.5.4. Concentration of Analytes before and after Intake of Vitamin D

In the male sample after intake of vitamin D the mean concentration of vitamin D increased from 93 nmol/l–194 nmol/l. But no significant changes in the concentration of analytes assayed were seen except a non significant decrease in PTH, EPO and C peptide (Table 9.6). The absence of significance may be due to lower sample number. These results indicate that increase in vitamin D decreases C peptide due to decrease in EPO levels. EPO and C peptide levels were decreased probably due to improvement of insulin resistance in the male sample. Decrease in insulin resistance decreases insulin secretion and C peptide.

In the female sample intake of vitamin D increased vitamin D levels from 74.45 nmol/l–255.71 nmol/l. In this sample hemoglobin concentration decreased resulting in a non significant decrease of EPO and PTH. But fasting insulin and C peptide increased non significantly in the sample (Table 9.7).

9.5.5. Correlations of EPO and PTH, Before and After Intake of Vitamin D

In the male sample the non significant negative correlation of EPO with total calcium was not influenced by vitamin D intake. The non significant negative correlation of EPO with iron, C peptide and positive correlation with PTH were lost after intake of vitamin D. Similarly, the negative correlation of PTH with calcium was lost after intake of vitamin D (Table 9.8).

In the female sample the strong negative correlation of EPO with hemoglobin, iron and ferritin were lost after intake of vitamin D (Table 9.9). There was a negative correlation of EPO with insulin and C peptide which was not influenced by intake of vitamin D. The negative correlation of PTH with hemoglobin was lost after intake of vitamin D. The negative correlation of PTH with vitamin D was not influenced by increase in vitamin D. These results were difficult to interpret.

9.6. Discussion

Plasma concentration of vitamin D is assayed to evaluate its nutritional status and deficiency state. This is best determined by the measurement of 25(OH)D, rather than vitamin D for the following reasons. 25(OH)D is the main circulating form of vitamin D. It has longer half-life and because of this, 25(OH)D is less affected by day to-day variation, exposure to sunlight, or food intake. Measurement of 25(OH)D is relatively easy compared with the more technically complicated methods used to measure vitamin D (Hollis, 2007; Hollis, 2008; Lensmeyer et al, 2006).

Causes of deficiency of 25(OH)D may be due to reduced availability of vitamin D which occurs with inadequate exposure to sunlight, dietary deficiency, malabsorption syndromes, and gastric or small bowel resection. The concentrations may be also decreased by inadequate conversion of vitamin D to 25(OH)D. Also, the accelerated metabolism of 25(OH)D, and urinary loss of 25(OH)D with its transport protein DBP can decrease 25(OH)D.

The analytes related to vitamin D and whose deficiency can occur in combination with vitamin D are hemoglobin, iron and ferritin. These deficiencies occur more in females and are related to vitamin D through EPO and PTH. Deficiencies in vitamin D in isolation or in combination with hemoglobin (anemia), iron and ferritin were found to influence concentrations of related analytes resulting in changes in their correlations with EPO and PTH. But the effects of these deficiencies are complex and many times difficult to explain. Therefore, the clinical interpretations of EPO and PTH serum concentrations require exclusion of these deficiencies or correction of the deficiencies.

In the vitamin D deficiency and anemia were the most common deficiencies seen in the female sample. The most abundant group was vitamin D deficiency in isolation followed by vitamin D deficiency in combination with ferritin and hemoglobin.

In the groups formed by the presence and absence of vitamin D deficiency, there were no major differences in concentrations of related analytes. In the male and female sample without deficiency, there were also no major gender differences seen in the correlations with EPO.

But in the samples with deficiency of vitamin D, there were significant differences in the correlations with EPO and PTH. Though interpretations of the influences of few of the deficiencies could be made, in most cases the interpretations were complex.

After correction of vitamin D deficiency in the participant by taking vitamin D, there were no major changes in the concentrations of analytes. But there were major change in the correlations of PTH with calcium in the male sample. In the female sample, there were major changes in the correlations of EPO and PTH with hemoglobin, ferritin and iron, in whom the deficiencies were more.

9.7. Conclusion

Deficiencies of vitamin D in isolation or in combination with the deficiencies hemoglobin, iron and ferritin were more in the female sample population. These deficiencies influenced the correlations of EPO and PTH with their related analytes. The results indicate that minor changes in concentrations of EPO and PTH are difficult to clinically interpret when there are deficiencies of vitamin D, hemoglobin, iron and ferritin.

Table 9.1. Number of samples grouped according to the presence of **deficiencies (n = 84)** in Vitamin D (<50nmol/l; Vit D) ,Hemoglobin (<125 g/l; HB), Iron (<9.85 µmol/l; Fe) and Ferritin (<20 ng/ml); Vitamin D deficiency in isolation (n = 33: Male n = 17 and Female n =16) or vitamin D in combination with ferritin (n = 24), hemoglobin and iron from the study population (n = 148). Samples without any of these deficiencies (A) were taken as **control sample (n = 64)**.

Gender	(A) Without deficiency (n = 64)	Deficiency samples							
		(B) Vit D (n = 33)	(C) Vit D, HB, Fe and Ferritin	(D) Vit D and HB	Vit D, Hb and Fe	(E) Vit D, Ferritin (n = 24)	(F) Vit D, HB and Ferritin	(G) Vit D, Fe and Ferritin	(H) Total with deficiency (n = 84)
Male (n = 54)	37	17	0	0	0	0	0	0	17
Female (n = 94)	27	16	9	3	4	24	8	3	67

Table 9.2. Comparison of analytes in **male sample population** in groups **without (A)** and **with (B) vitamin D deficiency** by 95% CI of mean and Student t test (Mann Whitney U test). The selected sample did not have deficiencies of hemoglobin, iron and ferritin. Min-max levels are the minimum and maximum levels for a variable.

Variables in SI units	**A. Without deficiencies (n = 37) Mean±SD min-max levels 95% CI mean**	**B. With vitamin D deficiency (n = 17) Mean±SD min-max levels 95% CI mean**	**After log10 transformation**			
			Shapiro – Wilk test, P		**Levene's test, P**	**Student t test (Mann-Whitney U test), P**
			Without deficiency	**With deficiency**		
Age (>18 years)	28.14±14.20 18.00 – 81.00 23.40 – 32.87	24.29±9.23 18.00 – 59.00 19.55 – 29.03	--	--	--	--
BMI (kg/m^2)	22.68±3.64 16.42 – 29.80 21.47 – 23.89	21.72±3.53 15.32 – 28.20 19.91 – 23.54	0.111	0.587	0.588	0.380
Waist circum-ference (cm)	81.53±8.63 64.50 – 97.00 78.65 – 84.40	79.50±8.12 63.00 – 92.00 75.32 – 83.68	0.150	0.287	0.707	0.434
Fasting Glucose (mmol/l)	5.34±0.613 4.44 – 6.95 5.14 – 5.55	5.05±0.775 4.01 – 6.80 4.65 – 5.45	0.089	0.093	0.465	0.103
ALT (U/L)	33.03±12.64 16.00 – 60.00 28.81 – 37.24	32.76±14.75 16.00 – 60.00 25.18 – 40.35	0.257	0.131	0.420	0.803
Triglycerides (mmol/l)	1.05±0.342 0.57 – 1.93 0.937 – 1.17	0.982±0.445 0.37 – 2.17 0.753 – 1.21	0.446	0.557	0.059	0.286
S. Creatinine (µmol/l)	83.88±12.62 61.88 -106.08 79.68 – 88.09	88.40±14.43 53.04 – 106.1 80.71 – 96.09	0.020	<0.001	0.706	(0.191)
High sensitive CRP (mg/l)	0.852±0.726 0.1 – 3.3 0.61 – 1.09	1.34±1.32 0.10 – 3.70 0.657 – 2.02	0.168	0.201	0.201	0.333
Intact PTH (ng/l)	31.16±18.00 11.10 – 96.81 25.16 – 37.16	33.86±15.71 12.30 – 70.40 25.78 – 41.94	0.497	0.371	0.613	0.404
S. EPO (U/L)	7.88±3.99 3.64 – 20.11 6.55 – 9.21	7.85±2.42 4.98 – 13.91 6.60 – 9.09	0.058	0.732	0.128	0.640
Vitamin D (nmol/l)	89.90±34.11 50.00 – 184.9	34.99±12.81 12.46 – 47.30	0.016	0.001	0.027	(<0.001)

	78.53 – 101.3	28.41 – 41.58				
Total Calcium (mmol/l)	2.51±0.124 2.30 – 2.88 2.47 – 2.55	2.48±0.181 1.90 – 2.75 2.38 – 2.57	0.462	0.001	0.462	(0.896)
Blood Hemoglobin (g/l)	145.94±14.28 125.0 – 181.8 141.2 – 151.7	148.46±14.24 125.0 – 172.0 141.1 – 155.8	0.261	0.401	0.812	0.540
S. Iron (µmol/l)	21.77±7.03 10.20 – 39.20 19.42 – 24.11	22.62±4.99 11.10 – 28.82 20.05 – 25.19	0.681	0.039	0.169	(0.434)
S. Ferritin (ng/ml)	62.98±30.81 20.90 – 137.5 52.70 – 73.25	59.91±30.67 22.10 – 112.5 44.14 – 75.68	0.058	0.071	0.972	0.754
Fasting Insulin (pmol/l)	45.73±23.11 18.68 – 124.7 37.91 – 53.55	47.04±18.25 17.92 – 85.22 37.66 – 56.43	0.416	0.728	0.941	0.678
Fasting C peptide (nmol/l)	0.715±0.213 0.35 – 1.27 0.643 – 0.787	0.727±0.234 0.36 – 1.16 0.606 – 0.847	0.791	0.529	0.331	0.969

Table 9.3. Comparison of analytes in **female sample** from groups **without (A), with (B) vitamin D deficiency, and (C) with vitamin D and ferritin deficiency** by 95% CI of mean and Student t test (Mann Whitney U test). Samples in groups (A) and (B) did not have deficiencies of hemoglobin, iron and ferritin. Min-max levels are the minimum and maximum concentrations.

Variables in SI units	**A. Without deficiencies (n = 27) Mean±SD min-max levels 95% CI mean**	**B. With vitamin D deficiencies (n = 16) Mean±SD min-max levels 95% CI mean**	**C. with vitamin D and ferritin deficiency (n = 24) Mean±SD min-max levels 95% CI mean**	**after log_{10} transformation Student t test (Mann-Whitney U test), P**	
				Compa-rison between A and B	**Compa-rison between A and C**
Age (>18 years)	31.22±11.57 18.00 – 52.00 26.65 – 35.80	26.50±9.42 20.00 – 46.00 21.48 – 31.52	23.24±2.76 18.00 – 33.00 22.10 – 24.38	--	--
BMI (kg/m^2)	23.09±3.76 16.40 – 30.00 21.61 – 24.58	20.89±2.99 17.29 – 27.63 19.30 – 22.48	22.26±3.40 16.87 – 30.04 20.83 – 23.69	**0.057**	0.438
Waist circum-ference (cm)	81.32±9.04 61.00 – 100.0 77.75 + 84.90	75.63±7.79 65.00 – 91.00 71.47 – 79.78	78.44±7.25 61.00 – 99.00 75.38 – 81.50	**0.044**	(0.257)
Fasting Glucose (mmol/l)	5.19±0.684 4.27 – 6.73 4.92 – 5.46	4.83±0.477 4.05 – 5.92 4.58 – 5.09	4.89±0.496 3.86 – 5.87 4.68 – 5.10	**0.079**	**0.093**
ALT (U/L)	25.52±8.54 13.00 – 48.00 22.14 – 28.90	26.50±10.25 17.00 – 51.00 21.04 – 31.96	21.08±4.65 13.00 – 33.00 19.12 – 23.05	(0.990)	**0.042**
Triglycerides (mmol/l)	0.926±0.356 0.49 – 1.74 0.785 – 1.74	0.823±0.261 0.52 – 1.32 0.685 – 0.962	0.920±0.338 0.42 – 2.12 0.778 – 1.06	(0.309)	(0.763)
S. Creatinine (µmol/l)	67.45±14.12 42.20 – 106.1 61.86 – 73.03	64.64±7.01 53.04 – 79.56 60.91 – 68.38	61.14±9.37 44.20 – 88.4 57.19 – 65.10	(0.534)	**(0.058)**
High sensitive CRP (mg/l)	0.933±0.807 0.10 – 3.10 0.614 – 1.25	1.28±1.79 0.10 – 5.00 0.323 – 2.24	0.925±0.839 0.1 – 3.00 0.571 – 1.28	(0.307)	0.834

Intact PTH (ng/l)	36.97±15.21 10.40 – 66.30 30.95 – 42.99	41.14±21.14 12.60 – 82.50 29.87 – 52.40	35.13±19.42 5.20 – 77.70 26.93 – 43.33	0.717	0.415
S. EPO (U/L)	7.64±3.10 2.03 – 15.35 6.41 – 8.87	6.59±1.69 3.68 – 9.84 5.69 – 7.49	8.74±3.19 4.59 – 19.10 7.39 - 10.09	(0.309)	0.137
Vitamin D (nmol/l)	86.86±26.65 50.0 – 135.83 76.32 – 97.40	33.56±11.91 13.20 – 47.92 27.21 – 39.90	31.26±8.81 20.00 – 46.43 27.55 – 34.98	**(<0.001)**	**<0.001**
Total Calcium (mmol/l)	2.47±0.113 2.30 – 2.78 2.43 – 2.52	2.42±0.209 2.13 – 2.93 2.31 – 2.53	2.46±0.102 2.23 – 2.70 2.41 – 2.50	(0.198)	0.656
Blood Hemoglobin (g/l)	133.15±8.93 125.0 – 156.2 129.6 – 136.7	135.13±7.78 125.0 – 154.0 131.0 – 139.3	132.38±6.96 125.7 – 150.0 129.4 – 135.3	(0.213)	(0.887)
S. Iron (µmol/l)	16.82±4.95 10.20 – 31.15 14.86 – 18.78	20.93±5.84 13.07 – 34.55 17.82 – 24.04	17.50±4.92 9.85 – 27.57 15.43 – 19.58	0.016	0.607
S. Ferritin (ng/ml)	42.58±24.42 20.80 – 138.6 32.92 - 52.24	37.80±14.10 21.30 – 64.70 30.29 – 45.31	11.83±4.02 5.30 – 18.4 10.13 – 13.52	0.584	**(<0.001)**
Fasting Insulin (pmol/l)	44.73±28.12 19.31 - 160.78 33.67 - 56.08	44.97±20.89 16.18 - 90.22 33.84 - 56.1	42.45±20.76 14.17 - 96.54 33.69 - 51.22	0.875	0.804
Fasting C peptide (nmol/l)	0.67±0.277 0.19 - 1.28 0.558 - 0.782	0.626±0.192 0.34 - 0.99 0.519 - 0.732	0.621±0.194 0.19 - 0.93 0.539 - 0.703	0.850	(0.727)

Table 9.4. Correlation (r) and significance of correlation (P) in **male sample** of EPO **(A)** and Intact PTH **(B)** with related analytes in groups **without and with** Vitamin D deficiency in isolation. Groups without and with vitamin D deficiency had no **deficiencies of 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	Without deficiency (n = 37)		With vitamin D deficiency (n = 17)	
	Correlation coefficient, r	P	Correlation coefficient, r	P
A. Correlations of EPO				
Intact PTH	**0.463**	**0.004**	0.011	0.966
Vitamin D	0.144	0.501	0.152	0.561
Total Calcium	-0.207	0.218	0.261	0.312
Blood Hemoglobin	**-0.355**	**0.031**	-0.135	0.604
S. Iron	**-0.435**	**0.007**	-0.152	0.561
S. Ferritin	-0.015	0.928	-0.016	0.952
Fasting Insulin	**0.518**	**0.001**	-0.023	0.930
Fasting C peptide	**0.361**	**0.031**	-0.005	0.986
B. Correlations of Intact PTH				
S. EPO	**0.463**	**0.004**	0.011	0.966
Vitamin D	-0.063	0.712	**-0.408**	0.104
Total Calcium	-0.215	0.201	**-0.638**	**0.006**
Blood Hemoglobin	-0.073	0.668	-0.260	0.314
S. Iron	-0.208	0.217	-0.266	0.302
S. Ferritin	0.266	0.112	0.141	0.588
Fasting Insulin	**0.567**	**<0.001**	**0.361**	0.155
Fasting C peptide	**0.516**	**0.001**	0.238	0.358

Fig.9.1. X-Y scatter diagram, of Intact PTH in **male sample** with EPO **(A)**, fasting insulin **(B)** and fasting C peptide **(C)** in groups **without Vitamin D deficiency** (n = 37) and **(D)** with total calcium in groups **with vitamin D deficiency (n = 17).** Both groups had no deficiencies of hemoglobin, iron and ferritin).

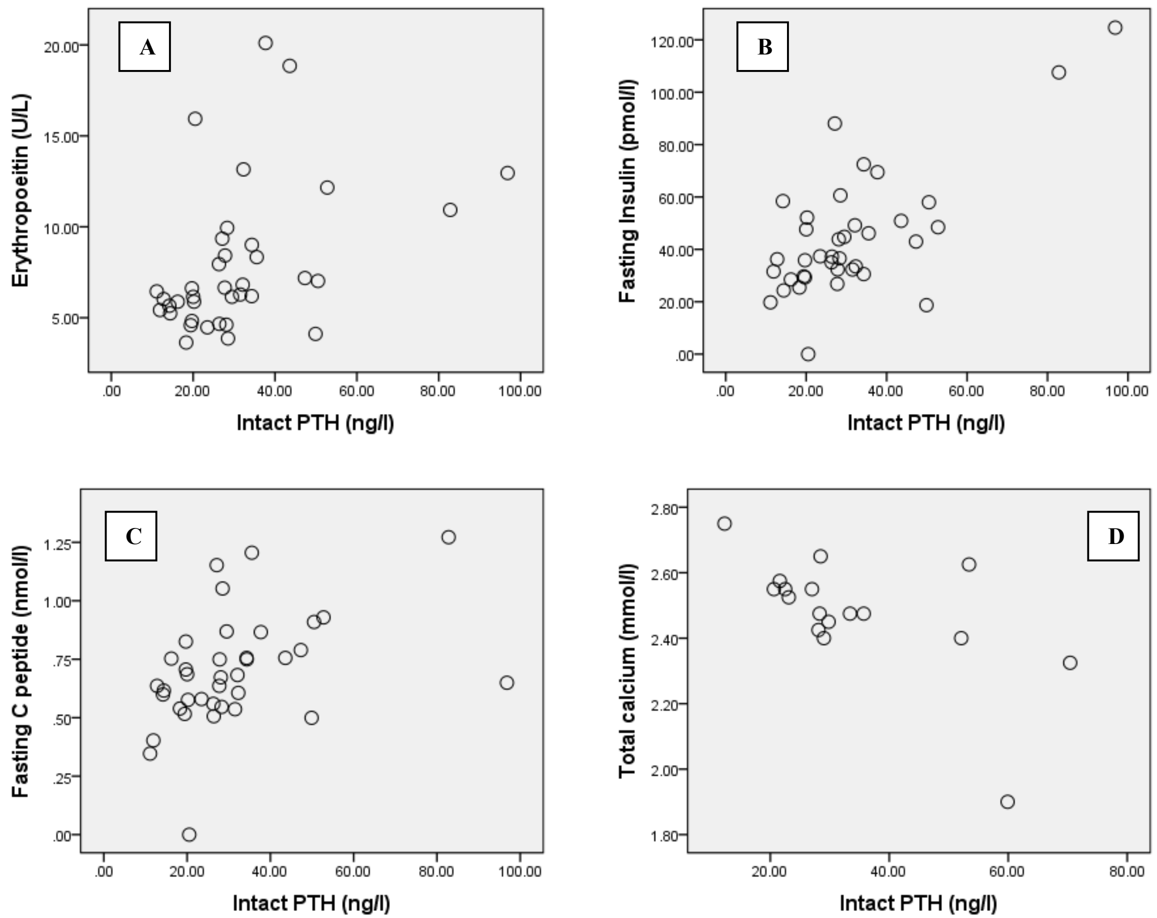


Table 9.5. Correlation (r) and significance of correlation (P) in **female sample** of EPO **(A)** and intact PTH **(B)** with related analytes in groups **without and with** vitamin D deficiency in isolation. Both groups had no deficiencies of hemoglobin, iron and ferritin. Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	**Without deficiency (n = 27)**		**With vitamin D deficiency (n = 16)**		**With vitamin D and ferritin deficiency (n = 24)**	
	Correlation coefficient, r	**P**	**Correlation coefficient, r**	**P**	**Correlation coefficient, r**	**P**
A. Correlations of EPO						
Intact PTH	**0.575**	**0.002**	0.191	0.478	**0.548**	**0.006**
Vitamin D	0.180	0.368	**-0.571**	**0.021**	-0.169	0.431
Total Calcium	-0.163	0.417	-0.282	0.290	-0.151	0.481
Blood Hemoglobin	**-0.773**	**<0.001**	**-0.375**	0.148	-0.085	0.693
S. Iron	**-0.508**	**0.007**	-0.073	0.788	-0.158	0.460
S. Ferritin	0.064	0.750	0.279	0.295	0.060	0.779
Fasting Insulin	**0.313**	0.120	0.481	0.059	**0.581**	**0.003**
Fasting C peptide	**0.379**	**0.056**	**0.563**	**0.029**	**0.410**	**0.047**
B. Correlations of Intact PTH						
S. EPO	**0.575**	**0.002**	0.191	0.478	**0.548**	**0.006**
Vitamin D	0.054	0.789	**-0.696**	**0.003**	-0.084	0.697
Total Calcium	-0.214	0.284	**-0.398**	0.126	-0.285	0.176
Blood Hemoglobin	**-0.467**	**0.014**	**-0.352**	0.181	-0.074	0.731
S. Iron	-0.197	0.324	0.295	0.268	0.000	0.999
S. Ferritin	0.180	0.368	0.365	0.165	-0.032	0.882
Fasting Insulin	-0.083	0.686	0.449	0.081	**0.562**	**0.004**
Fasting C peptide	0.107	0.604	0.497	0.060	0.292	0.166

Fig.9.2. X-Y scatter diagram of **Intact PTH** with EPO **(A)** and hemoglobin **(B)** in groups **without** Vitamin D, hemoglobin, iron and ferritin deficiencies (n = 27) and **(C)** with vitamin D in groups **with vitamin D deficient** but without deficiencies of hemoglobin, iron and ferritin) (n = 16) and (D) with fasting insulin in Vitamin D and ferritin deficient (n = 24) **female** samples.

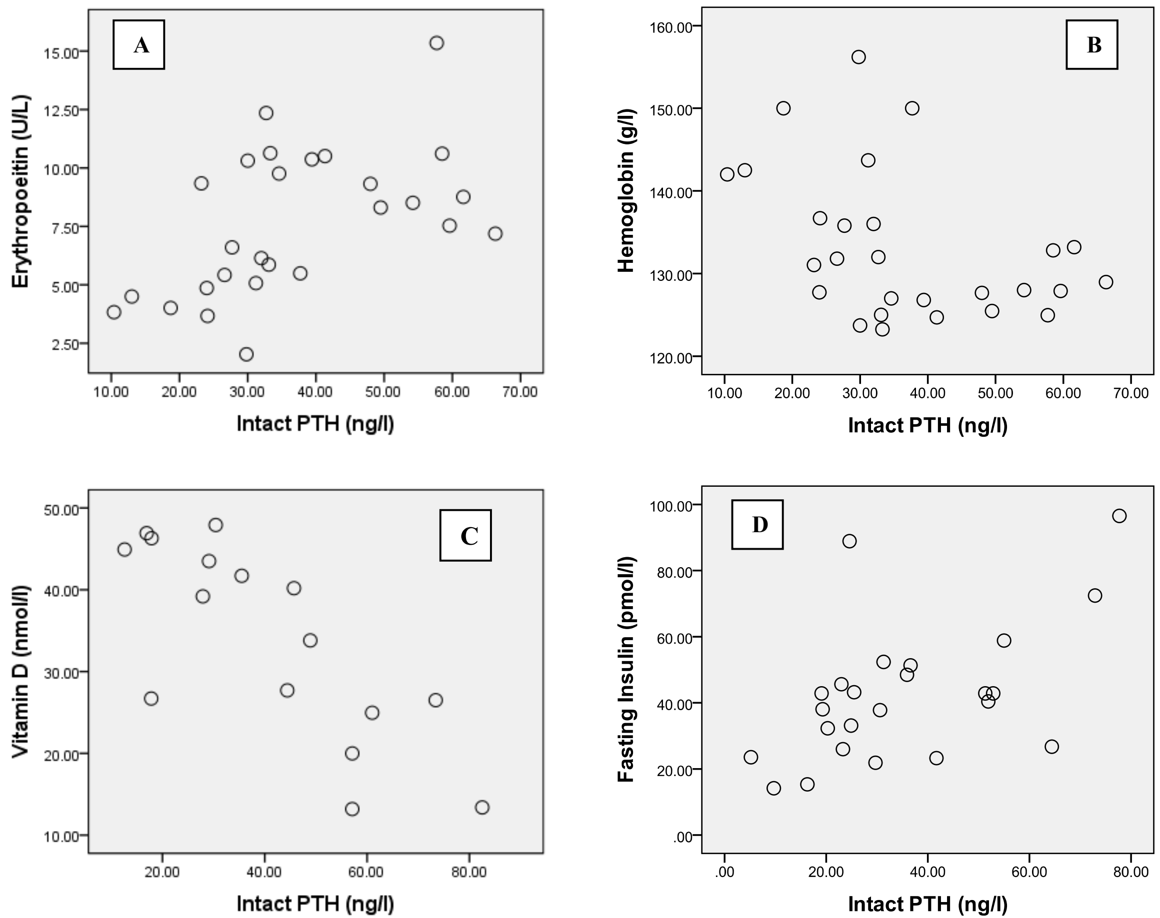


Table 9.6. Comparison of analytes in **male sample population** in groups **(A) before and (B) after taking cholecalciferol D3 60K units by** 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	A. Before intake (n = 19) Mean±SD Range 95% CI mean	B. After intake (n = 19) Mean±SD Range 95% CI mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			Before intake	After intake		
Intact PTH (ng/l)	33.62±14.69 8.40 – 71.00 26.54 – 40.70	28.77±12.13 9.50 – 57.90 22.92 – 34.62	0.144	0.823	0.999	0.365
S. EPO (U/L)	8.98±3.73 3.21 – 17.74 7.18 – 10.78	8.02±3.42 1.94 – 17.32 6.38 – 9.67	0.988	0.269	0.957	0.410
Vitamin D (nmol/l)	93.00±36.51 24.96 – 150 75.40 – 110.6	194.0±105.47 64.15 – 551.6 143.2 – 244.8	0.095	0.574	0.951	**<0.001**
Total Calcium (mmol/l)	2.49±0.149 2.20 – 2.90 2.41 – 2.56	2.48±0.135 2.20 – 2.75 2.42 – 2.55	0.110	0.411	0.962	0.829
Blood HB (g/l)	147.17±16.01 114.7 – 186.0 139.5 – 154.9	144.35±13.55 121.6 – 168.5 137.8 – 150.9	0.831	0.485	0.642	0.592
S. Iron (µmol/l)	18.73±8.14 1.79 – 32.58 14.81 – 22.65	18.61±7.42 7.34 – 35.08 15.03 – 22.18	**0.001**	0.676	0.186	(1.00)
S. Ferritin (ng/ml)	111.78±86.65 23.50 – 366.8 70.02 – 153.6	109.53±65.07 30.80 – 273.1 78.17 – 140.9	0.773	0.747	0.899	0.889
Fasting Insulin (pmol/l)	57.73±33.42 19.45-126.89 41.63-73.84	55.16±39.89 12.22-200.36 35.93-74.38	0.576	0.600	0.713	0.735
Fasting C peptide (nmol/l)	0.921±0.383 0.45-1.76 0.737-1.11	0.804±0.294 0.31-1.48 0.662-0.945	0.358	0.924	0.996	0.306

Table 9.7. Comparison of analytes in **female sample** in groups **(A) before and (B) after taking cholecalciferol D3 60K units by** 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	A. Before intake (n = 8) Mean±SD Range 95% CI mean	B. After intake (n = 8) Mean±SD Range 95% CI mean	after log_{10} transformation			
			Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			Before intake	After intake		
Intact PTH (ng/l)	37.04±15.27 22.80 – 61.70 24.27 – 49.80	32.22±18.37 15.19 – 72.15 16.86 – 47.58	0.260	0.725	0.707	0.417
S. EPO (U/L)	9.24±2.33 6.81 – 13.10 7.29 – 11.18	8.54±1.97 5.68 – 11.80 6.90 – 10.19	0.256	0.986	0.657	0.535
Vitamin D (nmol/l)	74.45±35.67 24.80 – 125.1 44.63 – 104.3	255.71±188.6 51.60 – 556.6 98.08 – 413.3	0.338	0.543	0.438	**0.007**
Total Calcium (mmol/l)	2.42±0.069 2.33 – 2.53 2.36 – 2.48	2.39±0.082 2.30 – 2.53 2.32 – 2.46	0.850	0.322	0.447	0.464
Blood HB (g/l)	131.45±12.74 115.6 – 158.8 120.8 – 142.1	119.07±9.59 97.50 – 128.5 111.1 – 127.1	0.307	**0.012**	0.911	(0.027)
S. Iron (µmol/l)	14.59±4.92 4.83 – 21.66 10.48 – 18.70	12.64±2.36 10.20 – 16.11 10.67 – 14.61	**0.020**	0.275	0.320	(0.172)
S. Ferritin (ng/ml)	21.12±16.44 4.00 – 57.50 7.38 – 34.87	23.33±14.45 11.40 – 55.80 11.25 – 35.41	0.767	0.207	0.344	0.494
Fasting Insulin (pmol/l)	48.04±24.04 18.06-92.09 27.94-68.14	67.63±50.54 20.07-177.10 25.37-109.88	0.495	0.886	0.554	0.424
Fasting C peptide (nmol/l)	0.749±0.232 0.49-1.15 0.555-0.943	0.823±0.476 0.39-1.94 0.425-1.22	0.409	0.280	0.469	0.895

Table 9.8. Correlation (r) and significance of correlation (P) of EPO **(A)** and Intact PTH **(B)** with related analytes, **before and after taking cholecalciferol D3 60K units** in **male sample population.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Before taking cholecalciferol D_3 (n = 19)		After taking cholecalciferol D_3 60K units (n = 19)	
	Correlation coefficient, r	P	Correlation coefficient, r	P
A. Correlations of EPO				
Intact PTH	0.316	0.188	0.129	0.599
Vitamin D	0.143	0.559	-0.275	0.255
Total Calcium	-0.308	0.199	-0.257	0.289
Blood Hemoglobin	-0.212	0.384	-0.125	0.611
S. Iron	-0.325	0.175	-0.173	0.478
S. Ferritin	-0.150	0.541	0.047	0.847
Fasting Insulin	0.024	0.924	0.029	0.905
Fasting C peptide	-0.222	0.361	0.033	0.895
B. Correlations of Intact PTH				
S. EPO	0.316	0.188	0.129	0.599
Vitamin D	0.163	0.506	-0.235	0.333
Total Calcium	**-0.440**	**0.060**	-0.167	0.495
Blood Hemoglobin	-0.138	0.574	0.096	0.696
S. Iron	-0.174	0.477	-0.195	0.423
S. Ferritin	-0.171	0.485	0.158	0.518
Fasting Insulin	-0.161	0.511	-0.170	0.486
Fasting C peptide	-0.134	0.586	-0.095	0.698

Table 9.9. Correlation (r) and significance of correlation (P) of EPO **(A)** and Intact PTH **(B)** with related analytes, **before and after taking cholecalciferol D3 60K units** in **female sample population.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	Before taking cholecalciferol D_3 (n = 8)		After taking cholecalciferol (D_3) 60K units (n = 8)	
	Correlation coefficient, r	**P**	**Correlation coefficient, r**	**P**
A. Correlations of EPO				
Intact PTH	0.723	0.043	0.599	0.117
Vitamin D	-0.025	0.952	-0.042	0.921
Total Calcium	0.056	0.895	0.043	0.920
Blood Hemoglobin	**-0.538**	**0.169**	**0.025**	**0.953**
S. Iron	**-0.700**	**0.053**	**-0.049**	**0.907**
S. Ferritin	**-0.503**	**0.204**	**0.228**	**0.587**
Fasting Insulin	**-0.708**	**0.049**	**-0.734**	**0.038**
Fasting C peptide	-0.397	0.330	-0.327	0.429
A. Correlations of Intact PTH				
S. EPO	**0.723**	**0.043**	0.599	0.117
Vitamin D	0.273	0.512	-0.306	0.461
Total Calcium	-0.336	0.416	-0.414	0.308
Blood Hemoglobin	**-0.629**	**0.095**	-0.009	0.983
S. Iron	-0.632	0.093	-0.630	0.094
S. Ferritin	-0.208	0.621	0.453	0.259
Fasting Insulin	-0.608	0.110	-0.579	0.133
Fasting C peptide	-0.215	0.609	-0.163	0.700

NESHEERA K. K. “ASSOCIATION OF HUMAN PLASMA ERYTHROPOIETIN LEVELS WITH OTHER ANALYTES IN HEALTH AND IN DISEASE STATES”. THESIS. AMALA CANCER RESEARCH CENTRE, UNIVERSITY OF CALICUT, 2018.

Chapter 10.

Influence of Fasting Hyperglycemia on the Erythropoietin and the Related Analytes in the Presence and Absence of Deficiencies

10.1. Abstract

Insulin resistance and β cell dysfunction are two fundamental biochemical changes seen in diabetes mellitus. In type 2 diabetes mellitus, glucose concentrations increase when insulin resistance could not be compensated by increased insulin secretion. In the male and female sample glucose, insulin and C peptide increased sequentially from control sample to impaired fasting glucose (IFG) to diabetes mellitus. This indicated increased insulin resistance from control to diabetes mellitus resulting in increase in all the parameters related to insulin resistance, such as fasting triglyceride, waist circumference, ALT and hsCRP. The deficiencies in hemoglobin, iron, ferritin and vitamin D had no influence on the relative concentration of fasting glucose, fasting insulin, fasting C peptide and EPO. The gender difference in PTH, which was earlier demonstrated in this study, was also present. As before, there were strong influences by the deficiency on the correlation of EPO with the related analytes. The influences were complex and could not be explained. These results indicated that interpretations of the relationships of EPO with other related analytes are complex and could not be easily interpreted.

10.2. Introduction

10.2.1. Insulin Resistance

Regulation of blood glucose is one of the targets of insulin action. Insulin resistance is where a normal or elevated insulin level produces an attenuated biological response, which refers to impaired sensitivity to insulin mediated glucose disposal. Compensatory hyperinsulinaemia occurs when pancreatic β cell secretion increases to maintain normal blood glucose levels in the setting of peripheral insulin resistance in muscle and adipose tissue (Wilcox, 2005). Insulin resistance can be seen in type 2 diabetes, obesity, hypertension, polycystic ovarian disease and a variety of genetic syndromes, and in physiologic conditions such as puberty and pregnancy (Kahn, 1978). Insulin resistance is also present in many states of stress, in association with infection and secondary to treatment with a variety of drugs, particularly glucocorticoids. Most

longitudinal studies showed that Insulin resistance can be seen early in life before any evidence of glucose tolerance, whereas β-cell failure develops later, in association with impaired glucose tolerance (IGT).

10.2.2. Insulin Resistance and EPO

EPO secreted by the kidneys in adults results in erythropoiesis. EPO has been shown to have actions on non erythropoietic cells (Brines and Cerami, 2006; Hand and Brines, 2011). These multiple targets of EPO have been mediated through different cell signalling pathways (Broxmeyer, 2013).

Deficiency of EPO in kidney dysfunction resulted in anemia. When EPO was used in such patients to treat anemia, it has been shown to decrease insulin resistance (Khedr et al, 2009; Tuzcu et al, 2004). In transgenic mice EPO lowers blood sugar, insulin and HBA1c (Katz, et al, 2010; Hojman, et al, 2009). EPO receptor in null mice develops insulin resistance (Teng, et al, 2011). Therefore, it was assumed that EPO may regulate glucose tolerance and insulin sensitivity. EPO was also found to regulate glucose metabolism (Saltiel and Kahn, 2011). The potential mechanism by which EPO improves glucose tolerance and regulates glucose metabolism was found to be by improving glucose intolerance by inhibiting gluconeogenesis and the inflammatory response from liver (Cornier, et al, 2008). EPO was also found to protect against diabetes by cytoprotection of pancreatic β-cells (Choi, et al, 2010).

10.2.3. Consequence of Insulin Resistance

Studies showed that insulin resistance in muscle leads to increased accumulation of fat and secondary insulin resistance, hyper triglyceridemia and increased levels of free fatty acids. Insulin resistance in liver leads to increased hepatic glucose output. Insulin resistance in brain leads to increased appetite, more obesity and further defect in hepatic glucose output. Finally insulin resistance in β-cell leads to defect in glucose sensing and thereby leads to relative insulin deficiency. Thus insulin resistance in multiple tissues could produce all of the defects associated with type 2 diabetes and treatment that improves insulin sensitivity would be expected to improve all defects.

10.2.4. β-Cell Function and Dysfunction

Lifestyle and environmental factors determine the development of glucose intolerance (Hamman, 1992). β-cell dysfunction plays a crucial role in type 2 diabetes mellitus.

The storage and metabolism of cellular fuels are regulated through secretion of insulin by pancreatic β-cells. The crucial function of β-cell is insulin secretion, proinsulin biosynthesis, processing of proinsulin to insulin and β-cell replication. The secreted insulin lowers glycemia by inhibiting hepatic and renal glucose production and increasing glucose uptake into target organs, primarily skeletal muscle. Glucose regulation of insulin secretion occurs directly (glucose induced insulin secretion) and also through modulation of insulin response to insulinotropic hormones, nutrients and neurotransmitters (glucose potentiation of nonglucose secretagogues). These dual aspects of glucose-regulated insulin secretion are a potent modulatory system that ensures that the tissue's need for insulin is exactly met in the fasting and postprandial states. The need for insulin is for the most part determined by the sensitivity of tissue to insulin - a curvilinear relationship exists with insulin secretion (Kahn et al, 1993).

In this chapter, the relationships of variations in the levels of glucose from healthy to impaired fasting and then to diabetes mellitus and evaluated for their relationships to EPO, PTH and related analytes. Gender differences and changes in correlations of EPO, glucose and their related analytes were evaluated in this study.

10.3. Objectives

10.3.1. Aspects of the Original Objectives Addressed in this Chapter

Fasting glucose, EPO, PTH and their related analytes which are regulated in the healthy state, may vary in relation to their regulatory factors in diseases states. Fasting glucose, insulin, EPO and PTH are involved in a number of overlapping regulatory functions. They may cause or result in variations in the factors that are regulated by glucose, EPO and PTH. In this study, we examine the influence of fasting hyperglycemia on EPO and related analytes,

1. Grouping samples according to fasting glucose concentrations as <100 mg/dl, ≥100 – 125 mg/dl and ≥126 mg/dl.
2. Correlation and significant variations in the concentrations of EPO and PTH along with other related analytes in these groups.

10.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine

1. Correlation and significant variations of erythropoietin with other analytes in healthy individuals,

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment

10.4. Materials and Methods

10.4.1. StudySsetting and Research Design

Please refer 2.1. Study setting and Research design in chapter 2

10.4.2. Selection of Participants, Inclusion and Exclusion Criteria

Healthy cross section of participants 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and

alcoholics. Participants included were on regular diet, exercise, rest and sleep, and all female participants were in the pre gestational period (Chapter 2; Table 1; Stage I).

10.4.2.1. Exclusion of subclinical diseases and deficiencies by Clinical Biochemistry assays

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of individuals with diseases and deficiencies (Stage III). Exclusion criteria for healthy controls 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. These exclusion criteria reduced the number of control sample (male, n = 23, female, n = 16).

Fasting EPO, PTH, fasting glucose, vitamin D, fasting Insulin, fasting C peptide and analytes related to iron metabolism have been estimated for all samples selected for the study. Samples with glucose 5.55 – 7.00 mmol/l (impaired fasting glucose, IFG) and samples with glucose >7 mmol/l (Diabetes) were also collected (male, n = 60, female, n = 38). After selection of study samples, they were subjected to groupings as controls, samples with glucose 5.55 – 7.00 mmol/l (IFG); (C) samples with glucose >7 mmol/l (Diabetes) (Table 10.1 and Table 10.2). IFG and diabetes samples were also evaluated after exclusion of deficiencies in vitamin D (<50nmol/l), hemoglobin <125 g/l, iron <9.85 µmol/l (55 µg/dl) and ferritin <20 ng/ml (Table 10.3 and Table 10.4).

10.4.3. Sample Collection and Sample Preparation

Please refer 2.3. Sample collection in section chapter 2

10.4.4. Analytical Control and Assays

Analytical measurements of **ALT, triglycerides, high sensitive CRP, EPO, fasting glucose, fasting insulin, fasting C peptide, intact PTH, vitamin D, hemoglobin, iron, and ferritin** assays were done. Please refer detailed description of these assays in chapter 2.

10.4.5. Diagnostic Criteria

Please refer 2.27. Diagnostic criteria section in chapter 2.

10.4.6. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer 2.27. Statistical analysis section in chapter 2

10.5. Results

Fasting EPO was earlier reported to be directly related to the concentration of fasting plasma insulin and C peptide, indicating the relationship of EPO to fasting hyperinsulinemia and fasting insulin secretion. In this section, the influence of deficiencies in hemoglobin, iron, ferritin and vitamin D on the relationship of EPO with various analytes in IFG and diabetes were evaluated.

In the male sample, concentration of various analytes related to EPO and blood glucose were analysed in participants with impaired fasting glucose (IFG) and type 2 diabetes mellitus and compared with healthy controls (Table 10.1). There was a serial increase of various analytes from healthy controls to IFG and diabetes mellitus. This pattern of results were seen in fasting glucose, fasting insulin, fasting C peptide, EPO, BMI, waist circumference, ALT, triglycerides, ferritin and hsCRP. There was a serial decrease in concentration seen in intact PTH from healthy control to IFG and to diabetes mellitus. Similar serial increase or decrease in the concentration analytes were not seen with vitamin D, hemoglobin and iron. As glucose level increased hyperinsulinemia and insulin secretion increased, indicating increase in insulin resistance. Secondary to increase in insulin resistance, there was increase in ALT (fatty liver), BMI, waist

circumference and triglycerides, indicating the increase in lipid synthesis in liver and peripheral tissues. The increase in obesity resulted in increased cytokine production leading to retention of ferritin at the iron stores and increase in hsCRP.

In the female sample, there was a similar increase in fasting glucose, insulin, C peptide, BMI, waist circumference, triglycerides and ALT indicating increased insulin resistance and increased lipid metabolism from control sample to IFG and then to diabetes mellitus (Table 10.2). In these samples, EPO and intact PTH increased from control sample to IFG and then decreased to diabetes mellitus sample, unlike in the male sample. Ferritin and iron decreased in IFG, but further increased in diabetes mellitus. These results indicated a difference in the consequences of hyperinsulinemia in males and females. In the female sample, PTH and EPO increased and decreased in a similar pattern.

The influence of deficiencies on the concentration of analytes related to EPO and glucose were studied. Samples without any of the deficiencies were grouped in control to IFG and then to diabetes mellitus. As before fasting glucose, EPO, fasting insulin, fasting C-peptide and ferritin levels were increased from control to IFG and then to diabetes mellitus. Intact PTH concentration was decreased from control sample to diabetes mellitus. The concentration of analytes seen in the presence and absence of deficiencies in the three groups were similar in the male sample. But the significance level of difference were decreased (Table 10.3).

In the female sample most of the analytes showed a similar pattern of increase in the absence of deficiencies (Table 10.4). But the level of significance was lower. The changes were seen in the pattern of increase or decrease of PTH. Ferritin may be related to the increase or decrease of vitamin D.

The correlation of EPO with analytes related to EPO and blood gucose were analysed. In healthy control EPO was directly related to C-peptide and PTH, and inversely related to hemoglobin and iron. The presence and absence of deficiencies did not seriously affect the correlation of EPO in IFG sample (Table 10.5). In the diabetes sample the correlation of EPO with PTH was losed.

In the female sample, EPO was directly correlated with PTH and inversely correlated with hemoglobin and iron, as in the male sample (Table 10.6). But in the IFG with and without deficiency the negative correlation of EPO with iron was losed. But the correlation with intact PTH increased.

These result indicated that there were significant changes in the concentration of EPO and insulin resistance in participants with IFG and diabetes mellitus.

10.6. Discussion

In the earlier part of this study, it was observed that EPO was directly correlating with fasting insulin and fasting C peptide, indicating increased insulin secretion with increase in EPO (Ref. Chapter 5 and 6). In this study, the influence of impaired fasting glucose and diabetes mellitus on EPO and their related analytes were evaluated. Healthy controls sample were compared with impaired fasting glucose and type 2 diabetes mellitus. Both in male and female sample glucose, insulin and C peptide increased from control sample to impaired fasting glucose and diabetes mellitus. These results further confirmed the role of EPO in increasing insulin secretion (Table 10.1 and Table 10.2).

In these three groups, BMI, waist circumference, triglycerides and ALT also increased in sequence. These results indicated that there is increased insulin resistance from control to diabetes mellitus. The increase in insulin resistance increases triglyceride synthesis in liver resulting in fat accumulation and fatty liver, and leading to increased fasting triglyceride from VLDL secreted by liver (Table 10.1 and Table 10.2). The sequential increase in ALT and hsCRP supported the presence of fatty liver and the consequent increase in inflammatory cytokines, increasing acute phase reactant hsCRP.

In these samples, hemoglobin and iron levels decreased with reference to the concentration of these analytes in the control sample. But ferritin in both males and females increased sequentially indicating the influence of inflammatory cytokines preventing the release of iron from ferritin stores.

There was a gender difference in the concentration of EPO and PTH in these samples. EPO increased in sequence from control samples to diabetes mellitus in male

and females. PTH decreased in the male sample in sequence from control to diabetes mellitus. But in the female sample PTH increased from control to diabetes mellitus. This gender difference in the activity of PTH has been reported earlier in this study (Chapter 4). Even after exclusion of deficiencies in hemoglobin, iron, ferritin and vitamin D, there was sequential increase in insulin, C peptide and glucose in both males and females (Table 10.3 and Table 10.4). The changes in hemoglobin, iron, ferritin and EPO from control sample to diabetes mellitus were also not influenced by the deficiencies. The gender difference in PTH was also observed. These observations indicated the role of EPO in increasing insulin secretion was not under the influence of deficiencies.

Though the deficiency did not influence the relative concentration of EPO, PTH and their related analytes, the deficiencies did influence the correlation of EPO with fasting C peptide in the male sample. The deficiencies did not significantly influence the correlation of EPO with PTH. In the male sample, the deficiencies did not influence the negative correlation of EPO with hemoglobin. But the deficiency influenced the correlation in the female sample. These observations indicated that the influence of deficiency of correlations of EPO are complex and cannot be analysed easily.

10.7. Conclusion

In the male and female sample glucose, insulin and C peptide increased sequentially from control sample to IFG to diabetes mellitus. This indicated increased insulin resistance from control to diabetes mellitus resulting in increase in all the parameters related to insulin resistance, such as fasting triglyceride, waist circumference, ALT and hsCRP. The deficiencies in hemoglobin, iron, ferritin and vitamin D had no influence on the relative concentration of fasting glucose, fasting insulin, fasting C peptide and EPO. The gender difference in PTH, which was earlier demonstrated in this study, was also present. As before, the influence of the deficiency on the correlation of EPO was complex and could not be explained.

Table 10.1. Comparison of analytes in **male sample** (n = 83) between (A) **Controls with glucose <5.55 mmol/l, (B) samples with glucose 5.55 – 7.00 mmol/l (impaired fasting glucose, IFG); (C) samples with glucose >7 mmol/l (Diabetes)** by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	**A. Controls with glucose <5.55 mmol/l (n = 23) Mean±SD Min - Max 95% CI of mean**	**B. IFG (n = 50) Mean±SD Min - Max 95% CI of mean**	**C. Diabetes (n = 10) Mean±SD Min - Max 95% CI of mean**	**after log_{10} transformation Student t test (Mann-Whitney U test), P**	
				Compare A and B	**Compare A and C**
Age (years)	26.83±9.71 18.00 – 51.00 22.63 – 31.02	33.98±16.64 18.00 – 81.00 29.25 – 38.71	42.50±10.17 29.00 – 62.00 35.23 – 49.77	(0.071)	**(0.001)**
BMI (kg/m^2)	22.12±3.08 16.61 – 26.87 20.78 – 23.45	24.61±4.06 16.42 – 34.90 23.46 – 25.77	27.02±4.19 20.30 – 33.33 24.02 – 30.14	**0.013**	**0.001**
Waist circum-ference (cm)	81.82±9.85 64.50 – 99.00 77.56 – 86.08	86.95±10.62 66.0 – 115.0 83.93 – 89.97	92.95±9.54 69.50 – 103.00 86.13 – 99.78	**0.054**	**(0.007)**
ALT (U/L)	33.09±10.52 18.00 – 59.00 28.54 – 37.64	52.26±34.52 16.00 – 164.0 42.45 – 62.07	112.80±82.64 35.00 – 290.0 53.68 – 171.92	**(0.021)**	**(<0.001)**
Triglycerides (mmol/l)	1.00±0.283 0.57 – 1.46 0.878 – 1.12	1.46±0.627 0.59 – 2.97 1.29 – 1.64	2.14±0.718 1.50 – 3.86 1.63 – 2.65	**0.001**	**<0.001**
hsCRP (g/l)	1.20±1.36 0.10 – 5.00 0.560 – 1.83	1.25±1.41 0.10 – 7.30 0.836 – 1.66	2.71±2.92 0.39 – 9.60 0.471 – 4.96	0.983	0.071
S. EPO (U/L)	6.30±1.72 3.64 – 10.02 5.56 – 7.04	9.16±4.70 1.94 – 27.09 7.82 – 10.49	11.02±6.72 5.98 – 28.39 6.21 – 15.83	**0.008**	**0.001**
Fasting Glucose (mmol/l)	4.88±0.284 4.42 – 5.51 4.76 – 5.01	6.04±0.349 5.55 – 6.95 5.94 – 6.14	10.04±3.95 7.07 – 18.51 7.21 – 12.87	**(<0.001)**	**<0.001**
Fasting Insulin (pmol/l)	39.88±16.47 12.22 – 75.84 32.76 – 47.00	64.21±48.71 19.45 – 300.30 50.37 – 78.05	78.21±33.40 26.25 – 140.36 54.32 – 102.11	**0.006**	**0.001**
Fasting C peptide (nmol/l)	0.628±0.184 0.31 – 1.04 0.548 – 0.708	0.952±0.379 0.45 – 2.32 0.844 – 1.06	1.14±0.412 0.60 – 1.84 0.846 – 1.44	**<0.001**	**<0.001**

Intact PTH (ng/l)	38.41±33.22 8.40 – 147.58 23.68 – 53.14	36.37±17.68 9.50 – 93.90 31.35 – 41.40	24.66±8.22 12.10 – 35.30 18.34 – 30.97	0.532	0.334
Vitamin D (nmol/l)	84.60±25.89 50.00 – 143.0 73.40 – 95.80	102.42±40.82 24.96 – 187.2 90.82 – 114.0	65.00±16.90 47.58 – 88.11 49.37 – 80.63	0.133	**0.050**
Hemo-globin (g/l)	150.33±14.28 131.9 – 181.8 144.2 – 156.5	139.61±6.12 115.0 – 158.8 137.9 – 141.4	145.00±11.92 121.0 – 157.0 136.5 – 153.5	**(0.002)**	(0.583)
Ferritin (ng/ml)	69.34±34.66 21.60 – 192.9 54.36 – 84.33	99.36±93.32 5.30 – 401.8 72.84 – 125.9	172.88±116.67 33.70 – 359.10 83.19 – 262.56	(0.342)	**(0.018)**
S. Iron (µmol/l)	23.04±7.06 12.53 – 39.20 19.98 – 26.09	19.01±6.58 5.01 – 34.91 17.14 – 20.88	23.75±6.35 12.89 – 30.97 19.21 – 28.30	**(0.051)**	0.746

Table 10.2. Comparison of analytes in **female** sample (n = 54). **(A) Healthy controls with glucose <5.55 mmol/l; (B) samples with glucose 5.55 – 7.00 mmol/l (impaired fasting glucose, IFG); (C) samples with glucose >7 mmol/l (Diabetes)** by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	A. Controls (glucose <5.55 mmol/l) (n = 16) Mean±SD Min - Max 95% CI of mean	B. IFG (n = 27) Mean±SD Min - Max 95% CI of mean	C. Diabetes (n = 11) Mean±SD Min - Max 95% CI of mean	After log_{10} transformation Student t test (Mann-Whitney U test), P	
				Compare A and B	Compare A and C
Age (years)	28.75±10.41 20.00 – 52.00 23.20 – 34.30	29.85±9.69 18.00 – 49.00 26.02 – 33.69	49.91±12.43 26.00 – 74.00 41.56 – 58.26	(0.900)	**(<0.001)**
BMI (kg/m2)	22.51±3.99 17.48 – 29.75 20.38 – 24.64	23.44±5.09 14.20 – 37.20 21.43 – 25.46	24.83±1.40 23.00 – 27.20 23.89 – 25.77	0.607	**(0.030)**
Waist circum-ference (cm)	81.32±9.33 68.00 – 98.00 76.35 – 86.29	82.60±9.73 61.00 – 101.00 78.75 – 86.45	91.86±3.81 85.50 – 100.00 89.31 – 94.42	0.700	**(0.003)**
ALT (U/L)	25.88±9.45 13.00 – 48.00 20.34 – 30.91	28.41±15.10 3.00 – 71.00 22.43 – 34.38	48.82±23.86 15.00 – 91.00 32.79 – 64.85	(0.801)	**0.003**
Triglycerides (mmol/l)	0.837±0.263 0.49 – 1.66 0.696 – 0.976	1.27±0.749 0.63 – 4.19 0.975 – 1.57	2.08±0.760 1.18 – 3.24 1.57 – 2.59	**(0.001)**	**(<0.001)**
hsCRP (g/l)	0.544±0.410 0.10 – 1.40 0.325 – 0.762	1.52±1.34 0.10 – 6.40 0.991 – 2.05	3.17±2.05 0.90 – 7.00 1.79 – 4.55	**0.002**	**<0.001**
S. EPO (U/L)	5.85±1.94 2.03 - 9.32 4.82 – 6.88	12.08±8.44 3.83 - 41.72 8.74 – 15.43	9.83±2.50 6.03 – 13.35 8.15 – 11.51	**<0.001**	**<0.001**
Fasting Glucose (mmol/l)	4.78±0.358 4.27 – 5.54 4.59 – 4.97	5.95±0.334 5.55 – 6.70 5.81 – 6.08	9.45±1.86 7.01 – 11.55 8.21 – 10.70	**(<0.001)**	**(<0.001)**
Fasting Insulin (pmol/l)	35.61±14.71 18.06 – 59.80 27.77 – 43.45	51.05±26.67 18.06 – 136.61 40.50 – 61.60	75.93±48.80 31.88 – 175.15 43.14 – 108.71	**0.033**	**0.002**
Fasting C peptide (nmol/l)	0.561±0.223 0.19 – 0.98 0.442 – 0.679	0.796±0.298 0.33 – 1.43 0.678 – 0.913	0.959±0.33 0.74 – 1.82 0.734 – 1.18	**0.008**	**(0.001)**

Intact PTH (ng/l)	34.73±16.69 8.00 – 66.30 25.84 – 43.63	53.70±38.17 10.40 – 157.70 38.60 – 68.80	42.26±13.92 28.90 – 73.40 32.30 – 52.22	0.107	0.158
Vitamin D (nmol/l)	81.85±21.84 50.00 – 117.56 70.21 – 93.48	76.29±51.40 7.74 – 269.57 55.95 – 96.62	43.17±15.89 19.60 – 66.39 28.48 – 57.87	(0.321)	**<0.001**
Hemo-globin (g/l)	136.79±9.78 125.0 – 156.2 131.58 – 142.0	128.55±6.83 110.0 – 143.0 125.85 – 131.3	126.93±8.28-9 110.0 – 141.2 121.4 – 132.5	**0.002**	**0.011**
Ferritin (ng/ml)	42.76±28.69 20.80 – 138.60 27.47- 58.04	25.64±17.40 3.20 – 80.00 18.76 – 32.52	69.37±59.83 5.80 – 174.20 29.18 – 109.57	**0.007**	(0.490)
S. Iron (µmol/l)	18.25±5.48 10.20 – 31.15 15.32 – 21.17	13.01±5.07 4.83 – 25.42 11.01 – 15.02	16.07±5.04 11.28 – 25.24 12.19 – 19.95	**0.003**	0.319

Table 10.3. Comparison of analytes in **male** sample (A) healthy controls with glucose <5.55 mmol/l; (B) samples with glucose 5.55 – 7.00 mmol/l (impaired fasting glucose, IFG); (C) samples with glucose >7 mmol/l (Diabetes) **after excluding hemoglobin (HB) <125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l** by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	A. without deficiencies, IFG and diabetes (n = 23) Mean±SD Min - Max 95% CI of mean	After excluding HB<125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l			
		B. IFG (n = 40) Mean±SD Min - Max 95% CI of mean	C. Diabetes (n = 9) Mean±SD Min - Max 95% CI of mean	after log10 transformation Student t test (Mann-Whitney U test), P	
				Compare A and B	Compare A and C
S. EPO (U/L)	6.30±1.72 3.64 – 10.02 5.56 – 7.04	8.67±3.86 1.94 – 18.56 7.43 – 9.90	9.09±2.98 5.98 – 13.69 6.80 – 11.38	**0.017**	**0.003**
Fasting Glucose (mmol/l)	4.88±0.284 4.42 – 5.51 4.76 – 5.01	6.07±0.360 5.56 – 6.95 5.95 – 6.18	9.97±4.19 7.07 – 18.51 6.75 – 13.18	**<0.001**	**(<0.001)**
Fasting Insulin (pmol/l)	39.88±16.47 12.22 – 75.84 32.76 – 47.00	67.76±53.07 19.45 – 300.30 50.79 – 84.74	79.74±35.06 26.25 – 140.36 52.79 – 106.69	**0.005**	**0.001**
Fasting C peptide (nmol/l)	0.628±0.184 0.31 – 1.04 0.548 – 0.708	0.989±0.402 0.45 – 2.32 0.860 – 1.12	1.15±0.434 0.60 – 1.84 0.821 – 1.49	**<0.005**	**<0.001**
Intact PTH (ng/l)	38.41±33.22 8.40 – 147.58 23.68 – 53.14	35.68±18.28 9.50 – 93.00 29.83 – 41.53	25.88±7.86 12.10 – 35.30 19.30 – 32.45	0.672	0.477
Vitamin D (nmol/l)	84.60±25.89 50.00 – 143.02 73.40 – 95.80	108.77±38.46 58.66 – 187.20 96.47 – 121.07	65.00±16.90 47.58 – 88.11 49.37 – 80.63	0.008	0.050
Hemo-globin (g/l)	150.33±14.28 131.9 – 181.8 144.2 – 156.5	140.85±4.50 132.0 – 158.8 139.4 – 142.3	147.67±8.93 130.0 – 157.0 140.8 – 154.5	**(0.009)**	0.650
Ferritin (ng/ml)	69.34±34.66 21.60 – 192.90 54.36 – 84.33	108.24±98.39 20.90 – 401.80 76.77 – 139.71	190.28±111.55 36.40 – 359.10 97.01 – 283.54	(0.134)	**<0.001**
S. Iron (µmol/l)	23.04±7.06 12.53 – 39.20 19.98 – 26.09	19.54±6.50 10.38 – 34.91 17.46 – 21.62	24.18±6.58 12.89 – 30.97 19.13 – 29.24	**0.043**	0.660

Table 10.4. Comparison of analytes in **female** sample **(A) healthy controls with glucose <5.55 mmol/l**; **(B) samples with glucose 5.55 – 7.00 mmol/l (impaired fasting glucose, IFG)**; **(C) samples with glucose >7 mmol/l (Diabetes) after excluding hemoglobin (HB) <125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l** by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	**A. healthy controls with glucose <5.55 mmol/l (n = 16) Mean±SD Min - Max 95% CI of mean**	**After excluding HB<125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l**			
		B. IFG (n = 10) Mean±SD Min - Max 95% CI of mean	**C. Diabetes (n = 5) Mean±SD Min - Max 95% CI of mean**	**after log10 transformation Student t test (Mann-Whitney U test), P**	
				Compare A and B	**Compare A and C**
S. EPO (U/L)	5.85±1.94 2.03 - 9.32 4.82 – 6.88	9.24±3.33 3.83 – 15.35 6.86 – 11.63	10.33±2.49 7.02 – 13.05 7.24 – 13.41	**0.008**	**0.004**
Fasting Glucose (mmol/l)	4.78±0.358 4.27 – 5.54 4.59 – 4.97	5.87±0.280 5.55 – 6.29 5.67 – 6.07	9.16±1.90 7.04 – 11.35 6.80 – 11.52	**<0.001**	**(0.001)**
Fasting Insulin (pmol/l)	35.61±14.71 18.06 – 59.80 27.77 – 43.45	53.01±21.36 24.31 – 92.09 37.73 – 68.29	67.67±22.70 39.86 – 89.31 39.48 – 95.86	**0.028**	**0.005**
Fasting C peptide (nmol/l)	0.561±0.223 0.19 – 0.98 0.442 – 0.679	0.859±0.267 0.40 – 1.18 0.668 – 1.05	0.970±0.218 0.79 – 1.32 0.699 – 1.24	**0.010**	**0.008**
Intact PTH (ng/l)	34.73±16.69 8.00 – 66.30 25.84 – 43.63	48.34±42.26 10.40 – 157.70 18.11 – 78.57	45.88±17.67 30.40 – 73.40 23.94 – 67.82	0.479	0.210
Vitamin D (nmol/l)	81.85±21.84 50.00 – 117.56 70.21 – 93.48	85.15±29.06 50.00 – 133.59 64.36 – 105.94	56.79±6.88 50.00 – 66.39 48.25 – 65.34	0.859	**0.017**
Hemo-globin (g/l)	136.79±9.78 125.0 – 156.2 131.6 – 142.0	130.66±4.73 126.2 – 142.0 127.3 – 134.0	129.80±4.55 125.0 – 136.0 124.2 – 135.5	(0.155)	(0.172)
Ferritin (ng/ml)	42.76±28.69 20.80 – 138.60 27.47- 58.04	38.31±18.22 21.50 – 80.00 25.28 – 51.34	83.34±67.15 20.60 – 174.20 -0.038 – 166.72	0.747	(0.248)
S. Iron (µmol/l)	18.25±5.48 10.20 – 31.15 15.32 – 21.17	14.84±2.80 10.56 – 21.30 12.84 – 16.84	16.07±5.29 11.81 – 25.24 9.51 – 22.64	0.098	0.431

Table 10.5. Correlation (r) and significance of correlation (P) in **male sample population,** of EPO with related analytes in groups with **(A) healthy controls with glucose <5.55 mmol/l; (B) samples with glucose 5.55 – 7 mmol/l (impaired fasting glucose) and glucose >7 mmol/l (Diabetes) with deficiencies (C) samples with glucose 5.55 – 7 mmol/l (impaired fasting glucose) and glucose >7 mmol/l (Diabetes) after excluding hemoglobin (HB) <125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	A. healthy controls with glucose <5.55 mmol/l (n = 23)		B. With deficiencies of HB, iron, ferritin and vitamin D				C. After excluding HB<125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l			
			IFG (n = 50)		Diabetes (n = 10)		IFG (n = 40)		Diabetes (n = 9)	
	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P
Fasting Glucose	0.266	0.220	-0.143	0.323	0.294	0.409	-0.084	0.605	0.317	0.407
Fasting Insulin	0.224	0.305	0.031	0.833	0.039	0.918	0.198	0.222	0.139	0.721
Fasting C peptide	**0.442**	**0.035**	-0.030	0.834	-0.073	0.841	0.087	0.594	-0.051	0.896
Intact PTH	**0.439**	**0.041**	**0.305**	**0.031**	-0.148	0.705	**0.341**	**0.031**	0.375	0.360
Vitamin D	0.244	0.261	-0.002	0.991	-0.513	0.239	-0.088	0.588	-0.513	0.239
Hemoglobin	**-0.329**	0.125	**-0.560**	**<0.001**	**-0.755**	**0.012**	**-0.412**	**0.008**	-0.434	0.243
Ferritin	0.281	0.194	-0.026	0.858	-0.571	0.109	0.154	0.344	-0.175	0.678
S. Iron	**-0.380**	0.074	**-0.394**	**0.005**	-0.143	0.549	**-0.282**	0.077	-0.019	0.961

Table 10.6. Correlation (r) and significance of correlation (P) in **female sample population,** of EPO with related analytes in groups with **(A) healthy controls with glucose <5.55 mmol/l; (B) samples with glucose 5.55 – 7 mmol/l (impaired fasting glucose) and glucose >7 mmol/l (Diabetes) with deficiencies (C) samples with glucose 5.55 – 7 mmol/l (impaired fasting glucose) and glucose >7 mmol/l (Diabetes) after excluding hemoglobin (HB) <125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Variables	A. without deficiencies, IFG and diabetes (n = 16)		B. With deficiencies				C. After excluding HB<125g/l, iron <9.85 µmol/l, ferritin <20 ng/ml and Vitamin D <50 nmol/l			
			IFG (n = 27)		Diabetes (n = 11)		IFG (n = 10)		Diabetes (n = 5)	
	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P	Correlation coefficient, r	P
Fasting Glucose	-0.176	0.514	0.319	0.104	-0.335	0.313	0.520	0.124	-0.503	0.387
Fasting Insulin	-0.016	0.954	0.096	0.633	0.018	0.958	0.380	0.279	0.397	0.508
Fasting C peptide	0.027	0.922	0.178	0.376	-0.345	0.294	0.377	0.283	0.488	0.405
Intact PTH	**0.483**	**0.058**	**0.593**	**0.001**	0.486	0.155	**0.938**	**<0.001**	0.379	0.529
Vitamin D	-0.118	0.662	0.157	0.435	-0.177	0.704	0.365	0.299	0.346	0.568
Hemoglobin	**-0.768**	**0.001**	-0.284	0.151	0.205	0.544	**-0.891**	**0.001**	0.847	0.070
Ferritin	0.085	0.753	-0.236	0.235	0.290	0.387	0.161	0.657	0.814	0.094
S. Iron	**-0.513**	**0.042**	-0.232	0.245	0.099	0.799	-0.081	0.824	0.793	0.109

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Chapter 11.

Influence of Increased Troponin I in Patients Clinically Suspected of Myocardial Injury on Erythropoietin and Its Related Analytes

11.1. Abstract

Atheromatous lesions leading to myocardial injury can influence EPO concentration resulting from the influence of cytokines and myocardial failure. Cardiac injury and infarction may cause fall in blood pressure resulting in hypoxia and increase in EPO. Cytokines produced from atheromatous lesions can also influence EPO through anemia of chronic inflammation and retention and increase of ferritin at the stores. EPO, PTH and their related analytes which are regulated in the healthy state, may vary in relation to their regulatory factors in diseases states. The influence of cytokines from the atheromatous plaque leads to anemia and iron deficiency, with retention of ferritin at the stores. These influences of anemia and iron deficiency can further complicate the outcome of myocardial injury. Myocardial injury may or may not lead to hypoxia. Hypoxia resulting from myocardial injury further increases EPO. It was observed that correlation of EPO with troponin I was influenced anemia and iron deficiency.

11.2. Introduction

Troponin is a regulatory protein of the thin filament of striated muscle. This protein consists of three subunits I, T and C, which are implicated together in muscle contractions. Troponin I have a cardiac isoform, which rapidly released after acute myocardial infarction (AMI) and is highly specific for the detection of myocardial injury. This isoform can be detected in blood between the 4th and 8th hour after the onset of chest pain, with a peak between the 14th and 36th hour; concentrations in blood remain high for 3 to 7 days (Christenson and Azzazy, 1998). Cardiac troponin is the more specific and sensitive biomarker for the detection of myocardial necrosis than the cardiac enzymes.

Troponin I is an automated quantitative test used for the determination of human cardiac troponin I in human serum or plasma using the ELFA (Enzyme-Linked Fluorescent Assay) technique. Troponin I is intended to be used as an aid in the diagnosis of myocardial infarction. The European Society for Cardiology (ESC) and the American College of Cardiology (ACC) recommended that the diagnosis of myocardial necrosis can be made when the concentration of cardiac troponin in the blood is greater than the 99th percentile of a healthy population, in the clinical setting of acute ischemia. The

acceptable imprecision (coefficient of variation) at the 99th percentile for this assay should be defined as ≤10% (Alpert et al, 2000).

11.2.1. Anemia in heart failure

Chronic heart failure (CHF) is a multi-organ syndrome which causes changes in the periphery affecting muscle and blood vessels, neurohormonal activation and metabolic and hormonal changes. It is now recognized that anemia is frequently a part of this syndrome and has been found to affect nearly 25% of heart failure patients. The cause of anemia in chronic heart failure is not known and it also shows a defect in iron utilisation.

Anemia is common in congestive heart failure and is associated with a marked increase in mortality and morbidity and also worsens congestive heart failure (Tang et al, 2008; Anand et al, 2005). Correction of the anemia with subcutaneous erythropoietin and intravenous iron has caused a great improvement in congestive heart failure patient and cardiac function and a marked reduction in the need for hospitalization and for high-dose diuretics. In addition to this, quality of life and exercise capacity of patients also improved with the correction of the anemia (Silverberg et al, 2005; Manolis et al, 2005).

Several studies have showed that the erythropoietin receptor is widely distributed in the cardiovascular system, including endothelial cells, smooth muscle cells and cardiomyocytes. Erythropoietin may have a direct positive effect on the heart by reducing cell apoptosis, and by increasing neovascularization, both of which could prevent tissue damage (Timmer et al, 2009). This could have profound therapeutic implications not only in congestive heart failure but also in the future treatment of myocardial infarction, coronary heart disease and strokes (Roger et al, 2004)

Chronic heart failure is also thought to include bone marrow dysfunction secondary to poor perfusion, impaired renal function and the effects of cytokine activation -especially TNF-alpha- which can induce both reduced erythropoietin production and increased resistance to its effects. In severe heart failure patients, the immune activation and pro-inflammatory cytokines strongly correlate with the severity of anemia.

Patients with CHF apparently suffer from **tissue hypoperfusion**, which causes internal environmental **hypoxia** and subsequently leads to the overexpression of EPO. Tissue hypoperfusion may lead to other types of stimulation such as oxidative stress that result in the production of oxygen radicals and upregulate the expression of EPO. So in patients with CHF and in those with CHF complicated by anemia, EPO expression is increased and the increase correlates with the severity of CHF. Thus, serum EPO expression performs an important role in the progression of CHF and therefore, is clinically significant for the prediction of the development, outcome and prognosis of CHF (Guo et al, 2013).

11.2.2. EPO and Cardiovascular protection

Several clinical studies showed that EPO has an important protective role in the cardiovascular system (Maiese et al, 2005). Experimental study results from knockout mice showed that the heart expressesfunctional erythropoietin receptors and injections of large doses of EPO protect from the consequences of transient or permanent coronary artery occlusion. EPO treated animals exhibit a reduction in myocardial damage and were associated with decreased apoptotic cell death (Parsa et al, 2003).Administration of EPO in patients with congestive heart failure or diabetes combined with congestive heart failure showed an improvement in the cardiac output and a decrease in medical resource utilization (Silverberg et al, 2006). Patients with anemia and congestive heart failure can improve exercise tolerance, renal function, and left ventricular systolic function by the administration of EPO (Palazzuoli et al, 2006). Clinical studies showed that administration of EPO in patients with anemia or on chronic hemodialysis can increase left ventricular ejection fraction, stroke volume, and cardiac output, indicating improved cardiac function secondary to the correction of anemia (Maiese et al, 2005; Silverberg et al, 2006). Recently, several studies showed that patients with acute myocardial infarction have an increased plasma EPO levels, and were raised within seven days of the cardiac insult. These results suggest that the raise in EPO levels may be a possible protective response from the body (Ferrario et al, 2007).Serum EPO levels may use as a biomarker for cardiovascular injury (Fu and Van Eyk, 2006).

11.2.3. Anemia of Chronic inflammation

Anemia of chronic disease (ACD) is usually normocytic, normochromic anemia which occurs in patients with chronic infection, inflammation, or neoplasia. Serum iron levels are usually decreased due to hepcidin activation by cytokines. Transferrin saturation is low in ACD, but ferritin levels are high due to inflammation. In ACD, oral supplementation of iron is not effective as elevated hepcidin levels impair the intestinal absorption of iron. But treatment with intravenous iron is effective. This marginally improves the anemic state as intravenous iron is trapped in the storage sites like macrophages and hepatocytes as a consequence of ferroportin degradation by hepcidin. ACD is categorized as anemia of heart failure, anemia of chronic kidney disease, anemia in inflammatory rheumatic diseases, anemia of the elderly (Weinstein et al, 2002).

11.3. Objectives

11.3.1. Aspects of the Original Objectives addressed in this Chapter

Cardiac injury and infarction may cause fall in blood pressure resulting in hypoxia and increase in EPO. EPO, PTH and their related analytes which are regulated in the healthy state, may vary in relation to their regulatory factors in diseases states. Patients with increased troponin I from those with clinical suspicion of myocardial injury were selected for the study.

3. Grouping samples according to troponin I concentrations indicative of myocardial injury, acute coronary syndrome and healthy controls.
4. Correlation and significant variations in the concentrations of EPO and PTH along with other related analytes in these groups.

11.3.2. Original Objectives (aspects referred above are in bold)

An analyte related to a disease condition varies significantly from the levels in a healthy state. These analytes, which are almost always regulated in the healthy physiological state, may also vary in relation to their regulatory factors. EPO is a cytokine involved in a number of regulatory functions and, therefore, may cause variations in factors that are regulated by EPO. In this study, we examine

1. **Correlation and significant variations of erythropoietin with other analytes in healthy individuals,**

2. Identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment

11.4. Materials and Methods

11.4.1. Study Setting and Research design

Please refer 2.1. Study setting and research design section in Chapter 2

11.4.2. Selection of Participants, Inclusion and Exclusion Criteria

Healthy cross section of participants 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. Clinical evaluation of volunteers in this study was done for exclusion of clinical and subclinical disease states, deficiencies and for partitioning the sample. Volunteers of this study who gave informed oral consent underwent an evaluation by clinical history and examination for exclusion of individuals with disease states, injury, infection, inflammation, allergic reactions, diabetes, hypothyroidism, stressed states and hypertension at the time of examination or in the past one month, and alcoholics. Participants included were on regular diet, exercise, rest, sleep, had no drugs for one week and all female participants were in the pre gestational period (Chapter 2; Table 1; Stage I).

11.4.2.1. Exclusion of subclinical diseases and deficiencies by Clinical Biochemistry assays

Informed written consent was obtained from each participant at stage II who donated blood and urine samples. Volunteers underwent Clinical Biochemistry laboratory evaluation for further exclusion of individuals with diseases and deficiencies (Stage III). Exclusion criteria for these were: BMI >30 kg/m^2, serum triglyceride >200 mg/dl (2.26 mmol/l), waist circumference ≥100 cm, 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. These samples were used as controls (n = 30). Samples with increased troponin I and with clinical symptoms of myocardial injury and Acute Coronary syndrome were also collected (n = 65) and most of the above mentioned analytes were assayed in these samples. After selection of study samples, they were subjected to groupings as controls, samples with Troponin I in the myocardial injury(MI) range (0.002 - 0.1µg/l) and samples with troponin I in the acute coronary syndrome (ACS) range (>0.1µg/l) (Table 11.1A). The samples with increased troponin I were also grouped according to presence and absence of deficiencies in hemoglobin <125 g/l and iron <9.85 µmol (Table 11.1B).

11.4.3. Sample collection and sample preparation

Please refer detailed description of sample collection in Chapter 2.

11.4.4. Analytical control and assays

Analytical measurements of **troponin I, ALT, triglycerides, creatinine, EPO, intact PTH, vitamin D, hemoglobin, iron, and ferritin** assays were done. Please refer detailed description of these assays in chapter 2.

11.4.5. Diagnostic criteria

Range of expected values: The value of troponin I is **<0.002µg/l** in patients with **no cardiac symptoms.** But when the concentration of troponin I is **0.002 - 0.1 µg/l**, it indicate **myocardial injury** and **>0.1 µg/l** is seen in **Acute coronory syndrome**.

11.4.6. Statistical Analysis

Statistical software used for this study was SPSS version 23.0. Please refer 2.27. Statistical analysis in chapter 2

11.5. Results

Troponin I is an indication of myocardial injury or acute coronary syndrome resulting from varying degree of obstruction of the coronary arteries. Samples were grouped into subgroups with or without increased troponin I, hemoglobin, and iron in isolation or in combination. The groups were also designed such that there were sufficient

numbers in each group for statistical analysis. Partitioning for grouping was done according to gender and according to the concentration troponin I (Table 11.1). The healthy control samples were without vitamin D, hemoglobin, iron and ferritin deficiencies.

The samples with increased troponin I were more in males than in females (Table 11.1A). Ferritin deficiencies were almost absent in sample with myocardial injury. Therefore the sample with increased troponin I were subgrouped into those with and without hemoglobin and iron deficiencies (Table 11.1B and 11.1C).

Male sample with myocardial injury and acute coronary syndrome were subgrouped and compared with control sample population. Both the groups with myocardial injury had increased EPO, ferritin and PTH (Table 11.2). These groups had decreased hemoglobin ferritin and vitamin D. For all the analytes with significant variations the increase and decrease were graded in sequence from control to myocardial injury to acute coronary syndrome. The increase in EPO may be due to the myocardial damage resulting in hypoxia or may be due to deficiencies in hemoglobin and iron. Increase in PTH in patients with myocardial injury was due to increase in EPO or due to decrease in vitamin D. The increase in ferritin in patient with myocardial injury and acute coronary syndrome can be due to retention of intracellular ferritin resulting from inflammatory cytokine from the atheromatous lesions. This inhibition of release of iron from ferritin source may lead to deficiencies of hemoglobin and iron, anemia of chronic inflammation.

Similar finding were also obtained in the female sample with increase of EPO, ferritin and PTH in groups with myocardial damage. Hemoglobin and iron decrease in groups with myocardial damage (Table 11.3).

Sample with increased troponin I were grouped into those with deficiencies in hemoglobin and iron in isolation and in combination (Table 11.4). These groups were compared with control group. EPO, ferritin and PTH increased in the group with myocardial infarction with hemoglobin and/or iron deficiencies (Table 11.4). In these groups with myocardial injury hemoglobin, iron and vitamin D concentrations were decreases. These results indicate that almost all myocardial injury patients had deficiency

in hemoglobin and/or iron. Therefore the increase in concentration of EPO and PTH may be due to myocardial injury, hemoglobin and iron deficiencies.

In most of the group with increased troponin I, EPO positively correlated with troponin I indicating that there is a direct relationship between EPO and troponin I, in addition to the negative correlation of EPO with vitamin D, hemoglobin and iron (Table 11.5). In the healthy control group EPO positively correlated with PTH and negatively with iron. In the presence of deficiency or myocardial injury these correlations were altered or were absent (Table 11.5).

Gender differences in analytes related to EPO were analysed in the sample with indication of myocardial injury (troponin I 0.002-0.1µg/l). Mean EPO, PTH and ferritin were higher in the female sample. Of the three analytes only PTH was significantly higher in the female sample (P = 0.015). EPO and ferritin in the female sample were more than twice the concentration present in the male sample (Table 11.6). But the significant were border line (P = 0.131 and P = 0.173). The decrease in significance was due to the high variation seen in the female sample. There was no significant gender difference in the concentration of hemoglobin and iron. These results indicate that the increase EPO in female sample may be due to hypoxia resulting from myocardial injury.

The gender differences in acute coronary syndrome showed higher EPO, ferritin and troponin I in the female sample. The concentration of iron was lower in the female sample, but none of the analyst showed significant gender difference (Table 11.7). The lack of significance was due to higher variation in the female sample. The increase in mean was 50% to more than 100% and the decrease in iron was almost 40%. These results indicate that there were variable increases in the level of troponin I, EPO and ferritin in the female sample. In both male and female sample hemoglobin and iron level decreased but ferritin level increased probably due to action of inflammatory cytokines. Therefore anemia and iron deficiency are present in both male and female. The higher level of EPO and PTH in the female sample may be due to the higher risk of hypoxia in females resulting from lower hemoglobin (Table 11.6 and 11.7).

11.6. Discussion

Troponin I is the marker of myocardial damage resulting from obstruction of coronary artery by atherosclerosis. Atherosclerosis is formed by deposition of lipid, inflammatory cell and fibrosis. The inflammatory cells secrete cytokines that influence insulin resistance and iron metabolism. These influences results in anemia and iron deficiency decrease in the release of iron from ferritin source and absorption of iron from the intestine. In myocardial failure can result in hypoxia leading to increase in EPO secretion. Therefore, myocardial injury leads to changes in analytes related to EPO, hemoglobin and iron metabolism.

Deficiency of hemoglobin, iron and ferritin were more prevalent in the female sample than in the male sample (Chapter 3, Table 3.2). Therefore, samples with increased troponin I and immediate clinical history of indicative myocardial injury were grouped along with samples with deficiency of hemoglobin and iron in combination and in isolation. This was done to see the EPO levels resulting from myocardial injury along with or without these deficiencies (Table 11.1). Ferritin deficiency was absent or rare in these patients, as an inflammatory condition leading to cytokine production retain ferritin at the iron source.

The sequential decrease of hemoglobin and iron from control to acute coronary syndrome is associated with sequential increase in ferritin (Table 11.2 and 11.3), indicating the action of cytokine released from the active atheromatous plaque. Consequent to the decrease in hemoglobin and iron EPO level increased sequentially. The differentiation of the increase in EPO level due to anemia and iron deficiency from that due to myocardial injury is important to determine the course of increased EPO. But this differentiation between anemia and myocardial injury results in increase in EPO can be made to certain extent from the results of correlations of EPO.

From the influence on EPO, it was observed that there is sequential increase in EPO from controls to the group with increase troponin I (TI) and then with increased (TI + Hb) to increased (TI + iron) to increased (TI + iron + Hb) (Table 11.4). These results indicate

that acute myocardial injury is associated with anemia which further complicates the hypoxia resulting from myocardial failure.

The correlations of EPO in patients with increased Troponion I indicated correlation statistic above 0.299 to 0.702 in all the four group (Table 11.5). The significance of correlations was highly varying indicating the influences of hemoglobin and iron in all the four group except the two group without hemoglobin deficiency. In the two groups with anemia the relatively high P value indicate interferences from hypoxia resulting from anemia. Therefore, it can be informed that EPO correlates with troponin I.

After portioning of the sample with myocardial injury into males and female, the sample number decreased and the correlation and their significance were difficult to interpret

11.7. Conclusions

Atheromatous lesions leading to myocardial injury can influence EPO concentration resulting from the influence of cytokines and myocardial failure. The influence of cytokines from the atheromatous plaque leads to anemia and iron deficiency, with retention of ferritin at the stores. These influences of anemia and iron deficiency can further complicate the outcome of myocardial injury. Myocardial injury may or may not lead to hypoxia. Hypoxia resulting from myocardial injury further increases EPO. It was observed that correlation of EPO with troponin I was influenced anemia and iron deficiency.

Table 11.1. Two groupings done for comparing samples with and without increased Troponin I. **A.** Male and female samples with increased Troponin I and healthy samples without Troponin I. **B.** Samples [with (male + female) and (Myocardial injury + ACS) combined] with increased Troponin I were further split into those with isolated and combined hemoglobin (HB) and Iron deficient samples. These Groups were also designed so that there were sufficient numbers in each group for statistical analysis. Cut off levels for deficiencies were as given in Methods (Chapter 2). **C.** Complete partitioning of samples as in **B.** above but with partitioned males & females, and partitioned myocardial injury & ACS. Ferritin partitioning was not done as most samples with myocardial damage had high ferritin. Samples with myocardial injury and ACS are with vitamin D deficiency.

A. Gender	Samples without Vitamin D, HB, Iron, Ferritin deficiencies	Samples with Troponin I (0.002 - 0.1µg/l) Indicates Myocardial injury	Samples with Troponin I (>0.1µg/l) Indicates Acute Coronary Syndrome
Male (n)	20	24	20
Female (n)	10	10	11

B.	Samples without Vitamin D, HB, Iron, Ferritin Deficient	Samples with troponin I > 0.002 µg/l (both MI & ACS)			
		Healthy HB & Iron	HB Deficient	Iron Deficient	HB, Iron Deficient
Total Sample (n)	30	14	13	17	21

C. Gender	Without Vit D, HB, Iron, Ferritin deficient	Samples with Troponin I (0.002 -0.1µg/l), Indication of Myocardial Injury (male: n = 24, female: n = 10)				Samples with Troponin I (>0.1µg/l) Acute Coronary Syndrome (male: n = 20, female: n = 11)			
		HB >125g/l, Iron >9.845 µmol/l	HB <125g/l	HB <125g/l, Iron <9.845 µmol/l	Iron deficient	HB >125g/l, Iron >9.845 µmol/l	HB Deficient	HB, Iron Deficient	Iron Deficient
Male (n)	20	6	7	5	6	5	3	9	3
Female (n)	10	3	2	2	3	0	1	5	5

Table 11.2. Comparison of analytes in **male** sample from **Table 11.1A male. (A) Healthy controls** (no deficiencies of Vitamin D, hemoglobin, iron and ferritin); **(B)** with Troponin I (0.002 - 0.1µg/l), indication of Myocardial Injury (MI); **(C) with Troponin I (>0.1µg/l)** with Acute Coronary Syndrome (ACS) by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	**(A) Without deficiencies (n = 20) Mean±SD Minimum - Maximum 95% CI mean**	**(B) Indicate MI (n = 24) Mean±SD Minimum - Maximum 95% CI mean**	**(C) ACS (n = 20) Mean±SD Minimum - Maximum 95% CI mean**	**After log_{10} transformation Student t test (Mann-Whitney U test), P**	
				Compare A and B	**Compare A and C**
Age (>32 years)	53.15±9.99 35 – 81 48.47 – 57.83	63.96±13.99 32.00 – 88.00 58.05 – 69.87	65.25±10.04 46.00 – 84.00 60.55 – 69.95	(0.002)	(<0.001)
ALT (U/L)	32.8±11.67 16.00 – 56.00 27.34 – 38.26	32.56±12.38 16.00 – 67.00 26.40 – 38.71	53.28±77.91 18 – 358 14.54 – 92.02	0.949	(0.682)
Triglycerides (mmol/l)	1.27±0.365 0.67 – 1.93 1.10 – 1.44	1.09±0.461 0.52 – 1.82 0.843 – 1.33	1.30±0.547 0.50 – 2.17 1.00 – 1.61	0.122	0.869
S. Creatinine (µmol/l)	88.4±12.17 70.72 – 114.92 82.71 – 94.09	123.76±95.28 61.88 – 415.48 66.18 – 181.34	131.92±81.14 61.88 – 335.9 82.89 – 180.9	(0.408)	(0.613)
S. EPO (U/L)	7.68±2.42 3.53 – 12.96 6.54 – 8.81	11.22±7.32 2.99 – 32.08 8.13 – 14.31	28.72±23.4 4.67 – 90.95 17.77 – 39.67	**(0.101)**	**(<0.001)**
Blood HB (g/l)	140.57±10.64 125 – 159.6 135.6 – 145.55	126.46±15.36 104 – 157 119.97 – 132.9	118.13±18.34 78.00 – 149.0 109.5 – 126.7	**(0.003)**	**(<0.001)**
S. Iron (µmol/l)	18.37±5.18 12.53 – 32.58 15.94 – 20.79	10.92±6.85 1.79 – 25.78 8.02 – 13.81	9.00±5.35 2.51 – 19.33 6.5 – 11.51	**(0.001)**	**(<0.001)**
S. Ferritin (ng/ml)	90.91±46.44 20.9 – 192.9 69.18 – 112.65	181.52±164.99 20.00 – 655.2 111.85 – 251.2	196.04±187.3 11.90 – 816.9 108.4 – 283.7	(0.063)	**(0.030)**
Vitamin D (nmol/l)	103.81±57.98 50 -250 74.98 – 132.64	26.95±12.04 8.00 – 49.6 19.68 – 34.23	17.85±9.36 6.73 – 37.70 12.44 – 23.25	**<0.001**	**<0.001**
Intact PTH (ng/l)	38.53±22.43 8.4 – 96.81 27.37 – 49.68	54.74±27.78 11.60 – 121.70 42.73 – 66.75	90.30±69.48 12.6 – 260.5 57.78 – 122.8	**(0.012)**	**(0.006)**

Table 11.3. Comparison of analytes in **female** sample from **Table 11.1A female. (A) Healthy controls** (no deficiencies of Vitamin D, hemoglobin, iron and ferritin); **(B) With Troponin I (0.002 - 0.1μg/l**), indication of Myocardial injury (MI); **(C) With Troponin I (>0.1μg/l)** Acute Coronary Syndrome (ACS) by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	**(A) No deficiencies (n = 10) Mean±SD Minimum - Maximum 95% CI mean**	**(B) Indicate MI (n = 10) Mean±SD Minimum - Maximum 95% CI mean**	**(C) ACS (n = 11) Mean±SD Minimum - Maximum 95% CI mean**	**After log_{10} transformation Student t test (Mann-Whitney U test), P**	
				Compare A and B	**Compare A and C**
Age (>32 years)	45.90±5.63 35.00 – 54.00 41.88 – 49.92	61.44±14.82 38.00 – 87.00 50.05 – 72.84	63.22±17.75 29.00 – 86.00 49.58 – 76.87	**0.007**	**(0.011)**
ALT (U/L)	27.2±11.00 13.00 – 51.00 19.33 – 35.07	30.00±10.18 17.00 – 43.00 19.32 – 40.68	39.55±21.37 19.00 – 89.00 25.19 – 53.90	0.579	0.091
Triglycerides (mmol/l)	0.975±0.349 0.68 – 1.74 0.725 – 1.23	1.32±0.48 0.72 – 1.86 0.557 – 2.09	1.19±0.414 0.75 – 2.06 0.874 – 1.51	0.166	0.191
S. Creatinine (μmol/l)	64.53±10.25 44.20 – 79.56 57.20 – 71.86	134.81±119.4 61.88 – 406.6 34.97 – 234.7	92.82±42.85 53.04 – 150.3 47.85 – 137.8	**(0.028)**	(0.297)
S. EPO (U/L)	7.82±2.7 2.03 – 10.37 5.89 – 9.75	24.67±24.53 4.72 – 79.70 7.13 – 42.22	52.78±81.88 2.32 – 289.39 -2.23 – 107.79	**(0.059)**	**(0.007)**
Blood Hemoglobin (g/l)	135.63±12.87 125.0 – 156.20 126.43 – 144.8	115.10±19.68 84.00 – 138.0 101.0 – 129.2	112.45±16.93 84.00 – 132.0 101.1 – 123.8	(0.089)	**(0.011)**
S. Iron (μmol/l)	17.13±4.10 11.64 – 23.99 14.19 – 20.07	10.04±5.34 1.79 – 19.15 6.22 – 13.86	5.86±2.38 3.04 – 10.02 -7.8 – 24.22	**(0.005)**	**(<0.001)**
S. Ferritin (ng/ml)	47.23±34.37 22.70 – 138.60 22.64 – 71.82	432.04±574.2 9.90 – 1500 21.29 – 842.8	281.58±438.9 13.20 – 1500 -13.24 – 576.4	**(0.023)**	(0.260)
Vitamin D (nmol/l)	74.14±24.32 50.00 – 116.06 55.44 – 92.83	37.96±36.20 11.70 – 132.8 12.06 – 63.85	22.08±18.72 8.00 – 62.60 7.70 – 36.47	**0.003**	**(0.001)**
Intact PTH (ng/l)	34.90±10.05 17.88 – 49.48 27.17 – 42.61	108.363±92.1 13.30 – 347.6 42.72 – 174.5	92.64±57.53 14.70 – 245.1 53.98 – 131.3	**0.007**	**(0.003)**

Table 11.4. Comparison of analytes in groups **(A)** without deficiencies, **(B)** with HB >125g/l & Iron >9.85 µmol/l **(C)** with Iron deficient, **(D)** HB <125g/l, **(E)** HB, Iron deficient by 95% CI of mean and Student t test (Mann Whitney U test).

Variables in SI units	A.Without Vit D, HB, Iron, Ferritin Deficiencies (n = 30) Mean±SD Minimum - Maximum 95% CI mean	Samples with Troponin I > 0.002 µg/l (both MI and ACS)				Student t test (Mann-Whitney U test), P (Groups compared after log_{10} transformation)			
		B.HB >125g/l, Iron >9.85 µmol/l (n = 14) Mean±SD Minimum - Maximum 95% CI mean	C. Iron <9.85 µmol/l. (n = 17) Mean±SD Minimum - Maximum 95% CI mean	D. HB <125g/l (n = 13) Mean±SD Minimum - Maximum 95% CI mean	E. HB, Iron Deficient (n = 21) Mean±SD Minimum - Maximum 95% CI mean	A & B	A & C	A & D	A & E
S. EPO (U/L)	7.73±2.47 2.03 – 12.96 6.80 – 8.65	10.89±11.08 2.99 – 47.56 4.50 – 17.29	26.26±25.68 3.87 – 90.95 13.06 – 39.46	24.88±21.23 7.96 – 79.70 12.05 – 37.71	35.65±61.10 2.32 – 289.39 7.84 – 63.46	(0.588)	**(0.008)**	**(<0.001)**	**(<0.001)**
Blood HB (g/l)	138.9±11.45 125.0 – 159.6 134.6 – 143.2	137.0±10.40 125.0 – 157.0 131.0 – 143.0	132.1±7.41 125.0 – 153.0 128.3 – 135.9	108.7±11.40 84.0 – 121.0 101.8 – 115.6	105.2±13.15 78.0 – 124.0 99.2 – 111.2	(0.668)	**(0.055)**	**(<0.001)**	**(<0.001)**
S. Iron (µmol/l)	17.95±4.81 11.64 – 32.58 16.16 – 19.75	14.36±4.40 10.02 – 25.78 11.82 – 16.90	6.08±2.33 2.15 – 9.31 4.88 – 7.29	15.72±4.07 10.02 – 22.20 13.26 – 18.18	4.67±2.00 1.79 – 8.41 3.76 – 5.58	**0.009**	**(<0.001)**	0.122	**(<0.001)**
S. Ferritin (ng/ml)	76.35±47.10 20.90 – 192.9 58.77 – 93.94	124.96±126.8 20.00 – 481.2 51.74 – 198.2	235.86±210.5 9.90 – 702.4 127.7 – 344.1	398.68±498.8 20.00 – 1500 93.26 – 696.1	228.8±338.5 11.9 – 1500 74.7 - 382.9	0.217	**(0.018)**	**(0.001)**	**(<0.011)**

Vitamin D (nmol/l)	93.92±50.82 50.0 – 250.0 73.81 – 114.0	33.69±35.84 9.12 – 132.75 8.05 – 59.33	27.03±18.79 8.00 – 62.60 16.62 – 37.44	19.37±8.32 11.70 – 37.70 12.97 – 25.76	21.83±12.49 6.73 – 44.80 13.89 - 29.76	**(<0.001)**	**(<0.001)**	**(<0.001)**	**(<0.001)**
Intact PTH (ng/l)	37.23±18.71 8.40 – 96.81 29.98 – 44.49	73.44±52.22 30.90 – 226.1 41.88 – 105.0	80.49±38.31 13.30 – 142.4 60.80 – 100.2	62.69±62.36 11.60 – 254.1 25.01 – 100.4	96.77±81.15 17.6 – 347.6 59.83 – 133.7	**(<0.002)**	**(<0.001)**	(0.153)	**(<0.001)**
Troponin I (µg/l)	<**0.002 µg/l**	1.46±2.78 0.002 – 10.13 -0.146 – 3.07	0.766±1.28 0.003 – 4.25 0.109 – 1.42	6.39±22.13 0.005 – 80.00 -6.99 – 19.78	1.83±4.79 0.008 – 22.28 -0.352 – 4.01	--	--	--	--

Table 11.5. Correlation (r) and significance of correlation (P) of EPO with related analytes in groups **(A) without deficiency (B) with HB >125g/l, Iron >9.85 µmol/l (C) with Iron deficient, (D) HB <125g/l** and **(E) HB and iron deficient** by parametric (Pearson's, r) and non parametric (Spearman's rho ρ) methods.

Correlation of EPO with related Variables	**A.Without deficiency (n = 30)**		**Samples with troponin I > 0.002 µg/l (both MI and ACS)**							
			B.HB >125g/l, Iron >9.85 µmol/l (n = 14)		**C.Iron deficient (n = 17)**		**D. HB <125g/l (n = 13**		**E. HB and iron deficient (n = 21**	
	Correlation coefficient, r (Spearman's rho ρ)	**P**	**Correlation coefficient, r**	**P**	**Correlation coefficient, r**	**P**	**Correlation coefficient, r**	**P**	**Correlation coefficient, r**	**P**
Intact PTH	**0.300**	0.120	0.272	0.370	0.225	0.324	0.296	0.326	0.010	0.967
Vitamin D	0.166	0.408	0.120	0.742	**-0.386**	0.156	**-0.352**	0.353	**-0.495**	0.102
Blood HB	-0.274	0.143	-0.235	0.418	**-0.431**	0.084	**-0.608**	**0.028**	-0.209	0.364
S. Iron	**-0.303**	0.103	-0.236	0.416	0.225	0.385	**-0.445**	0.128	-0.137	0.554
S. Ferritin	0.089	0.641	0.286	0.322	0.117	0.654	0.202	0.507	-0.282	0.215
Trponin I	--	--	**0.473**	0.088	**0.702**	0.002	**0.329**	0.273	0.238	0.299

Table 11.6. Gender differences in Troponin I, EPO and related parameters in participants **with indication of myocardial injury (troponin I 0.002 - 0.1µg/l).**

Variables in SI units	Indication of myocardial injury		after log_{10} transformation			
	Male Mean±SD Minimum - Maximum 95% CI of mean (n = 24)	Female Mean±SD Minimum - Maximum 95% CI of mean (n = 10)	Shapiro – Wilk test, P		Levene's test, P	Student t test (Mann-Whitney U test), P
			Male	Female		
S. EPO (U/L)	11.22±7.32 2.99 – 32.08 8.13 – 14.31	24.67±24.53 4.72 – 79.70 7.13 – 42.22	0.904	0.605	**0.034**	**(0.131)**
Blood HB (g/l)	126.46±15.36 104 – 157 119.97 – 132.9	115.10±19.68 84.00 – 138.00 101.0 – 129.18	0.290	0.070	**0.024**	**(0.173)**
S. Iron (µmol/l)	10.92±6.85 1.79 – 25.78 8.02 – 13.81	10.04±5.34 1.79 – 19.15 6.22 – 13.86	0.149	0.502	0.568	0.921
S. Ferritin (ng/ml)	181.52±164.99 20.00 – 655.2 111.85 – 251.2	432.04±574.20 9.90 – 1500 21.29 – 842.79	0.703	0.752	**0.038**	(0.705)
Vitamin D (nmol/l)	26.95±12.04 8.00 – 49.6 19.68 – 34.23	37.96±36.20 11.70 – 132.75 12.06 – 63.85	0.427	0.686	0.296	0.486
Intact PTH (ng/l)	54.74±27.78 11.60 – 121.70 42.73 – 66.75	108.363±92.14 13.30 – 347.60 42.72 – 174.54	**0.046**	0.905	0.893	**(0.015)**
Troponin I (µg/l)	0.022±0.018 0.002 – 0.07 0.014 – 0.029	0.029±0.025 0.003 – 0.07 0.011 – 0.047	0.722	0.322	0.383	0.612

Table 11.7. Gender differences in Troponin I, EPO and related parameters in participants **with acute coronary syndrome, (troponin I >0.1µg/l).**

Variables in SI units	**Acute coronary syndrome**		**after log_{10} transformation**			
	Male Mean±SD Minimum - Maximum 95% CI of mean (n = 20)	**Female Mean±SD Minimum - Maximum 95% CI of mean (n = 11)**	**Shapiro – Wilk test, P**		**Levene's test, P**	**Student t test (Mann-Whitney U test), P**
			Male	**Female**		
S. EPO (U/L)	28.72±23.4 4.67 – 90.95 17.77 – 39.67	52.78±81.88 2.32 – 289.39 -2.23 – 107.79	0.605	0.948	0.103	0.727
Blood HB (g/l)	118.13±18.34 78.00 – 149.00 109.54 – 126.7	112.45±16.93 84.00 – 132.00 101.08 – 123.8	0.070	0.176	0.935	0.446
S. Iron (µmol/l)	9.00±5.35 2.51 – 19.33 6.5 – 11.51	5.86±2.38 3.04 – 10.02 -7.8 – 24.22	0.502	0.594	0.097	0.145
S. Ferritin (ng/ml)	196.04±187.26 11.90 – 816.9 108.4 – 283.67	281.58±438.85 13.20 – 1500 -13.24 – 576.41	0.752	0.503	**0.014**	(0.650)
Vitamin D (nmol/l)	17.85±9.36 6.73 – 37.70 12.44 – 23.25	22.08±18.72 8.00 – 62.60 7.70 – 36.47	0.686	0.252	0.138	0.801
Intact PTH (ng/l)	90.30±69.48 12.6 – 260.5 57.78 – 122.81	92.64±57.53 14.70 – 245.10 53.98 – 131.29	0.905	**0.041**	0.137	(0.680)
Troponin I (µg/l)	3.19±4.99 0.11 – 22.28 0.855 – 5.52	8.21±23.84 0.15 – 80.00 -7.81 – 24.22	0.143	**0.052**	0.420	0.359

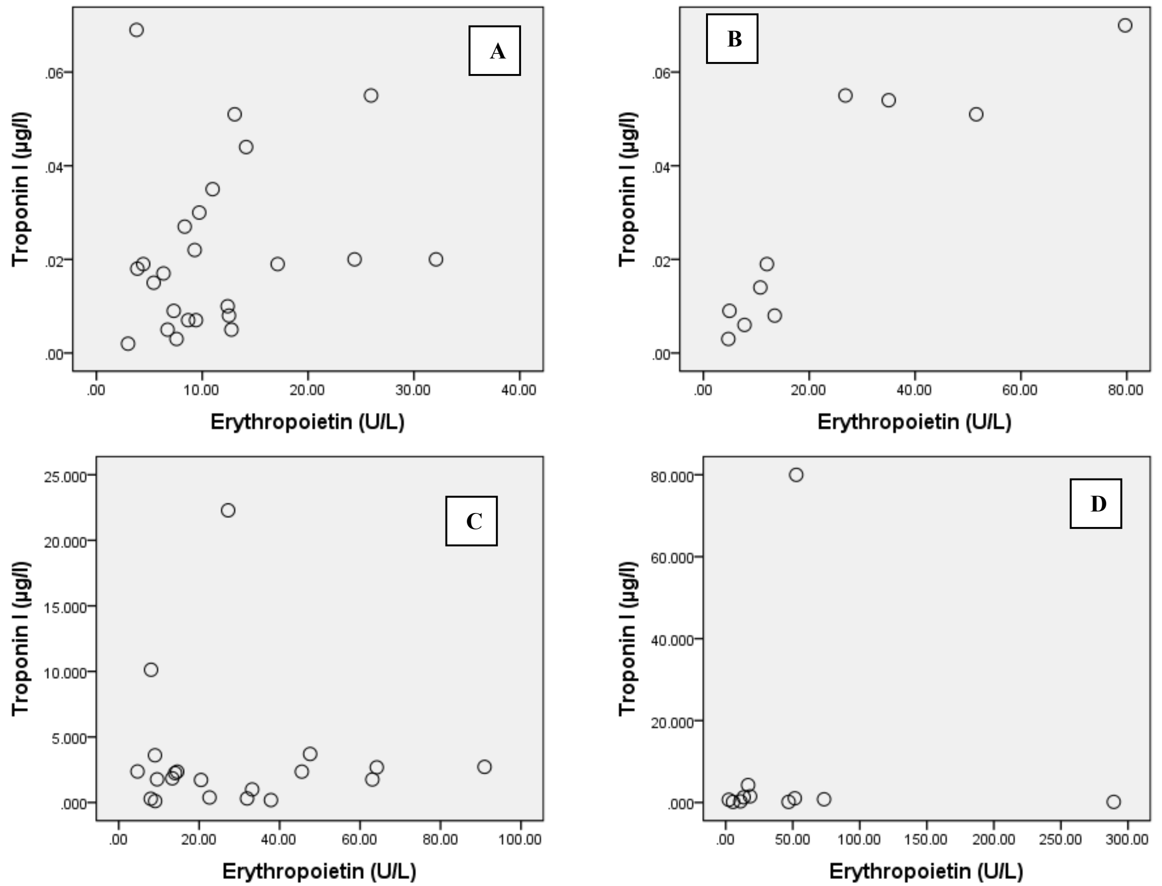
Table 11.8. Correlation (r) and significance of correlation (P) in **male sample population,** of EPO with related analytes in groups **(A) without** (Vitamin D, hemoglobin, iron and ferritin deficiencies)**, (B) with troponin I (0.002 - 0.1µg/l), indication of myocardial injury and (C) with troponin I (>0.1µg/l) Acute coronary syndrome.** Parametric (Pearson's, r) method was used as at least one of the two correlating variables had Gaussian distribution after log_{10} transformation.

Correlation of EPO with related variables	**(A) Without deficiency (n = 20)**		**(B) with indication of myocardial injury (n = 24)**		**(C) with acute coronary syndrome (n = 20)**	
	Correlation coefficient, r	**P**	**Correlation coefficient, r**	**P**	**Correlation coefficient, r**	**P**
Intact PTH	0.233	0.336	**-0.578**	**0.004**	**0.411**	0.072
Vitamin D	**0.312**	0.207	**-0.346**	0.247	-0.157	0.592
Blood Hemoglobin	0.088	0.713	**-0.427**	**0.037**	-0.240	0.308
S. Iron	-0.082	0.730	0.044	0.837	**-0.405**	0.076
S. Ferritin	**0.304**	0.193	0.152	0.479	0.032	0.895
Trponin I	--	--	0.291	0.167	0.061	0.799

Table 11.9. Correlation (r) and significance of correlation (P) in **female sample population,** of EPO with related analytes in groups **(A) without** (Vitamin D, hemoglobin (HB), iron and ferritin deficiencies)**, (B) with troponin I (0.002 - 0.1µg/l), indication of myocardial injury and (C) with troponin I (>0.1µg/l) Acute coronary syndrome** by parametric (Pearson's, r) and non parametric (Spearman's rho ρ) methods.

Correlation of EPO with related variables	(A) Without deficiency (n = 10)		(B) with indication of myocardial injury (n = 10)		(C) With acute coronary syndrome (n = 11)	
	Correlation coefficient, r (Spearman's rho ρ)	P	Correlation coefficient, r	P	Correlation coefficient, r	P
Intact PTH	(0.200)	**0.606**	0.134	0.712	0.068	0.843
Vitamin D	(-0.109)	0.780	-0.193	0.593	**-0.486**	0.184
Blood HB	**(-0.772)**	**0.009**	**-0.644**	**0.044**	-0.065	0.849
S. Iron	**(-0.503)**	**0.138**	-0.111	0.759	-0.021	0.950
S. Ferritin	(0.067)	0.855	**0.771**	**0.009**	**-0.434**	0.182
Troponin I	--	--	**0.915**	**<0.001**	0.049	0.886

Fig. 11.1. X-Y scatter diagram, of troponin I with EPO in **(A,C) male** and **(B,D) female** patients **with indication of myocardial injury (troponin I, 0.002 - 0.1µg/l) (A,B) and with Acute coronary syndrome (troponin I>0.1µg/l) (C,D).**



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Summary and Conclusion

Introduction: Erythropoietin (EPO) is a cytokine that is known to regulate erythropoiesis. Kidney is the major contributor for maintenance of erythropoietin (EPO) concentrations in blood plasma (Mujais et al, 1999), and may be in minor amounts by liver and brain (Fisher, 2003). Hypoxia and iron deficiency increase synthesis of EPO by kidneys and absorption of iron by intestine. Hypoxia results from anemia, high altitude, heart failure, lung diseases and certain deficiencies.

Research Problem: This study examined the correlation and significant variations of EPO with other analytes in healthy individuals, identification of such correlations and significant variations in disease conditions and the changes in these analytes during treatment. From these data obtained, a Baseline Reference Interval of EPO was calculated.

Importance of this study and its significance: EPO is rarely estimated for clinical evaluation of any disease condition except for investigation of the cause of anemia in patients with kidney diseases and for the treatment of the anemia using rhEPO. But EPO has been found to be involved in a large number of disease conditions imparting protective roles and better prognostic values such as near normal hemoglobin when there is severe iron deficiency, decreased damage to brain and heart during ischemia, and anti-apoptotic role during oxidative stress conditions such as diabetes mellitus. Deficiencies in hemoglobin, iron, ferritin and vitamin D were found to influence the concentrations and correlations of EPO and related analytes. These deficiencies were more in the female sample. Though interpretations of the influences of few of the deficiencies could be made in this study, in most cases the interpretations were complex.

Results and Discussions:

Chapter 3: Gender differences in EPO. There were gender differences in the correlations of EPO with related analytes, and in their concentrations in healthy young adults. There were gender differences in the correlations of EPO with hemoglobin, iron and ferritin with **strong negative correlations in females.** The residual correlation of EPO with iron in males, after exclusion of subclinical hypoxia/anemia, indicated that in addition to hypoxia, iron **deficiency also increased EPO. In males,** hemoglobin is increased by

androgens; in females, hemoglobin, iron and ferritin are decreased by **monthly menstrual blood loss** leading to rise in EPO to increase erythropoiesis. Concentration of hemoglobin, iron and ferritin influence EPO. If subclinical anaemic and iron deficient samples which increased EPO levels are excluded with cut off for hemoglobin <125g/l, iron <36.9 µg/dl and ferritin <20 ng/ml, then a **more healthy reference interval for EPO** can be determined.

Chapter 4: Gender differences in PTH, EPO and analytes related to them were evaluated after excluding the deficiencies. The results showed that there were gender differences in the levels of PTH and EPO, and the parameters that are related to them. **In women**, the lower levels of hemoglobin and iron may be due to monthly blood loss, and the lower levels of vitamin D may be due to decreased exposure to sunlight. These deficiencies may be contributing to **higher levels of PTH and EPO in women.** There was **correlation of PTH and hemoglobin only after exclusion of deficiencies** in hemoglobin, iron and vitamin D. Thereby, indicating the roles of these causing both PTH and EPO resistance, inhibiting erythropoiesis. **In men**, after excluding all deficiencies, **hemoglobin was much higher than in women** and may be due to androgens. In men, **PTH did not correlate with EPO but negatively correlated with hemoglobin and iron** after excluding vitamin D deficiency or all four deficient analytes. This may be due to PTH resistance resulting from vitamin D deficiency.

Chapter 5: Gender difference in the relationship of EPO with C peptide was analysed. Insulin secretion is increased in the presence of insulin resistance. EPO has been shown to have a beneficial role in type 2 diabetes and was proposed to be due to decrease in insulin resistance. Insulin resistance is higher in the male sample. Hemoglobin was also higher in the male sample resulting in lower EPO. The **lower EPO in the male sample did not decrease C peptide and EPO was not inversely related to C peptide**. In the **female sample**, it was observed that **EPO directly correlated with C peptide** which indicated increased C peptide with increased insulin secretion and EPO. As insulin resistance is lower in females, EPO probably increased insulin secretion.

Chapter 6: Gender differences in concentrations and correlations of fasting insulin, before and after exclusion of deficiencies in hemoglobin, iron and ferritin were evaluated.

One of the causes of increase in fasting insulin or hyperinsulinaemia is insulin resistance. Insulin resistance increases insulin secretion as a compensatory mechanism. In males insulin resistance is higher than in females resulting in increased fasting insulin in males. EPO has been shown to be beneficial for type 2 diabetes, probably due to decrease in insulin resistance. In this study we have shown that **fasting insulin correlates with EPO in the female sample and not in the male sample.** This gender difference may be due to EPO decreasing insulin resistance in males, resulting in decrease of insulin secretion. But increase in insulin secretion directly by EPO may be confounding the correlation between insulin, C peptide and EPO in the male sample. This confounding was less in the female sample.

Chapter 7: Baseline reference interval of EPO in the sample population, and partitioned male and female groups were calculated. Clinical exclusion, Clinical Biochemistry exclusion and partitioning of samples were used to exclude samples with disease states and deficiencies to obtain the baseline reference sample. **Reference Interval of fasting EPO sample population (n = 411)** calculated by non parametric percentile method with bootstrap sampling was 3.84 – 26.7 U/L. This reference interval was very similar to the reported reference interval of 4 – 27 U/l (Kratz et al, 2015**).** **Baseline Reference Interval of fasting EPO sample,** after exclusion of subclinical deficiencies in vitamin D, hemoglobin, iron and ferritin, was calculated by non parametric minimum-maximum levels. In the male sample it was 4.11 – 12.73 U/L. In the female sample it was 3.67 – 13.41 U/L. When values were compared with reported reference interval, it was seen that the lower limits were similar but the upper limits of the baseline reference intervals were much lower.

Chapters 8 to 11: Influences of the following disease states on EPO and its related analytes were evaluated. Differences in concentrations of analytes and in their correlations with EPO were observed with and without deficiencies or diseases. Differences in the correlations influenced the regulation of analytes and caused alterations in the biochemical functions. In many cases the methods by which these influences take place could not be explained.

8. Deficiencies of hemoglobin, iron and ferritin: In samples with anemia and iron deficiency, there were major differences in the correlations of EPO with C peptide. In the female sample, correlation of EPO with hemoglobin was better in the absence of deficiency. In the male sample, EPO did not correlate with hemoglobin in presence or absence of deficiencies.

9. Vitamin D deficiency in combination with the deficiencies hemoglobin, iron and ferritin: These were more in the female sample population and influenced the correlations of EPO and PTH with their related analytes. The results indicate that minor changes in concentrations of EPO and PTH are difficult to clinically interpret when there are deficiencies of vitamin D, hemoglobin, iron and ferritin.

10. Fasting hyperglycemia in the presence and absence of deficiencies: In the male and female sample glucose, insulin and C peptide increased sequentially from control sample to IFG to diabetes mellitus. This indicated increased insulin resistance from control to diabetes mellitus resulting in increase in all the parameters related to insulin resistance, such as fasting triglyceride, waist circumference, ALT and hsCRP. The deficiencies in hemoglobin, iron, ferritin and vitamin D had no influence on the relative concentration of fasting glucose, fasting insulin, fasting C peptide and EPO. The gender difference in PTH, which was earlier demonstrated in this study, was also present. As before, the influence of the deficiency on the correlation of EPO was complex and could not be explained.

11. Increased troponin I in patients clinically suspected of myocardial injury: Atheromatous lesions leading to myocardial injury can influence EPO concentration resulting from the influence of cytokines and myocardial failure. The influence of cytokines from the atheromatous plaque leads to anemia and iron deficiency, with retention of ferritin at the stores. These influences of anemia and iron deficiency can further complicate the outcome of myocardial injury. Myocardial injury may or may not lead to hypoxia. Hypoxia resulting from myocardial injury further increases EPO. It was observed that correlation of EPO with troponin I was influenced by anemia and iron deficiency.

Conclusions: In addition to erythropoiesis, EPO influences a large number of functions; a large number of biochemical states can influence EPO. Examples are the concentrations of glucose, insulin, C peptide, hemoglobin, iron, ferritin and vitamin D. A number of disease states can cause minor to major changes in concentrations and correlations of EPO and its related analytes. They are anemia, iron deficiency, vitamin D deficiency, diabetes mellitus, heart disease and hypoxic states. The minor influences can be evaluated from the Baseline Reference interval of EPO, which was obtained in the absence of subclinical disease states. Interpretations from concentrations and correlations of EPO can be made only in the absence of other influences such as deficiencies and sub clinical disease states or after giving allowances for these influences on EPO. But interpreting the method of influences of diseases and deficiencies on EPO is complex.

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LIST OF PUBLICATIONS

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2. **Nesheera KK**, Sindu PC, Jacob J. 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 International journal of Scientific Research. 2018; 7:76-80.