# Influence of Oxygen Sensitive Vascular Endothelial Growth Factor(VEGF) Gene Expression in Pulmonary Tuberculosis and its Correlation with Erythropoietin and Tumor Necrosis Factor-alpha (TNF-α)

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By

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## DECLARATION BY THE CANDIDATE

I here by declare that the thesis entitled **"Influence of Oxygen Sensitive Vascular Endothelial Growth Factor (VEGF) Gene Expression in Pulmonary Tuberculosis and its Correlation with Erythropoietin and Tumor Necrosis Factor-alpha (TNF-α)"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. J. G. Ambekar**, Professor of Biochemistry, BLDE (Deemed to be University)'s Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka. No part of this thesis has been formed the basis for the award of any degree or fellowship previously.

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| | |
|---|---|
| ACD | Anemia of chronic disease |
| ADA | Adenosine deaminase |
| AFB | Acid fast bacilli |
| AIDS | Acquired immunodeficiency syndrome |
| ANOVA | Analysis of variance |
| ATT | Antituberculous treatment |
| BC | Before Christ |
| BCG | Bacille Calmette Guerin |
| BFU-E | Burst forming unit- erythroid |
| *B.henselae* | *Bartonella henselae* |
| BMI | Body mass index |
| CBNAAT | Cartridge based nucleic acid amplification test |
| CBP | Cyclic adenosine monophosphate response element-binding protein |
| CD3 | Cluster of differentiation 3 |
| CD40 | Cluster of differentiation 40 |
| CD80 | Cluster of differentiation 80 |
| CD86 | Cluster of differentiation 86 |
| *C.difficile* | *Clostridium difficile* |
| Cells/cumm | Cells per cubic millimeter |
| CFU-E | Colony forming unit-erythroid |
| cm | Centimeter |
| cumm | Cubic millimeter |
| CXR | Chest x-ray |
| DC | Dendritic cells |
| DOTS | Directly Observed Treatment Short-course |
| *E.coli* | *Escherichia coli* |
| EDTA | Ethylene diamine tetra acetic acid |
| ELISA | Enzyme linked immunosorbent assay |

| | |
|---|---|
| EPO | Erythropoietin |
| EPOR | Erythropoietin receptor |
| EPTB | Extra pulmonary tuberculosis |
| Erk | Extracellular signal-regulated kinase |
| ESR | Erythrocyte sedimentation rate |
| $Fe^{+2}$ | Ferrous ion |
| FIH | Factor inhibiting hypoxia inducible factor |
| fL | Femtolitre |
| g/dl | Gram per deciliter |
| Hb | Hemoglobin |
| HIF | Hypoxia inducible factor |
| HIF-1 | Hypoxia inducible factor-1 |
| HIF-1α | Hypoxia inducible factor-1 alpha |
| HIF-1β | Hypoxia inducible factor-1 beta |
| HIV | Human immunodeficiency virus |
| *H.pylori* | *Helicobacter pylori* |
| HRE | Hypoxia response element |
| HRP | Horseradish peroxidase |
| IEC | Institutional Ethical Committee |
| IFN-γ | Interferon gamma |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| JAK- 2 | Janus kinase- 2 |
| Kg | Kilogram |
| KDa | Kilodalton |
| Lakhs/cumm | Lakhs per cubic millimeter |
| LPA | Line probe assay |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| LTBI | Latent tuberculosis infection |
| $m^2$ | Meter squared |
| MAPK | Mitogen-activated protein kinase |
| *M.africanum* | *Mycobacterium africanum* |
| *M.avium* | *Mycobacterium avium* |
| *M.balnei* | *Mycobacterium balnei* |
| *M.bovis* | *Mycobacterium bovis* |
| *M.bovis* BCG | *Mycobacterium bovis* Bacille Calmette Guerin |
| *M.butricum* | *Mycobacterium butricum* |
| *M.canetti* | *Mycobacterium canetti* |
| MCH | Mean corpuscular hemoglobin |
| MCHC | Mean corpuscular hemoglobin concentration |
| *M.chelonei* | *Mycobacterium chelonei* |
| MCV | Mean corpuscular volume |
| MDR-TB | Multi drug resistant tuberculosis |
| *M.fotuitum* | *Mycobacterium fortuitum* |
| *M.gordonae* | *Mycobacterium gordonae* |
| MHC II | Major histocompatibility complex II |
| millions/cumm | Millions per cubic millilitre |
| mIU/ml | Milli international units per millilitre |
| *M.kansasi* | *Mycobacterium kansasi* |
| ml | Millilitre |
| *M.leprae* | *Mycobacterium leprae* |
| *M.marinum* | *Mycobacterium marinum* |
| mm Hg | Millimeter of mercury |
| mm/hour | Millimeter per hour |
| *M.microti* | *Mycobacterium microti* |
| MMPs | Matrix metalloproteinases |
| MMP-1 | Matrix metalloproteinase-1 |
| MMP-8 | Matrix metalloproteinase-8 |

| | |
|---|---|
| *M.phlei* | *Mycobacterium phlei* |
| *M.pinnepedii* | *Mycobacterium pinnepedii* |
| mRNA | Messenger ribonucleic acid |
| *M.scrofulaceum* | *Mycobacterium scrofulaceum* |
| *M.simiae* | *Mycobacterium simiae* |
| *M.smegmatis* | *Mycobacterium smegmatis* |
| *M.tuberculosis* | *Mycobacterium tuberculosis* |
| MTB, Mtb | *Mycobacterium tuberculosis* |
| *M.ulcerans* | *Mycobacterium ulcerans* |
| *M.xenopi* | *Mycobacterium xenopi* |
| NF-κB | Nueclear factor-kappa B |
| NK cell | Natural killer cell |
| nm | Nanometer |
| NO | Nitric oxide |
| NSP | National Strategic Plan |
| NTB | No tuberculosis |
| OD | Optical density |
| *P.aeruginosa* | *Pseudomonas aeruginosa* |
| PCV | Packed cell volume |
| pg | Picogram |
| pg/cell | Picogram per cell |
| pg/ml | Picogram per millilitre |
| PHD | Prolyl hydroxylase |
| PHD-1 | Prolyl hydroxylase-1 |
| PHD-2 | Prolyl hydroxylase-2 |
| PHD-3 | Prolyl hydroxylase-3 |
| Pi3k | Phosphoinositide 3-kinase |
| PKC | Protein like kinase C |
| PLHIV | People living with Human immunodeficiency virus |
| PMDT | Programmatic management of drug resistant tuberculosis |

| | |
|---|---|
| PTB | Pulmonary tuberculosis |
| RBC | Red blood cells |
| Ref No. | Reference number |
| Rif | Rifampacin |
| RNTCP | Revised National Tuberculosis Control Program |
| rpm | Revolutions per minute |
| *S.aureus* | *Staphylococcus aureus* |
| *S.aureus peritonitis* | *Staphylococcus aureus peritonitis* |
| SD | Standard deviation |
| SPSS | Statistical package for the social sciences |
| STAT 5 | Signal transducer and activator of transcription-5 |
| TB | Tuberculosis |
| TLRs | Toll like receptors |
| TMB | Tetra methyl benzedine |
| TNF | Tumor necrosis factor |
| TNF-α | Tumor necrosis factor-alpha |
| TNFR-1 | Tumor necrosis factor receptor-1 |
| TNFR-2 | Tumor necrosis factor receptor-2 |
| TNFSF | Tumor necrosis factor super family |
| TNFRSF | Tumor necrosis factor receptor super family |
| TOG | Technical and operational guideline |
| TST | Tuberculin skin test |
| VEGF | Vascular endothelial growth factor |
| VEGF-A | Vascular endothelial growth factor-A |
| VEGF-B | Vascular endothelial growth factor-B |
| VEGF-C | Vascular endothelial growth factor-C |
| VEGF-D | Vascular endothelial growth factor-D |
| VEGF-E | Vascular endothelial growth factor-E |
| VEGFR | Vascular endothelial growth factor receptor |

| | |
|---|---|
| VEGFR-1 | Vascular endothelial growth factor receptor-1 |
| VEGFR-2 | Vascular endothelial growth factor receptor-2 |
| VEGFR-3 | Vascular endothelial growth factor receptor-3 |
| pVHL | Von Hippel-Lindau tumor suppressor protein |
| vs. | Versus |
| WBC | White blood cells |
| WHO | World Health Organization |
| WHR | Waist to hip ratio |
| XDR-TB | Extensive drug resistant tuberculosis |
| *Y.enterocolitica* | *Yersinia enterocolitica* |
| Z-N staining | Ziehl-Neelsen staining |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| κ | Kappa |
| μm | Micrometer |
| -ve | Negative |
| +ve | Positive |
| % | Percentage |
| Mø | Resident macrophages |

# ABSRACT

**Aim and Objectives:** The study aimed to evaluate the impact of oxygen sensing vascular endothelial growth factor protein (VEGF) expression and its correlation with erythropoietin (EPO) and inflammatory marker like tumor necrosis factor- alpha (TNF-α) and also association of these oxygen sensing molecular markers with bacterial burden measured in terms of sputum smear acid fast bacilli grading in pulmonary tuberculosis (PTB) patients.

**Material and Methods:** In the present cross sectional study demographic and anthropometric parameters were recorded from 197 newly diagnosed sputum positive PTB cases and 100 healthy controls. The hematological parameters, serum VEGF, EPO and TNF-α were estimated by ELISA methods. The results were analyzed with appropriate statistics using SPSS version 16.0 software.

**Results:** Results on BMI, hematological parameters, serum VEGF and TNF-α showed significant difference between PTB cases and controls. We noted blunted response of EPO to hemoglobin concentration. We reported positive correlation between VEGF and TNF-α, no correlation between VEGF and EPO and negative correlation between TNF-α and EPO in PTB. Serum VEGF and TNF-α levels were positively correlated with bacterial burden while EPO showed negative correlation with bacterial burden in PTB patients.

**Conclusion:** Increased bacterial burden resulted in simultaneous induction of hypoxia and inflammation associated with increased VEGF and TNF-α and relative inadequacy of EPO to hemoglobin level in PTB. Thus we concluded that these oxygen sensing molecular markers could be used as early tool in diagnosing and predicting active PTB and also as marker to measure disease severity and bacterial burden in PTB patients.



**Key words:** Acid fast bacilli grading, Angiogenic marker, Bacterial burden, Erythroid progenitors, Hypoxia, Inflammatory cytokine, *Mycobacterium tuberculosis*, Oxygen sensing molecular marker.

# CHAPTER 1

# INTRODUCTION

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis* (MTB) and presently, it is one of the most prevalent infectious diseases in the world[1]. Mycobacterium has very ancient origin and it has been assumed that the genus Mycobacterium emerged more than 150 million years ago[2]. Mummies from the Egyptian pre-dynastic era and Peruvian pre-Columbian era, dating back to 2400 BC disclosed the vertebral lesions typical to TB[3, 4].

In human beings, MTB most frequently targets the lungs leading to pulmonary tuberculosis (PTB). However, it can also affect other body parts of the host resulting in extra pulmonary tuberculosis (EPTB). PTB has become paramount health burden worldwide because of its infectious nature, persistent advancement and the need for the long term treatment[5]. PTB is said to be one of the prime reason behind morbidity and mortality to the mankind[6]. Despite of major effective measures, every year millions of people continue to fall ill due to TB and globally, it is one of the top 10 causes of death. Worldwide, about 10 million people developed TB and approximately 1.3 million people have been reported to be died because of TB in 2017[7]. Geographically, high burden of TB incidence is noticed in Asia and Africa. In India, TB is an old disease and at present contributing as one of the major public health problems with very slow response in reduction even after many effective control programs. It has been calculated that, around one fourth of global TB burden is accounted by India alone[8].

The TB disease is transmitted from an individual to another through the air. Poverty, under nutrition, lack of education, poor housing, overcrowding, immigration and limited health care facility are some of the socioeconomic aspects leading to the development of TB[9, 10]. A low body mass index (BMI) is another individual risk factor for the occurrence of active TB[11]. BMI is an important tool to measure the status of nutrition and lower BMI is strongly associated with morbidity and mortality in PTB patients[12].

During inflammation associated with bacterial infection, occurrence of microenvironment is a typical feature of tissue hypoxia as observed in PTB. Probably, the

disproportion between the oxygen requirement and its supply leads to hypoxia which is seen during the course of bacterial infection[13]. In mammalian cells, hypoxia is sensed by hypoxia inducible factor- 1 alpha (HIF-1α), a transcriptional factor which in response induces the expression of vascular endothelial growth factor (VEGF), erythropoietin (EPO), basic fibroblast growth factor, glycolytic enzymes and glucose transporters required for the regulation of angiogenesis, apoptosis, cellular stress and metabolism[14, 15].

Vascular endothelial growth factor, the oxygen sensing angiogenic factor is a homodimeric heparin binding glycoprotein and is considered to be important in the mediation of angiogenesis and enhancing vascular permeability. In addition, it has many roles in lung maturation and has been indicated in various parts of the lungs and pleura[16]. In many lung diseases like chronic obstructive pulmonary disease, lung cancer, pulmonary hypertension, acute lung disease and in asthma, involvement of angiogenic molecular marker VEGF has been indicated[17], but very few studies have mentioned about the role of VEGF in PTB[18-20].

The cytokine tumor necrosis factor-alpha (TNF-α) is secreted early in PTB from infected macrophages, dendritic cells and T cells and is one of the major inflammatory cytokines essential for host defense against MTB and along with interferon gamma (IFN-γ) and interleukin-2 (IL-2) promotes granuloma formation and thus controls the further progression of disease in PTB[21, 22]. Documentary evidence from the earlier study showed that, HIF-1α which induces the expression of oxygen sensing VEGF and EPO can also increase the expression of TNF-α in hypoxic condition seen in bacterial infections[13].

Erythropoietin is an endogenous glycoprotein hormone which is essential for controlling red blood cell (RBC) production[23]. Under hypoxic condition, the kidney generates EPO through the mediation of HIF-1α to stimulate erythropoiesis. EPO also enhances the survival of erythroid progenitors and precursors found in the bone marrow by preventing their apoptosis[24]. In chronic diseases like PTB, occurrence of anemia is a typical hematological disorder with varying aetiology. Nevertheless, inflammatory

cytokine and/or deficiency of iron found to be the probable causative factors in the pathophysiology of anemia of chronic disease like PTB[25, 26]. Studies have reported that production of EPO is reduced and biological activity is impaired in the presence of increased TNF-α in chronic diseases.[27].

Various researchers have worked extensively on different molecular aspects of progression of PTB. However a definite link between oxygen sensing cellular signaling pathways and the degree of progression of PTB is under documented, which need to be studied comprehensively. So in the present study we aimed to evaluate the impact of oxygen sensing protein (VEGF) expression (measured quantitatively by ELISA) and its correlation with serum EPO and inflammatory marker like TNF-α in PTB patient.

Among the oxygen sensing molecular markers included in our study such as serum VEGF, EPO and TNF-α, earlier studies have reported about increased serum levels of VEGF and TNF-α in PTB and few countable studies have mentioned about inappropriate response of EPO in PTB. However, so far not much is known about the correlation among these oxygen sensing molecular markers in PTB and also their association with bacterial burden measured in terms of sputum acid fast bacilli (AFB) gradings in PTB. So in the present study, we made an attempt to demonstrate that, to know the disease severity, bacterial burden and extent of disease estimation of serum levels of oxygen sensing VEGF, TNF-α and EPO appears to be better predictive molecular markers in PTB.

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# CHAPTER 2

# AIM AND OBJECTIVES

## 2.1 Aim of the study:

To evaluate the impact of oxygen sensing protein (VEGF) expression and its correlation with EPO and inflammatory marker like TNF-α during PTB.

## 2.2 Objectives of the study:

1. To evaluate the quantitative assessment of serum VEGF, EPO and TNF-α in PTB patients.
2. To evaluate the correlation between serum VEGF and TNF-α, serum VEGF and EPO and also between serum TNF-α and EPO in PTB patients.
3. To evaluate the correlation of serum VEGF, EPO and TNF-α with bacterial burden measured in terms of sputum acid fast bacilli (AFB) gradings in PTB patients.
4. Overall evaluation of oxygen sensing pathway in relation to VEGF, EPO and TNF-α in PTB patients.

**2.3 Hypothesis:**

There is a relationship between PTB and oxygen sensitivity. Various genes related to oxygen sensing mechanism either in hypoxia or hyperoxia must have an influence on PTB. Hence our hypothesis is VEGF protein expression (measured quantitatively by ELISA) not only influences PTB but also it is correlated with serum EPO and TNF-α concentration in PTB.

# CHAPTER 3

# REVIEW OF LITERATURE

### 3.1 Historical perspective:

Although the history of species MTB dates back about 150 000 years, the germ mycobacterium is not as young as MTB and has been found somewhere around 150 million years ago[1]. Studies on Neolithic skeletons and Egyptian Mummies explored about TB, that has affected mankind for thousands of years[2, 3]. Ancient Greeks used the word Phthisis for TB meaning fatal disease[4]. TB has existed in India for several thousand years. From around 1500 BC, the word consumption was mentioned in Indian literature which is a Sanskrit word for wasting disease related to excessive fatigue, worries, hunger and chest wound[5]. Somewhere about 500 BC it was written in a Sanskrit manuscript about Sosha, a disease with symptoms like cough and blood spitting. In the same manuscript it was also mentioned about Brahman's king, the Moon-God, who was the first to suffer from this disease and hence also termed as Rajayakshama or Kings Disease[6].

In due course of time several scientists and researchers from all around the world worked extensively and found many interesting things regarding this disease but not the cause of this disease. Finally the German physician and microbiologist Heinrich Hermann Robert Koch announced on 24$^{th}$ March 1882 that he had identified MTB, the specific causative agent for the development of lethal PTB[7]. He was awarded Nobel Prize in 1905 in Physiology and Medicine for his great work with respect to TB[8].

### 3.2 Epidemiology of Tuberculosis:

Tuberculosis, a potentially serious infectious disease that mainly affects the lungs is becoming worldwide health problem. Every year, many people from all around the world becoming victim and sick due to TB. Globally, it is one of the top 10 causes of death and also predominant cause of death from a single infectious agent ranking above HIV/AIDS. About 1.3 million people have died due to TB in 2017 among HIV negative people and an additional 3 lakhs deaths from TB with HIV positive people[9].

In 2017, it has been reported by WHO that, globally about 10 million people developed TB disease. Out of these, 5.8 million men, 3.2 million women and 1.0 million

were children and 90% were adults with economically productive age groups of 15-54 years. Among global TB burden 87% were accounted by 30 high TB burden countries[9].

Failure of adequate treatment for TB leads to life threatening critical condition due to the emergence of drug- resistant MTB strains with the development of more severe drug- resistant TB which is found to be major disaster for public health. Worldwide in 2017, it was estimated that about 5,58,000 people developed drug- resistant TB, resistant to rifampacin and among these 82% had multidrug- resistant TB (MDR-TB). Among reported MDR-TB cases in 2017, 8.5% constituted extensively drug-resistant TB (XDR-TB). It has been mentioned in WHO global TB report 2018 that, 3.5% of new TB cases and 18% of previously treated cases had drug resistant TB worldwide[9].

Globally, health burden caused by TB is decreasing each year since from 2013 to 2017, but very slowly. Every year there is a decline of 2% TB incidence worldwide. The number of deaths from TB disease is also reducing, 1.8 million in 2013 to 1.3 million in 2017. There is downfall in TB deaths in people with HIV positive by 44% from 2000 to 2017. There is also fall in TB death rate in people without HIV by 42% in the period 2000-2017[9].

Among 1.7 billion people from worldwide reported to be infected with MTB and out of this about 5-10% will suffer from TB disease at some point during their life time. However, in people with HIV, diabetes, poor nutrition, smoking tobacco and alcohol abuse there is a high chance of getting TB disease thus increasing the probability of co-morbidity. There is a high rate of TB death without proper treatment. Studies have shown that 70% of untreated people with sputum positive PTB have died, 10 years after being diagnosed to have TB and 20% in the case of sputum negative (culture positive) PTB patients[10].

**3.3 Tuberculosis in India:**

In India, TB continues to be a predominant infectious disease leading to public health problem due to its potentiality to infect innumerable people, survive latent in the

host for an unknown period and then reappear as apparent disease[9]. According to global TB report 2018, India ranks first among 30 high TB burden countries. In 2017 version of global TB report it was mentioned that, incidence of TB in India was nearly 28,00,000 which is equivalent to one fourth of global TB burden and mortality due to TB (excluding HIV) was found to be approximately 4,23,000[11]. The incidence of drug-resistant TB in India is around 1,47,000 and out of 5,58,000 people notified globally to have drug-resistant TB in 2017, 24% is contributed by India alone[11].

India is the 2nd highest populated country in the world and crowded living conditions are very common. This could be one of the major risk factor for increased transmission of TB disease and probably this is the reason behind increased incidence and prevalence of TB disease in India[9]. Every day more than 1400 people develop TB disease, and more than 600 people die due to TB in India[12].

As a part of TB control activities, government of India launched Revised National TB Control Programme (RNTCP) in 1997 with a plan to detect and treat all the TB cases. The RNTCP thus established, adopted the globally recommended Directly Observed Treatment Short-course (DOTS) policy, as the most structured, efficient and economical method to strengthen the TB control strategy in India. RNTCP was covered entire nation in 2006 and the programme has since then been achieving global benchmarks in detection of cases and successful treatment[12, 13].

Inspite of involvement in TB control programme for more than 50 years, TB remains one of the major persistent causes of morbidity and mortality to general public in India. At the same time the rate of missing cases are also increasing which are not notified, either not diagnosed or poorly diagnosed and treated by private sectors[14]. Now, government of India is ready to tackle with TB better than earlier with sophisticated approach for diagnosis, treatment and care of TB. The proof for this activity was bold decision taken by government authorities to accept National Strategic Plan (NSP) 2017-2025 for TB elimination in India with vision of "TB free India with zero deaths, disease and poverty due to TB" and the goal "to achieve rapid decline in the burden, mortality

and morbidity due to TB". This was one of the significant milestones in the progress of TB control activities noticed in 2017[14].

### 3.4 Mycobacteria:

Mycobacteria are aerobic, facultative, immobile, non-capsulated, non-sporing, slow-growing, intracellular, gram positive slender rod shaped (bacilli) bacteria that forms mould-like pellicle in liquid culture (hence the name Mycobacteria, meaning fungus like bacteria). It is difficult to stain the bacilli but once stained, resists decolourisation with dilute mineral acids and hence the TB bacilli are also called acid fast bacilli (AFB) [15].

### 3.4.1 Classification of Mycobacteria:

The genus Mycobacteria can be classified into various major groups for the sake of diagnosis and treatment. The classification is illustrated in the Figure-3.1.

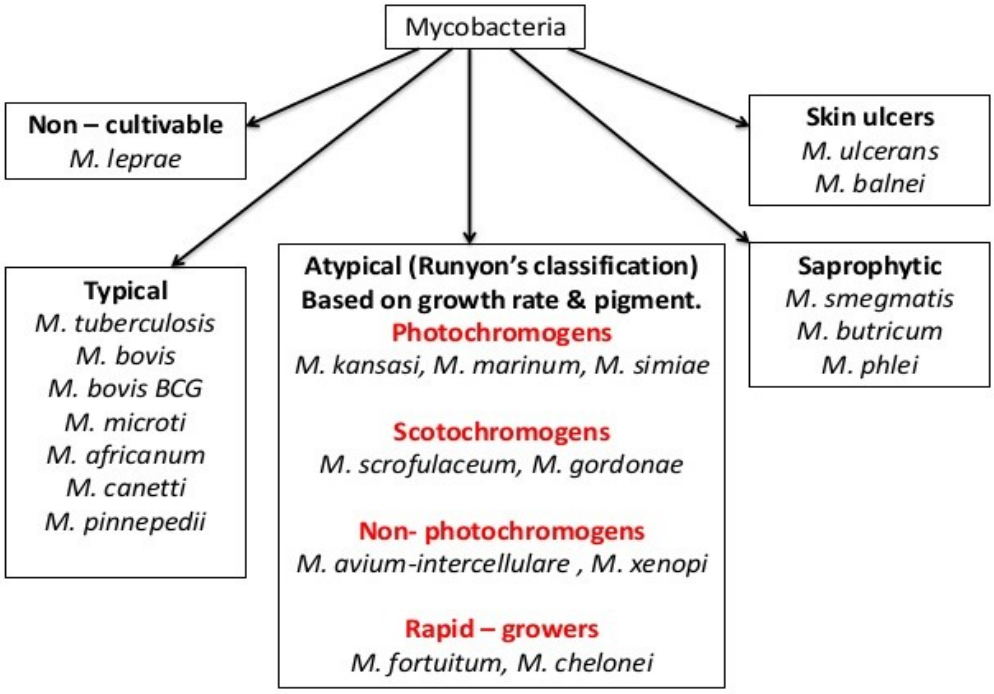


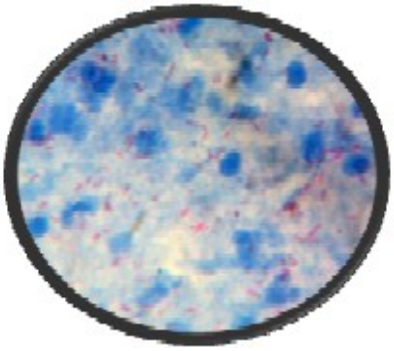

Figure -3.1: Classification of Mycobacteria

(Source: https://www.slideshare.net/lyallkamal/mycobacteria-48137623)

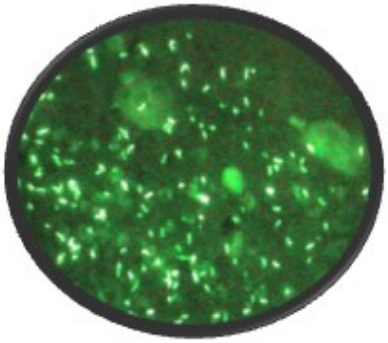
### 3.4.2 *Mycobacterium Tuberculosis* (*M. tuberculosis*/MTB):

#### 3.4.2.1 Morphology:

When viewed under microscope, the structure of *M.tuberculosis* looks like slender, straight or slightly curved rod measuring about 3 x 0.3µm in size. Their occurrence is single, in pairs or in small clumps. These bacilli are gram positive but the presence of an unusual waxy coating on their cell surface mainly due to the lipid, mycolic acid makes the cells resistant to gram staining. Acid fast stains such as Ziehl-Neelsen (Z-N staining) or Fluorescent stains such as auramine rhodamine are used to identify the bacilli with microscope. When observed under oil immersion objective the bacilli appear as bright red rods with blue background with Z-N staining. Under fluorescent microscopy the bacilli will appear as bright rods with dark background with auramine rhodamine stains[15]as illustrated in the Figure-3.2.



AFB (Z-N staining)



AFB (Auramine rhodamine staining)

Figure-3.2: AFB under microscopy
(Source:https//www.aphl.org/programs/infectious_disease/tuberculosis/TBCore/TB_AFB_Smear_Microscopy_TrainerNotes.pdf).

### 3.5 Types of tuberculosis (TB):

More than 85% of the globally notified TB cases are pulmonary tuberculosis (PTB) targeting mainly lungs, but in less than 15% cases the germ can affect many other sites of the body leading to extra pulmonary TB (EPTB) [16]. Figure-3.3 shows the types of TB.

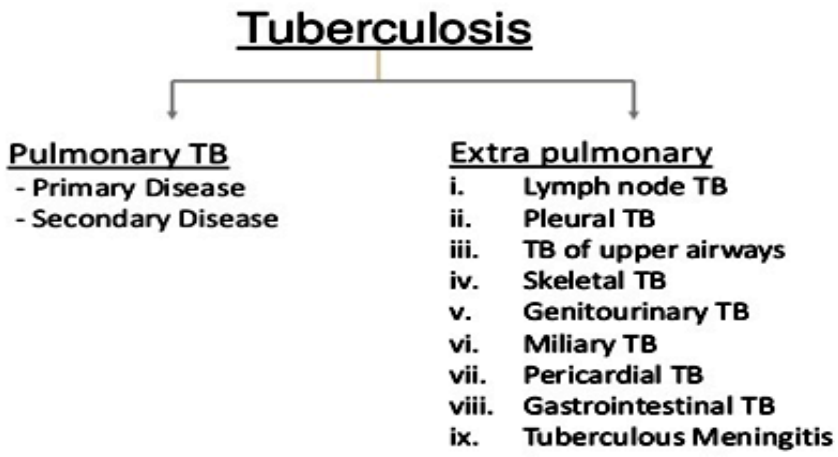



Figure -3.3: Types of TB.

(Source: https://www.slideshare.net/NikhilOza2/tuberculosis-51411487)

### 3.6 Transmission and pathogenesis of pulmonary tuberculosis (PTB):

The TB bacilli from a subject with untreated PTB is expelled out to the air during coughing, sneezing, laughing, shouting, singing or any other such activities of infected subject as tiny droplet nuclei and the transmission takes place when another subject breathes in these droplet nuclei and become infected[17]( Figure-3.4).

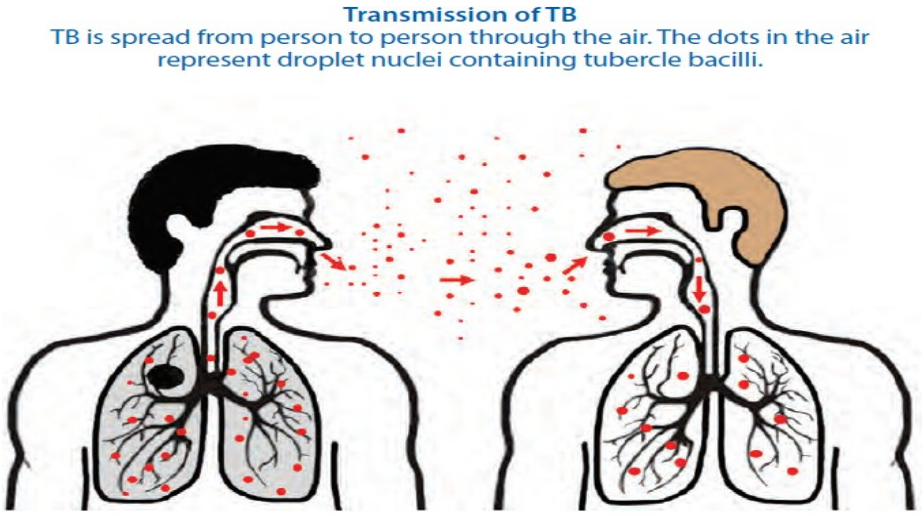



Figure-3.4: Transmission of TB

(Source:https://www.ncbi.nlm.nih.gov/books/NBK344406/figure/wt605814_ch2.F1/)

After reaching alveoli of new host, the bacilli are taken up by resident macrophages (Mø) leading to engulfment followed by phagocytosis of inhaled bacteria.

This initiates the pro-inflammatory response and placement of special cells of innate and adaptive immune systems, and subsequent formation of a granuloma.

The bacilli can be ceased within the necrotic granuloma for prolonged period provided hosts defense mechanism is intact. Once the host defensive mechanism fails for any reason, the bacilli will begin to multiply rapidly. The granuloma then breaks open and MTB is leaked into the airways as shown in the Figure-3.5 and might expelled out through coughing sneezing etc. leading to further transmission of the disease[18]. Some of these bacilli may disseminate by lymphatic route or through blood circulatory system to other parts of the body (including the regional lymph nodes, apex of the lung, kidneys, brain, bone etc) resulting in the development of EPTB [19].

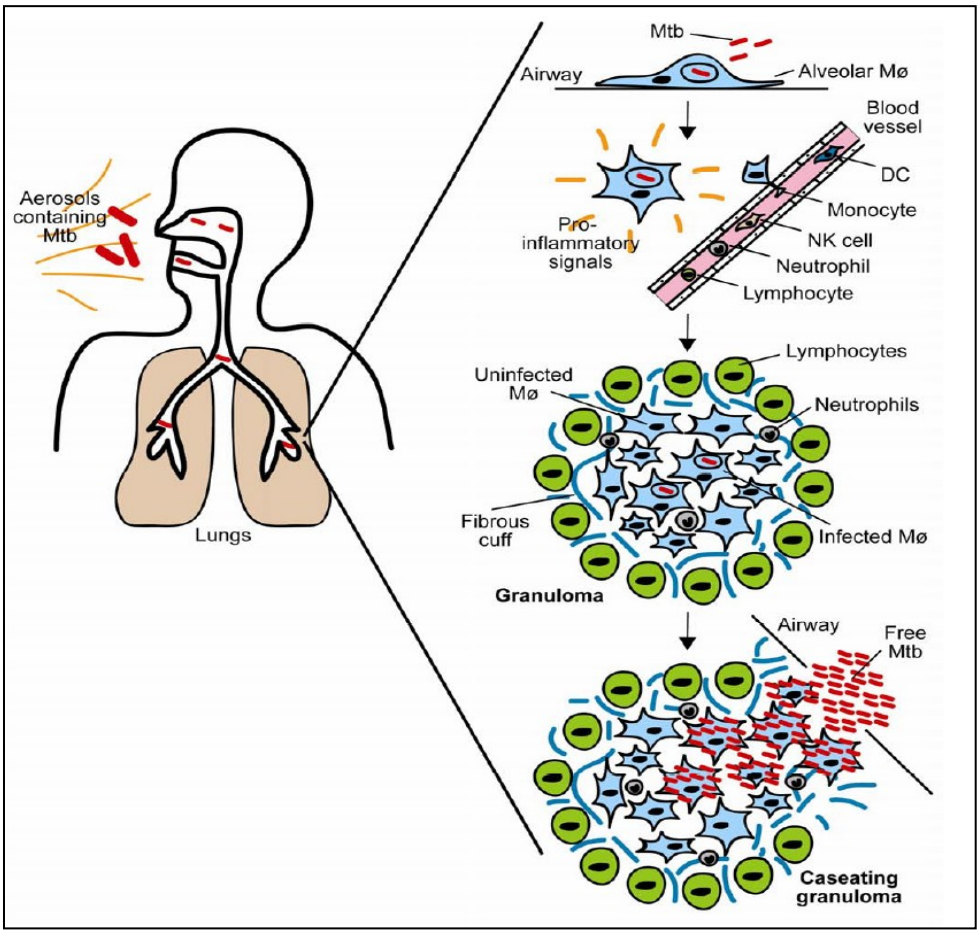



Figure-3.5: The infection route and pathogenesis of MTB in a human host.
(Source: Russell DG (2007). Who puts the tubercle in tuberculosis? *Nat Rev Microbiol* 5(1): 39-47).

### 3.7 Progression of tuberculosis (TB):

Figure-3.6 explains about the diagrammatic representation of progression of TB in human beings.

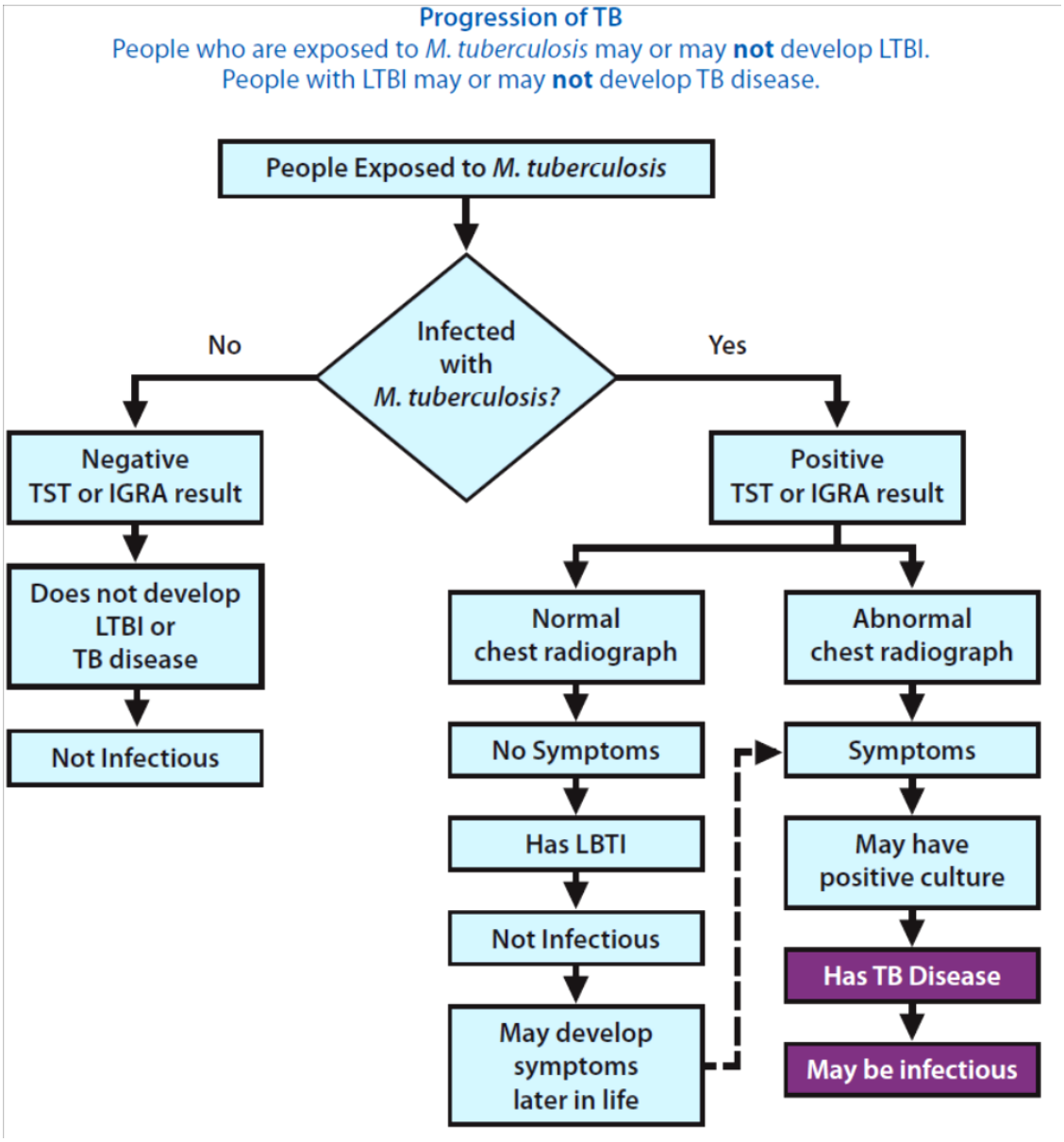



Figure-3.6: Progression of TB.

(Source: https://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf).

### 3.8 Latent tuberculosis infection (LTBI) and TB disease:

Not all the person infected with TB bacilli will develop TB disease. In some person, after infection the bacteria may stay dormant for a long period in host resulting in latent TB infection (LTBI). In some cases the progression of LTBI to TB disease may occur immediately or it may take very long time which is largely attributed to hosts immune system[20]. The following table (Table-3.1) shows the comparison between LTBI and TB disease;

Table-3.1: Comparison between LTBI and TB disease

| Person with LTBI (Infected) | Person with TB Disease (Infectious) |
|---|---|
| Has a small amount of TB bacteria in his/her body that are alive, but inactive | Has a large amount of active TB bacteria in his/her body |
| **Cannot** spread TB bacteria to others | May spread TB bacteria to others |
| Does **not** feel sick, but may become sick if the bacteria become active in his/her body | May feel sick and may have symptoms such as a cough, fever, and/or weight loss |
| Usually has a TB skin test or TB blood test reaction indicating TB infection | Usually has a TB skin test or TB blood test reaction indicating TB infection |
| Radiograph is typically normal | Radiograph may be abnormal |
| Sputum smears and cultures are negative | Sputum smears and cultures may be positive |
| Should consider treatment for LTBI to prevent TB disease | Needs treatment for TB disease |
| Does **not** require respiratory isolation | May require respiratory isolation |
| **Not** a TB case | A TB case |

(Source: https://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf

### 3.9 Symptoms of Pulmonary tuberculosis (PTB):

The major symptoms which are commonly observed in PTB patients can be diagrammatically illustrated in the Figure-3.7 as shown below;

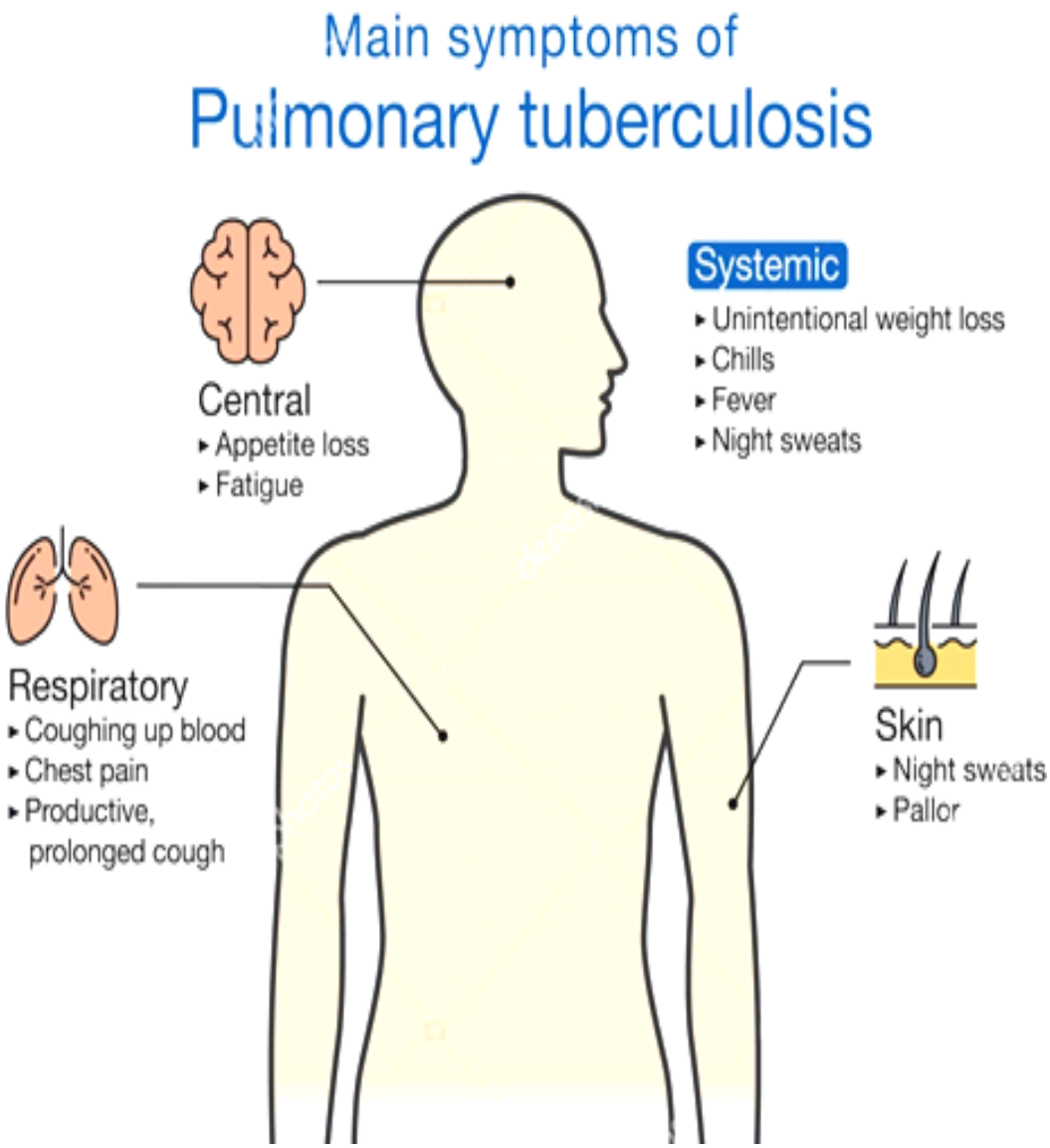



Figure-3.7: Major symptoms of PTB.

(Source: https://depositphotos.com/174491076/stock-illustration-main-symptoms-of-pulmonary-tuberculosis.html)

### 3.10 Types of pulmonary tuberculosis (PTB):

Based on the pattern, time of infection and nature of responses, PTB may be of primary or secondary.

#### 3.10.1 Primary PTB:

It is seen as initial infection especially in young children and it is usual findings in countries like India where TB is endemic. After bacilli are taken up by alveolar macrophages, they multiply and forms initial focus of infection with small sub pleural granuloma along with enlarged hilar lymph node together constitutes Ghon complex mainly located in the lower lobe or lower part of upper lobe. In most of the cases, these lesions resolve within 2-6 months leaving behind a calcified nodule and further there is no transmission of the infection[15, 21].

#### 3.10.2 Secondary PTB:

Also known as post-primary TB as it is due to reactivation of previous infection or adult TB as it is most probably seen in adults. The reinfection or endogenous reactivation is dependent on host immunity. Upper lobes of the lungs show the impact and necrotic lesions, tissue injury and cavitations may be seen. Outbreak of granuloma, leakage of bacilli and spread of infection are some of the other typical features of secondary TB[15, 21].

### 3.11 Diagnosis of pulmonary tuberculosis:

The new technical and operational guideline (TOG) framed by RNTCP in relation to diagnostic algorithm of PTB[22] is as follows;

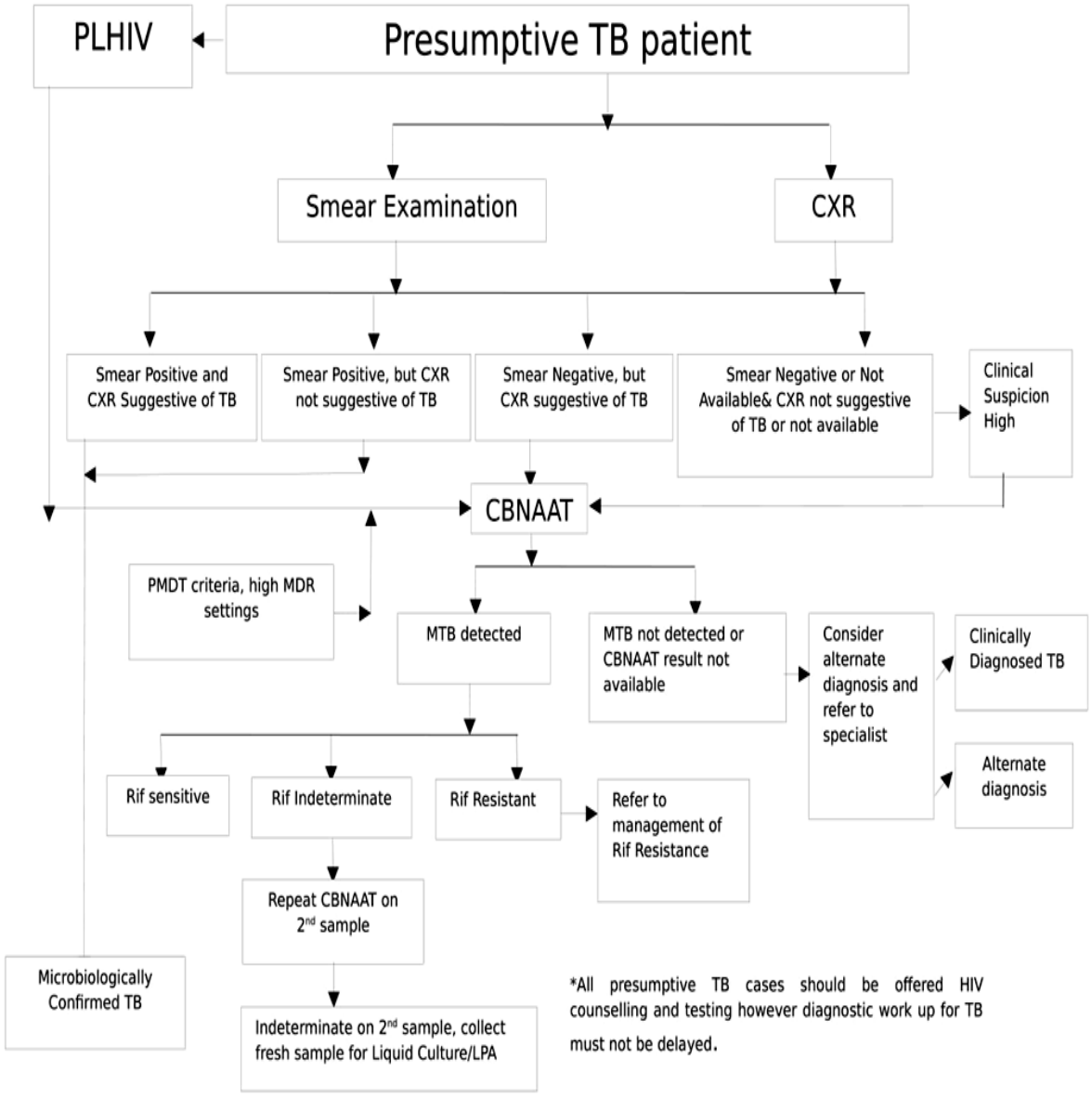



Figure-3.8: Diagnostic algorithm of PTB, RNTCP new guideline.

### 3.12 Treatment of tuberculosis (TB):

Successful anti tuberculous treatment (ATT) were first established in the 1940s. Until then the only known mode of therapy for TB was need of continuous rest, healthy food and mild exercise for very long period of time. At present chemotherapy proved TB is treatable and curable. The present approved regimen for drug susceptible TB is first

line drugs (Table-3.2). The treatment success rates of drug susceptible TB, reported routinely by member states of WHO is around 85% [9, 23].

Table-3.2: RNTCP treatment regimen for drug sensitive TB

| RNTCP treatment regimes | | | |
|---|---|---|---|
| **Treatment groups** | **Type of patient** | **Regimen** | |
| | | Intensive phase (IP) | Continuation phase (CP) |
| New | Sputum smear-positive<br>Sputum smear-negative<br>Extra-pulmonary<br>Others | $2H_3R_3Z_3E_3$ | $4H_3R_3$ |
| Previously treated | Smear-positive relapse<br>Smear-positive failure<br>Smear-positive treatment after default<br>Others* | $2H_3R_3Z_3E_3S_3$ / $1H_3R_3Z_3E_3$ | $5H_3R_3E_3$ |
| The number before the letters refers to the number of months of treatment. The subscript after the letters refers to the number of doses per week. The drugs are as follows: isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E), and streptomycin (S). | | | |

(Source: *Ind J Med Ethics* 2014; 11(1):48-52)

Second line drugs are used for the treatment of drug resistant TB which includes MDR-TB and XDR-TB. Details regarding treatment aspects of drug resistant TB as per PMDT guideline under RNTCP are shown in the following tables.

Table-3.3: RNTCP treatment regimen for drug resistant TB (PMDT guideline)

| Regimens under PMDT | | | |
|---|---|---|---|
| **Regimens under PMDT** | **Intensive phase (IP)*** | **Continuation phase (CP)**** | **Reserve/ substitute drugs** |
| Regimen for MDR-TB | 6–9 Km, Lvx, Eto, Cs, Z, E | 18 Lvx, Eto, Cs, E | PAS, Mfx, Cm |
| Regimen for XDR-TB | 6–12 Cm, PAS, Mfx, high-dose H, Cfz, Lzd, Amx/Clv | 18 PAS, Mfx, high-dose H, Cfz, Lzd, Amx/Clv | Clarithromycin, Thiacetazone |

*Intensive phase for MDR-TB: 6 to 9 months. Intensive phase for XDR-TB: 6 to 12 months.

**Continuation phase for MDR-TB and XDR-TB: 18 months.

Drugs: Km - kanamycin, Lvx - levofloxacin, Eto - ethionamide, Cs - cycloserine, Z - pyrazinamide, E - ethambutol, PAS - para amino salicylic acid, Mfx - moxifloxacin, Cm - capreomycin, H- isoniazid, Cfz - clofazimine, Lzd - linezolid, Amx/Clv - amoxyclav

(Source: *Ind J Med Ethics* 2014; 11(1):48-52)

### 3.13 Association of body mass index (BMI) with pulmonary tuberculosis:

BMI is a value derived from mass and weight of a person. It is defined as the "person's body weight in kilogram divided by the square of the height in meters". The unit of BMI is $kg/m^2$. It is a measure of body fat depending on height and weight applicable to adult male and females. It can be used to screen for weight categories leading to health problem. Based on BMI person can be grouped as underweight, normal weight, overweight or obese[24].

It was known from the centuries as TB is the disease of poor which in turn drives malnutrition. The harmful visual impact of TB is accompanied by significant social and economic insinuation for patients, certainly better considered in the bifacial relationship between poverty, poor nutrition and TB. The disease is five times more frequently seen

among people of low socioeconomic background which needs to be addressed for effective control of TB[25].

It is obvious that measurement of BMI gives an idea about the nutritional status of an individual[26]. It is necessary to highlight that, low BMI increases the risk of progression of LTBI to active TB indicating the importance of proper nutrition in TB[27].

Few studies described an inverse link between lower BMI and higher probability of mortality in PTB indicating lower BMI is one of the significant individual risk factor for the occurrence of PTB[25, 28, 29].

A study conducted in Taiwan during 2011-2012 by Yen et al. suggested that, among all the risk factors, low BMI seems to be predominant cause for TB specific and non-TB specific mortality during therapy[28].

The study from Zachariah et al. found that, the mortality rate was increased in TB patients with BMI <17.0 kg/m$^2$ within 4 weeks of chemotherapy [30]. In a similar type of study, a high rate of TB death during first 8 weeks of treatment among TB patients with BMI <18.5 kg/m$^2$ was reported by Lai et al[31].

A comparative study by Mupere et al. between men and women TB patients with same BMI reported about elevated death rate among male TB patients and further he clarified, this discrepancy was related to less fat body mass and little energy store in man compared to woman at the time of diagnosis[32].

In 15% of population, it was noticed to have congenital apical lung bullae and interestingly, in some young males with low BMI that may enlarge leading to target site for TB reactivation[33, 34].

According to Cegielski et al. the risk of getting infection due to TB bacilli decreases with increase in BMI and further, reduced incidence of TB was observed in overweight person[35].

The low BMI and increased susceptibility to PTB may be attributed to deficiencies of vital nutrients and biological mechanisms leading to reduced cellular immunity of the host indicating a relationship between body immune response and BMI[36, 37].

The inflammatory cytokine, TNF-α with a antimycobacterial activity is implicated in the induction of fever, loss of appetite and weight loss in PTB patients and further studies have shown inverse correlation between TNF-α and BMI in patients with PTB. However, to know the exact association between inflammatory response and low BMI, further research is required[38,-41].

**3.14 Hematology and pulmonary tuberculosis:**

Hematology is one of the medical branches which deals with the study of blood, blood forming organs and diseases related to the blood. The hematological tests are laboratory based measurement of blood to diagnose and monitor many clinical conditions. The commonly measured hematological parameters include complete blood picture count with blood indices and erythrocyte sedimentation rate (ESR).

Previous studies have reported that, hematological abnormalities are very common in chronic diseases like PTB[42-45]. There is a link between PTB and reversible changes in blood picture and these hematological changes may act as important tool in the diagnosis, monitoring and to know the treatment out come in PTB[46].

The incidence of anemia is most common in majority of PTB patients and mostly of normocytic normochromic type. However, microcytic and macrocytic anemia can also occur in PTB. The degree of anemia depends on the clinical severity of PTB[43, 47-50]. There are many causative factors for the development of anemia in PTB. However, studies have enlightened the frequent implication of iron deficiency and inflammatory cytokines as

causative agents in the pathophysiology of anemia of chronic disorders like PTB. Upon exposure to bacilli, as a defense mechanism, activated monocytes releases large amount of inflammatory cytokine TNF-α. Moreover, studies found that TNF-α interferes with erythropoietin (EPO) production and iron utilization leading to blunted EPO response to anemia of untreated or treatment failure cases of TB[42, 51, 52].

It is obvious to record leucocytosis with neutrophilia in PTB which is due to the inbuilt defense mechanism against invading TB bacilli resulting in the elevated polymorphonuclear leucocytes and macrophages[43]. Leucopenia with neutropenia was uncommon and less prevalent in PTB[42]. There is a lack of literature indicating exact cause for neutropenia but the implication of hypersplenism, granulopoietic inhibitory activity of T-lymphocyte, abundant migration of neutrophil or malnutrition cannot be ruled out. Results from many studies reported about lymphocytopenia in PTB and role of cytokines were suggested for the pathogenesis of lymphocytopenia[43, 44].

Earlier research work reported thrombocytosis in PTB patients. There is a positive correlation between thrombocytosis and inflammatory response and the increased production of IL-6 during inflammatory diseases like PTB promotes platelet production[46, 53].

Previous workers documented high ESR values in most of the PTB patients which confirms that, estimation of ESR is a regular practice in TB[45, 54]. Furthermore, it has been observed from the earlier literature that all the hematological parameters came back to normal range after successful treatment for TB[54-56].

**3.15 Hypoxia and bacterial infection:**

Normal concentration of oxygen in the atmosphere is 21% (159 mm Hg). Generally, healthy tissue demands 2.5-9% of oxygen (20-70 mm Hg), whereas markedly lower levels (less than 1%) have been identified in injured and necrotic sites[57]. The situation of decreased oxygen tension at the tissue level is termed as hypoxia either affecting whole body or certain areas of the body[58].

The functioning capacity of immune cells depends on constant supply of energy and in normoxic condition; mitochondrial oxidative phosphorylation assures the supply of energy. However, in hypoxic condition particularly observed in every site of inflammation, infected or injured tissues forces the immune cells to adapt to anaerobic pathway to fulfill their energy requirements[59].

Previous studies reported that, occurrence of tissue hypoxia during bacterial infection is characteristic feature of microenvironment associated with bacterial infection[60]. Moreover, the reason behind the development of hypoxia with respect to bacterial infection associated with inflammation depends on many factors including increased oxygen demand by inflamed cells, infiltrating neutrophils, multiplying microbes or some time vascular pathophysiology and thrombosis associated with decreased blood supply to tissues due to chronic infection[61, 62]. Probably, imbalance between demand and supply of oxygen leads to hypoxia as noted during bacterial infection.

In mammalian cells, response to hypoxia is governed by a transcriptional factor namely hypoxia inducible factor -1alpha (HIF-1α). It is a heterodimer with two basic helix-loop-helix protein subunits, HIF-1α and HIF-1β. HIF-1α is oxygen dependent whereas HIF-1β is constitutively expressed subunit[63-65]. The proof for existence of hypoxia during bacterial infection associated with inflammation is the expression of HIF-1α in most of the immune cell types including both innate and adaptive populations[66-68].

**3.16 Activation of hypoxia inducible factor-1 alpha (HIF-1α) by hypoxia:**

Now it is best understood that the mechanism, how HIF-1 pathway are activated and diagrammatically, the activation of HIF-1 pathways are shown in Figure-3.9. The family of prolyl hydroxylases (PHDs) namely PHD1, PHD2 and PHD3 and single asparagine hydroxylase known as factor inhibiting HIF (FIH)[69, 70] which are dependent on oxygen and iron hydroxylate two proline residues (P402 and P564) of the HIF-1α subunit during normoxic conditions. This leads to the ubiquitinylation of HIF-1α through

the mediation of von Hippel- Lindau tumor suppressor protein (pVHL) and further proteosomal degradation[69].

In contrast, hypoxia results in the inactivation of PHD and further stabilization of HIF-1α which translocates into nucleus where it dimerizes with HIF-1β and thus forms active HIF-1 heterodimer complex which then binds to hypoxia responsible element (HRE) found on the promoter region of the target gene. Additional proteins, either p300 or cyclic adenosine monophosphate response element-binding protein (CBP) bind the complex as co activators and further modulates the transcription of target gene[69, 71]. VEGF, Erythropoietin (EPO), basic fibroblast growth factor, glycolytic enzymes and glucose transporters are few examples of HIF-1 induced target gene products involved in the regulation of angiogenesis, apoptosis, cellular stress, immunity and metabolism[72].

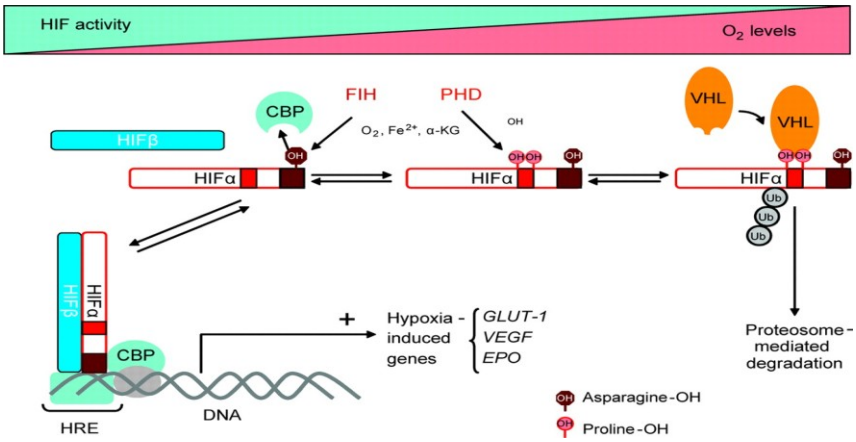



Figure-3.9: HIF-1α regulation by oxygen dependent pathway and expression of target genes.

(Source: Reshef Tal. The role of hypoxia and hypoxia-inducible factor-1 alpha in preeclampsia pathogenesis. *Biol Repro* 2012; 87(6):1-8)

Initial studies highlighted more about the role of HIF-1 in tumor pathology where it was found to be involved in increased tumor angiogenesis, progression and subsequent metastasis[63]. However, recent studies suggested important role for HIF-1 in the infectious and inflammatory diseases and also as master molecule in the regulation of immune response[66, 73]. HIF-1 plays a novel role in the activation of T cells, B cells, dendritic cells, macrophages and neutrophils during infection and inflammation[62, 63, 74].

The innate immune function of myeloid cells during bacterial infection relay on HIF-1α where it regulates the production of host immune defense factors such as granule proteases, antimicrobial peptides, TNF-α and nitric oxide (NO). Notably, the HIF-1α mediated generation of TNF-α and NO during bacterial infection not only inhibits microbial proliferation but also stabilizes HIF-1α in myeloid cells engaged at infection site leading to more elegant mechanism to control the infection[75].

On entry of pathogenic bacteria into the host cells, the toll like receptors (TLRs) are activated which in turn activates nuclear factor-kappa B (NF-κB) resulting in the expression of many genes and subsequent development of inflammation and hypoxia. Further, the NF-κB stimulate HIF-1α expression which is further stabilized by prevailing inflammatory hypoxia and thus the activity of HIF-1α increases exponentially resulting in the upholding of factors designed for promoting immunity and bacterial clearance[76] .

It may be noted that, during bacterial infection hypoxia seems to be beneficial in controlling the infection but the overall effects of hypoxia in bacterial infection depends on the nature of pathogen, its virulence factor and type of infection occured[77]. It is difficult to predict whether the enhanced activity of HIF-1α in hypoxia, induced by infection and inflammation is protective or harmful to the host. It has been shown that, HIF-1α seems to be protective to host during infection with Group A *streptococci*[78], *P.aeruginosa*[75], *Y.enterocolitica*[79], *C.difficile*[80] and *S.aureus*[81]. In contrast accumulated HIF-1α may be harmful to the host during infection with *S.aureus peritonitis*[82], *E.coli* LPS[83], *B.henselae*[84] and *H.pylori*[80].

The use of pharmacological modulators of HIF-1 which is currently under evaluation in cancer therapy should also be considered in future in case of fighting against infection caused by pathogens exploiting HIF-1 to aggravate the clinical condition particularly those infections involving multidrug resistant pathogens[85].

### 3.17 *Mycobacterium tuberculosis* (MTB) and hypoxia:

It was known from the fast that MTB has been associated with human TB disease for thousands of years and this became possible due to the capability of the bacilli to survive in the host for decades without exhibiting any symptoms residing in the complex cellular aggregates called granuloma[86]. TB granulomas are hall mark structure of the MTB infection and studies have indicated granulomas are hypoxic in nature[87, 88]. Within this granuloma, TB bacilli remain as inactive non replicating state of persistence leading to LTBI[89].

The association between hypoxia and immune defense found to be important in the immunopathology of TB which depends greatly on the complex interaction between HIF-1α and NF-κB[88]. It has been noticed that during MTB infection, the expression of NF-κB is up regulated in macrophages of granuloma which in turn increases HIF-1α concentration and further promoting the immune mechanism effectively[90].

The balance between antimicrobial and anti-inflammatory response in the hypoxic environment of TB granulomas plays a major role in restricting the pathogenesis of TB bacilli[91]. Further, the microenvironment in TB granuloma induces the expression of HIF-1α which plays an important role for the production of many vital immune effector molecules with antimycobacterial activity thereby controlling MTB replication[92].

Earlier it was thought that formation of granuloma is an effective host immune response against MTB by locking bacilli in the complex necrotic microenvironmental structure and reducing the infection burden. But recent research activities in animal model suggested that MTB can survive and escape from granuloma using host factors leading to reactivation and further spread of infection [91].

Recently, Oehlers et al. studied TB granuloma in the zebra fish – *Mycobacterium marium* model and found increased activity of angiogenic factor VEGF formed as a result of stimulation from hypoxia induced HIF-1α. Further they concluded, granuloma associated angiogenesis mediated by VEGF promotes bacterial growth leading to increased spread of infection to other parts of the host[93]. In a similar type of study

conducted in mouse infected with MTB, revealed extensively vascularized granuloma mediated by HIF -1α dependent VEGF and finally author reported that bacilli take advantage of the formation of new blood vessels to disseminate[94].

Matrix metalloproteinases (MMPs), especially MMP-1 with collagenase activity are implicated in the tissue destruction in PTB and is activated by hypoxia induced HIF-1α[94]. The research outcome of Belton et al. explored that human TB lesions are highly hypoxic and increased expression of MMP-1 resulted in the cavitations and lung tissue destruction with increased spread of infection[88].

More recently it was reported that, during bacterial infection hypoxia can stimulate neutrophil proteases such as MMP-8 and elastase which resulted in the destruction of lung extracellular matrix components like collagen and elastin leading to loss of structural frame work of lungs[95].

So from the host point of view, occurrence of hypoxia during bacterial infection seems to be protective in one angle and destructive from other angle.

**3.18 Vascular endothelial growth factor (VEGF):**

VEGF is a basic, heparin binding, homodimeric glycoprotein of 45 KDa which was initially purified from a fluid produced by a tumor[96]. The VEGF and VEGF receptors (VEGFR) plays an efficient role in controlling vasculogenesis during embryonic development and angiogenesis at a later stage[97]. The pro-angiogenic activity of VEGF is essential to regulate tissues at physiological level and also leads to formation of new blood vessels to surpass ischemic disease. They also function as vascular permeability factor, stimulator of cell migration in macrophage lineage and endothelial cells and regulator of lymphangiogenesis[98].

There are at least 7 members of the VEGF gene family have been identified, among them 5 genes are found in human genome namely VEGF–A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. In human beings, the most frequently studied VEGF family is VEGF-A which is also known as VEGF[99]. The isoform of human

VEGF-A that is VEGF, contains 121, 145, 165, 183, 189 and 206 amino acid residues and they are formed as a result of alternative splicing of VEGF gene[99]. Among all the isoforms, $VEGF_{165}$ is the most important because of its biological activity and bioavailability[100].

### 3.19 Vascular endothelial growth factor receptors (VEGFRs):

VEGFRs are typical tyrosine kinase receptors including an immunoglobulin like extracellular domain for binding of the ligand, a transmembrane domain, and a cytoplasmic domain with single tyrosine kinase domain. The potential activity of VEGF is associated with specific receptors. There are 3 types of VEGFRs including VEGFR-1, VEGFR-2 and VEGFR-3[101].

VEGF-A activates both VEGF-R1 and VEGFR-2 by binding with them and regulates angiogenesis, vascular permeability, cell migration and gene expression[102]. VEGF-C and D binds to VEGFR-3 and mainly regulates lymphangiogenesis and also involved in angiogenesis observed mainly during embryonic stage development[103]. VEGF-B receptor is VEGFR-1 and is implicated in pathological and stressful condition[104].

Many factors can regulate VEGF gene expression such as hypoxia, inflammation, growth factors, cytokines and other extracellular components. Among them hypoxia seems to play predominant role by inducing HIF-1α (Figure-3.9). The promoter region of VEGF mRNA contains HIF-1α binding site. Under hypoxia, upon binding with VEGF promoter site, HIF-1α induces the transcription of VEGF mRNA resulting in the production of VEGF protein[100, 105].

Figure-3.10 indicates the binding of VEGF to its corresponding receptors and subsequent cellular responses.

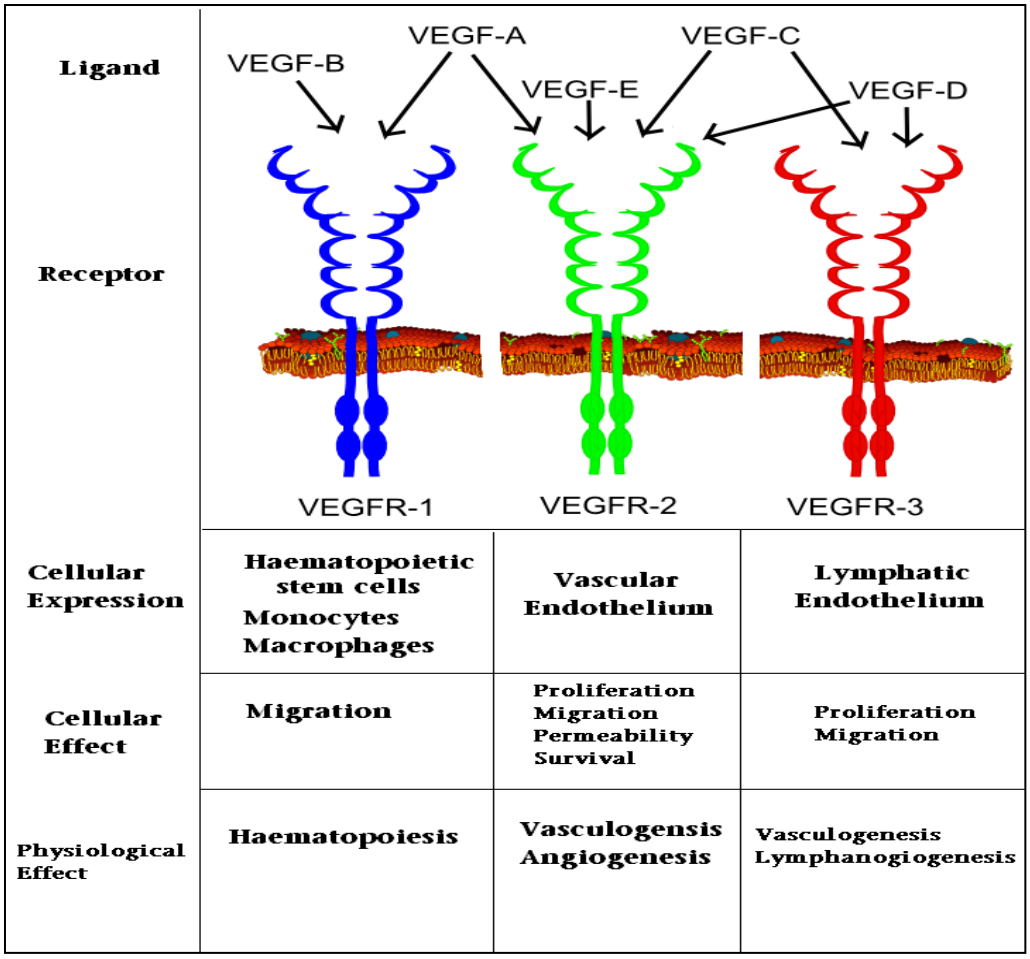



Figure-3.10: VEGF receptors, ligand binding and cellular responses.
(Source: https://proteopedia.org/wiki/index.php/Image:VEGF_effects.PNG)

### 3.20 Vascular endothelial growth factor (VEGF) in lung:

Extensive research work has been carried out in the field tumor pathology related to VEGF where it is known to stimulate tumor angiogenesis. Many literatures have shown the presence of higher circulatory levels of VEGF in patients with different cancers[106-110]. VEGF promotes tumor growth and metastasis by contributing in the formation of new blood vessels thereby feeding and nurturing the tumor and taking tumor cells to other body sites[111].

Comparatively very little is known about role of VEGF in lungs. It is required for lung development and also implicated in lung physiology. It is expressed in many parts of the lung. The developing lung demonstrated raised VEGF protein and VEGF mRNA

indicating the importance this angiogenic factor in lung vasculature[112]. Furthermore, in newborns VEGF may prevent the occurrence of respiratory distress syndrome by stimulating the production of surfactant by lung cells leading to lung maturation[113].

Among healthy tissues, lung tissue exhibits increased expression of VEGF and its receptors and are located in alveolar type II cells, neutrophils, macrophages, mesenchymal cells, vascular smooth muscle cells and air way epithelial cells[114]. The VEGF protein concentration in lung is 500 times more than plasma in normal subject contributed mainly by alveolar and air way epithelial cells and also by air way smooth muscle cells, In general the healthy lung alveolar macrophages secret very low VEGF. Though neutrophils produce large amount of VEGF, but their numbers are less in normal lung[115, 116].

In addition VEGF has been implicated in the pathophysiology of many lung disorders like chronic obstructive pulmonary disorders, asthma, pulmonary hypertension, lung cancer, acute lung injury and pleural disease. In these disorders, whether VEGF plays protective role or destructive role is still debatable[117].

**3.21 Vascular endothelial growth factor (VEGF) in pulmonary tuberculosis (PTB):**

Though not extensively studied, few previous researchers have reported higher values of serum VEGF in PTB patients[118-122].

According to Abe et al. elevated levels of VEGF in PTB patients may prevent the development of chest cavities and further he observed typical chest cavities in PTB patients having comparatively low levels of VEGF[119]. However, such findings are not carried in the subsequent study but reported increased serum VEGF could be used as marker of active PTB and disease monitoring[121].

Study from Amin et al. suggested that, serum VEGF in PTB cases could be a simple invasive test for the prediction of disease activity. They found serum level of VEGF with the cut off value of 197pg/ml was able to predict active PTB with 100%

sensitivity and 90.2% specificity[120]. Another two similar type of studies reported serum VEGF levels with cut off values of 250 pg/ml and 458.5 pg/ml with great sensitivity (but reduced specificity) may be used for the diagnosis of active PTB and concluded negative results decreases the chances of getting TB whereas positive results requires further confirmation as the specificity is low[118, 121].

In a study, serum levels of VEGF in PTB patients before starting the effective treatment, 3 months and 6 months after the initiation of the treatment were recorded and noticed gradual decrease in VEGF levels after the onset of treatment regimen indicating the role for VEGF in therapeutic response[122].

It is important to mention the significant involvement of VEGF in the differential diagnosis of TB and in discriminating between PTB, LTBI and non tuberculosis (NTB), serum concentrations of VEGF could be used[123]. To monitor the sputum culture conversion as a measure of treatment success in TB patients, evaluation of VEGF can be considered especially in HIV negative TB patients[124].

Some studies have also highlighted about the pathophysiological role of VEGF in extra pulmonary tuberculosis (EPTB). Disease severity in neurotuberculosis can be evaluated in patients using VEGF measurement and high level of VEGF is associated with clinical severity of neurotuberculosis[125].

Increased levels of serum VEGF in pleural effusions are indicative of malignant pleural effusion and could be used to distinguish between malignant pleural effusion and tubercular pleurisy and few studies have suggested the inclusion of VEGF estimation in the diagnostic panel could improve the differential diagnosis of effusions and thus helps the clinician to take appropriate decision[126-128].

Recent research work conducted using rabbit and zebra fish model infected with MTB observed the presence of high vasculature in TB granuloma mediated by increased

VEGF expression due to hypoxia which facilitated increased bacterial survival and further dissemination of infection to distant site[93, 129].

Furthermore, Papaioannou et al. in his review article commented that, VEGF could be beneficial to the host when it is present in the right quantity, in the right time and place and concluded that the requirement of future research to answer the exact role played by VEGF in health and diseases[130].

**3.22 Erythropoietin (EPO):**

The decisive factor for the regulation of red blood cell production is a glycoprotein hormone of renal origin that is erythropoietin (EPO). In the bone marrow, EPO is crucial for the erythroid precursor maturation by stimulating proliferation and differentiation and thus inducing erythropoiesis[131].

EPO belongs to class I cytokine family member with 30.4 KDa molecular mass with globular structure. It constitutes 165 amino acids which is adequate to bind with the receptors[132]. It has four complex carbohydrate chains. Three out of four oligosaccharide chains are attached to the asparagine residues at position 24, 38 and 83 by N-linkage and probably involved in the upholding of EPO in circulation. The remaining one sugar chain is joined to serine residue at position 126 by O-linkage with no proved biological function[133].

The following diagram (Figure-3.11) explains the primary structural features of EPO indicating number and sequence of amino acids and their linkage with sugar chains.

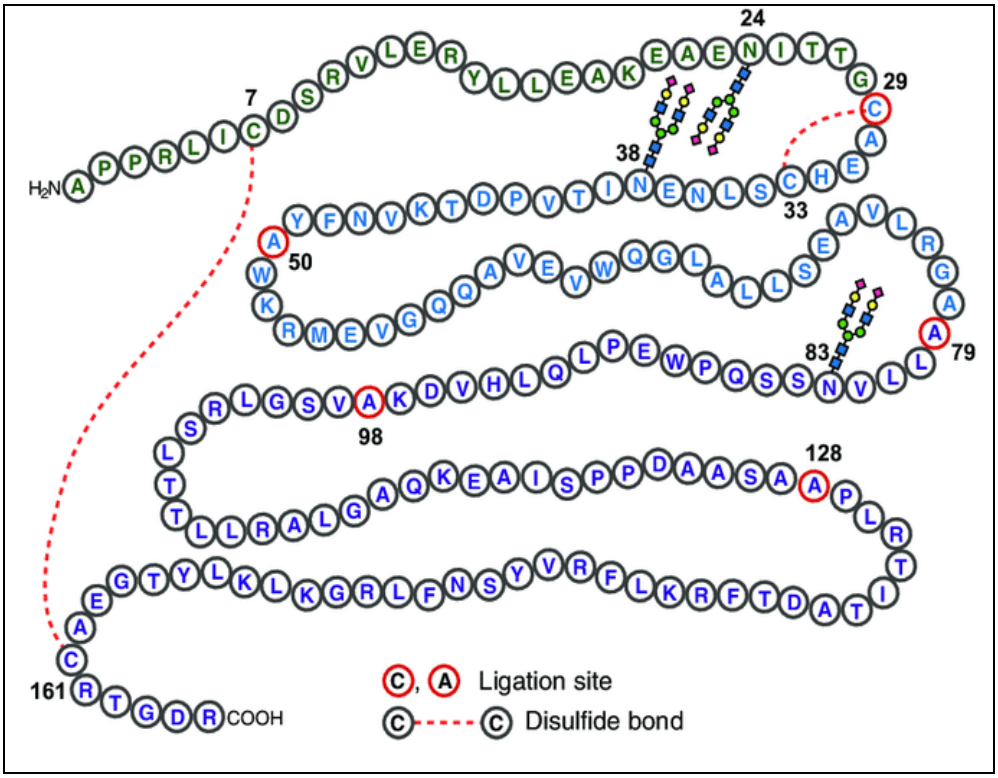



Figure-3.11**:** Primary structure of EPO showing the amino acid sequence, glycosylation sites, ligation sites (red circle), and disulfide bonds (red dotted line).

(Source: Figure on Research Gate. Available from: https://www.researchgate.net/ figure/ Structure-of-the-EPO-glycoforms-and-sialyloligosaccharide-A-Primary-structure-of-EPO -2_fig1_290649272)

The renal cortex contains peritubular fibroblast-like cells from where endogenous EPO originates after birth, but in fetus, EPO originates predominantly from liver hepatocytes[134, 135]. However, the timing of shifting from fetal liver to kidney as a source of EPO after birth depends on species[136, 137]. In general, the ratio between kidney and liver EPO levels in adults during normal erythropoiesis is 9:1[138]. In adults, other than kidney and liver, EPO mRNA expression has been noticed to occur in brain, where EPO probably function as neutrotrophic and neuroprotective[139].

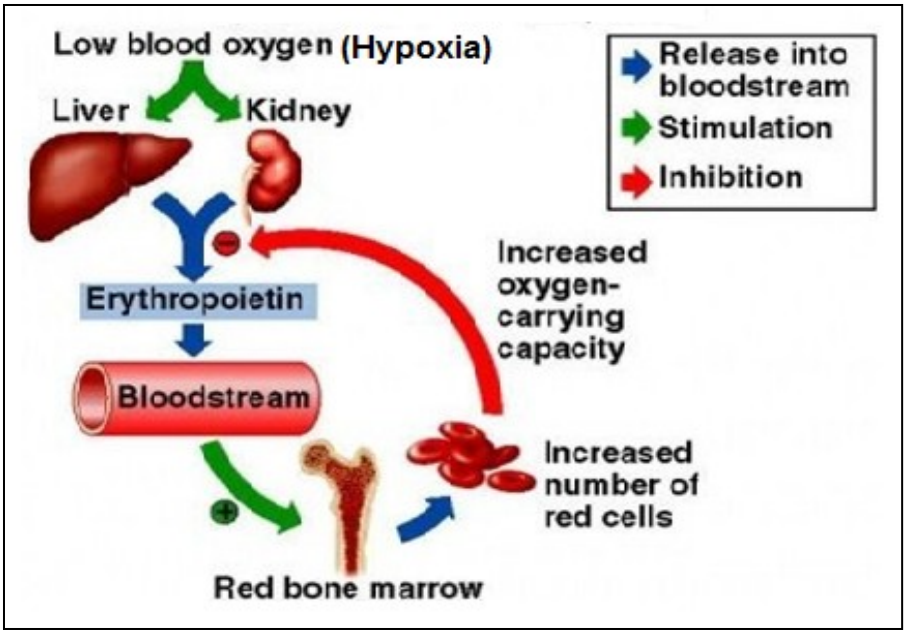



Figure-3.12: Hypoxia stimulated release of EPO

(Source: Erslev AJ. Erythropoietin. *N Engl J Med* 1991; 324(19):1339-44)

It is widely accepted that, the stimulus for augmented EPO gene transcription is tissue hypoxia mediated by HIF-1α transcription factor (Figure-3.9) indicating HIF-1α is the major regulator of EPO production[140]. It has been estimated that, in person with proper kidney function, more than 1000 fold increase in EPO values in circulation may be resulted by hypoxia[141]. Furthermore, liver can contribute significant amount of EPO during severe hypoxic stress[142] (Figure-3.12). In human serum, the concentration of EPO is not constant and studies have noted swing in the EPO values, higher at midnight compared to morning[143].

**3.23 EPO receptor (EPOR) and mechanism of action:**

The major target of EPO is erythroid progenitors such as burst forming unit erythroid (BFU-E) found in the bone marrow, where EPO promotes survival of these progenitors by preventing apoptosis. The BFU-E gives rise to many colony forming units erythroid (CFU-E), which are EPO responsive and contains large number of EPOR on their surface and in addition an important transcription factor required for RBC development that is GATA-1 is also present in CFU-E[144].

The mature EPOR is a 484 amino acid containing, 52KDa glycoprotein found on the surface of the erythroid progenitors of bone marrow. It belongs to cytokine class-I super family. Generally it is a dimer having single hydrophobic transmembrane sequence with cytoplasmic and extracellular domains[145].

After binding to ligand, the EPOR changes its homodimerized state resulting in the autophoshorylation of Janus kinase-2 (JAK-2) and subsequent activation of many signal transduction pathway as depicted in the Figure-3.13, leading to well defined physiological responses which promotes cell proliferation, differentiation and survival of erythroid progenitors[146-148].

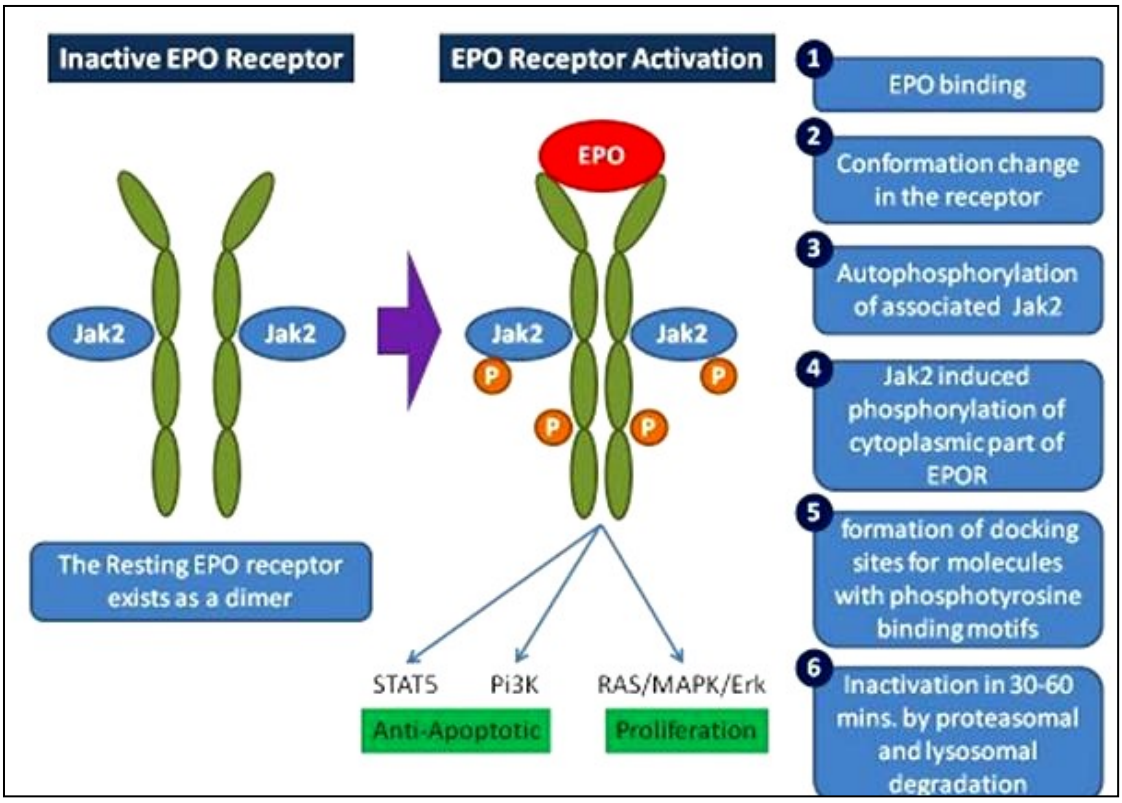



Figure-3.13: EPO/EPOR mediated signal transduction pathway and physiological responses.
(Source:https://allaboutblood.files.wordpress.com/2011/12/122311_0205_theerythrop21.jpg)

It has proved from in vitro studies that, the termination or returning of EPO mediated signal transduction mechanism to basal level takes about 30-60 minutes and dephosphorylation of receptor results in the internalization of EPO-EPOR complex[149].

**3.24 Erythropoietin (EPO) and chronic diseases like pulmonary tuberculosis (PTB):**

Chronic diseases like infectious disease, inflammatory conditions cancer, autoimmune disorder and chronic kidney disease are characterized by the presence of systemic illness and inflammation where anemia is a common finding and is termed as anemia of chronic disease (ACD) [150].

Many studies have documented occurrence of anemia in chronic disease like PTB[151-153]. Despite of increased EPO levels than controls, few studies have demonstrated relative deficiency of serum EPO concentration with respect to hemoglobin level in PTB patients [51, 151, 154].

In addition, studies have also showed in chronic diseases that, increased levels of inflammatory cytokine TNF-α inhibits erythropoietin production and interferes with iron metabolism resulting in the development of ACD[155-158].

Taken together, one can assume that, the inflammatory cytokine TNF-α increases in PTB which might inhibit the production of EPO leading to ACD like PTB. There are multiple reasons for the development of anemia in chronic diseases. However, TNF-α and other cytokines probably play predominant role in the pathophysiology of ACD[159].

In PTB, the invading bacilli activate immune cells and as a host response TNF-α and other cytokines are produced[52]. These cytokines in order to with hold the iron from being used by pathogens increases the synthesis of ferritin and facilitates the macrophagial iron storage and retention resulting in the functional hypoferremia. Moreover in kidney, TNF-α and IFN-γ interfere with EPO gene expression and results in the subsequent relative inadequacy of EPO in proportion to hemoglobin concentration.

Thus the combined effects resulted in the defective erythropoiesis and further development of anemia in chronic diseases[52, 160].

Studies have suggested that in erythroid cells, TNF-α along with other cytokines slow down the EPO induced signaling cascade and blocks the expression of specific transcriptional factors required for the erythrocyte differentiation control and directs the apoptosis of erythroid progenitor cells[161]. TNF-α and IFN-γ also affects the proliferation and differentiation of BFU-E and CFU-E[162]. Evidence from a recent study also indicated that TNF-α can inhibit the expression of EPOR proteins leading to EPO resistance in chronic diseases[163].

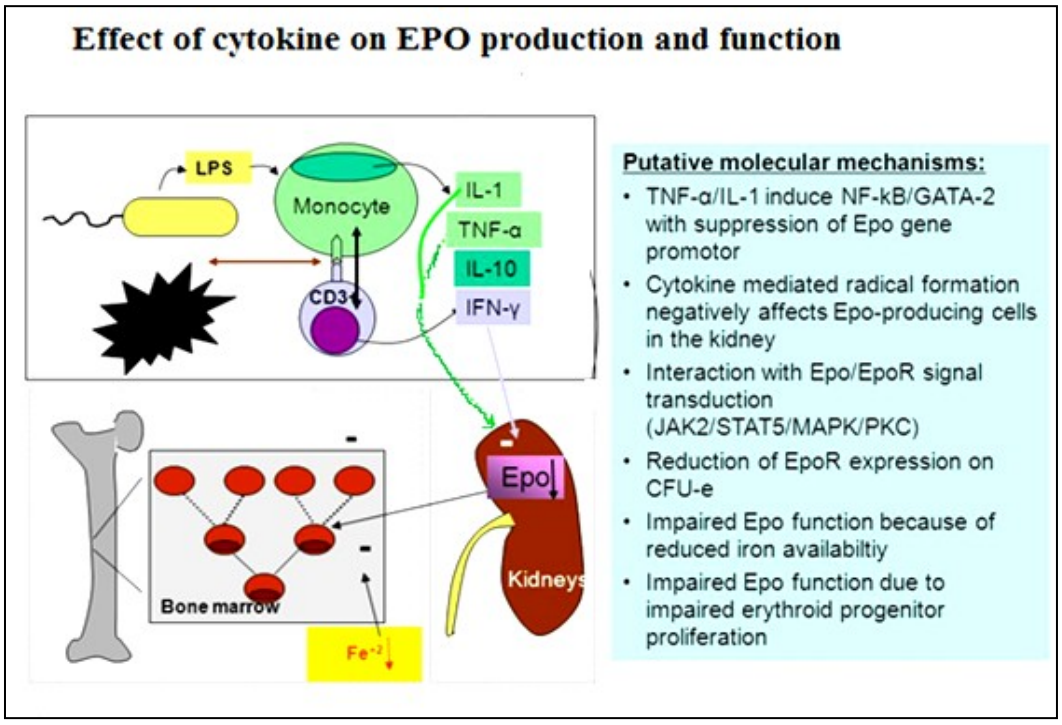



Figure-3.14: Inhibitory effect of TNF-α and other cytokines on EPO production.
(Source: Weiss G, Goodnough LT, *N Engl J Med* 2005; 352:1011-23)

Another important mechanism which leads to inappropriate synthesis of EPO during inflammatory condition is the cytokine mediated induction of free radicals. The main targets of these free radicals are peritubular cells, the major EPO producing cells of

kidney. The alteration in the binding affinities of EPO-inducing transcription factor was found to be mediated by TNF-α, IFN-γ and IL-1 through the production of free radicals[164, 165]. The Figure-3.14 illustrates the effect of cytokine, predominantly TNF-α on EPO production.

Observations from Ebrahim et al. showed comparatively high EPO values in iron deficient anemic patients than anemic PTB patients[51]. Similarly, another study observed the decreased serum EPO in anemia patients with chronic infection or malignancy compared to anemia patients with primary hematopoietic disease or iron deficiency suggesting the specific role of cytokines in the suppression of EPO and thus establishment of anemia in chronic diseases[166].

Thus from above literature survey it is well understood that, in fact there are many reasons in the development of ACD, but involvement of TNF-α seems to be the major causative factor in the pathophysiology of anemia in PTB patients by restricting EPO production and action.

**3.25 Tumor necrosis factor –alpha (TNF-α):**

Cytokines are small proteins including interferons, interleukins, chemokines, lymphokines, Tumor necrosis factor –alpha (TNF-α) and few growth factors. They are involved in cell signaling (autocrine, paracrine or endocrine signaling) by acting as mediator of intracellular communicator in immune system. Their immunomodulating potency has shown to be important in health and disease. Cytokines are produced by macrophages, B and T lymphocytes, mast cells, dendritic cells, endothelial cells, fibroblasts and stromal cells in response to infection, inflammation, tissue injury, trauma and many more[167, 168].

TNF-α is an important inflammatory cytokine produced mainly by macrophages, T and B lymphocytes, mast cells, dendritic cells, endothelial cells and natural killer cells[169, 170]. TNF-α belongs to tumor necrosis factor super family (TNFSF) and their corresponding receptors belongs to tumor necrosis factor receptor super family

(TNFRSF) which are found in variety of tissues and cells. The existence of both TNF and their receptors are critical because of their predominant role in embryonic development, immunity both cellular and adaptive and maintenance of cellular physiology[171, 172]. Now it has been postulated that there are 18 members in TNFSF and 28 members in TNFRSF[173].

Initially, TNF-α was discovered as anti-tumor activity product from bacterial endotoxin in 1975 which in tumor induced mice resulted in the necrosis of tumor and further killing of transformed cell lines[174]. Later research exposed its molecular characterization followed by purification and molecular cloning[175].

TNF-α exists in two forms, soluble and transmembrane forms. Generally it is produced as 212 amino acid containing transmembrane proteins which on proteolytic cleavage by TNF-α converting enzyme produces soluble homotrimeric form of TNF-α[176]. Soluble form of TNF-α can bind and activate TNF receptor-1 (TNFR-1) found in most tissues and stimulate apoptosis and proliferation. The transmembrane form TNF-α can bind and activate TNF receptor-2 (TNFR-2) found exclusively in immune cells and stimulate only proliferation[177].

**3.26 Tumor necrosis factor –alpha (TNF-α) in pulmonary tuberculosis (PTB):**

On encounter with MTB infection, the inflammatory cells such as polymorphonuclear neutrophils, macrophages, dendritic cells (DC), T and B lymphocytes, are direted to the primary site of infection mediated and regulated by TNF-α along with IFN-γ[178]. In MTB infection TNF-α activates macrophages and along with IFN-γ induces the production of reactive oxygen and nitrogen species which are antimycobacterial[179].

Some of the actively replicating mycobacterial components such as a 30 KDa antigen (Ag85B) and lipoarabinomannan have been implicated in the induction of TNF-α in the host[180]. Few studies have also indicated about cellular hypoxia seen during

bacterial infection can induce increased expression of TNF-α through the mediation of HIF-1α[75].

TNF-α is a key molecule performing many important functions during MTB infection including granuloma formation, DC maturation, activation of macrophages, release of chemokines, apoptosis of macrophages and along with IFN-γ, activation of phagocytosis[181-183] as represented schematically in the Figure-3.15.

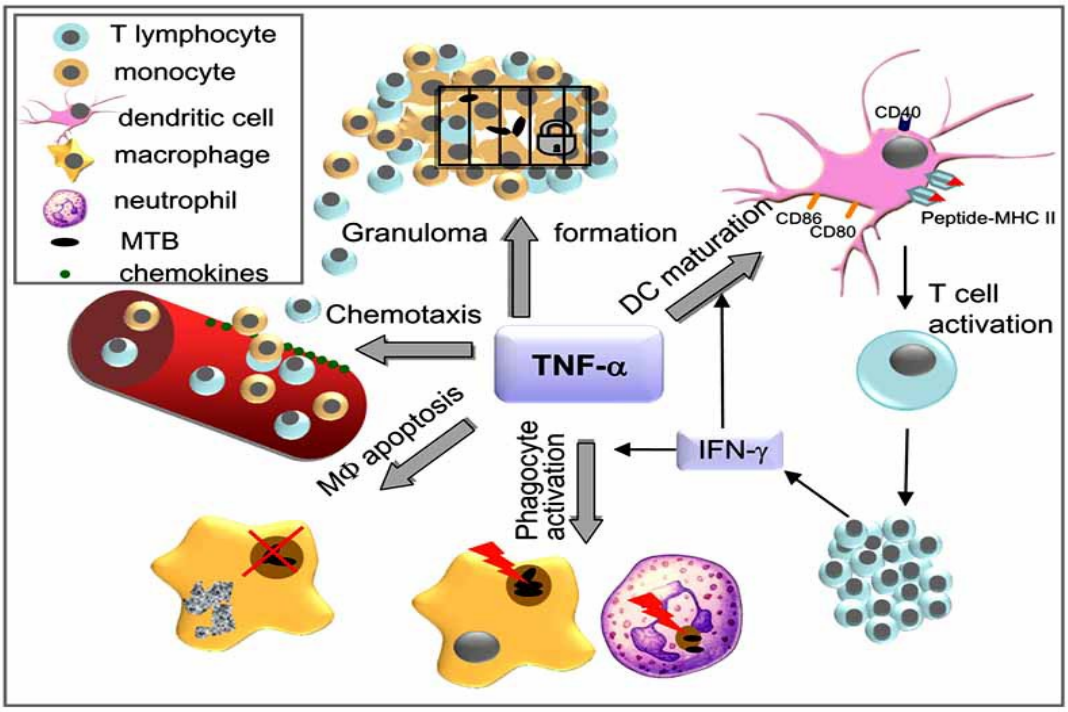



Figure-3.15: Multiple roles played by TNF-α in PTB.
(Source: TNF-alpha in tuberculosis: a cytokine with a split personality - Scientific Figure on Research Gate. Available from: https://www.researchgate.net/figure/Fig-1-Schematic-representation-of-multiple-roles-of-TNF-in-immune-response-to-MTB_fig1_24190887)

Previous studies have revealed that, TNF-α increase early in MTB infection and promote the migration of variety of immune cells to the infection foci resulting in the formation of complex structured granuloma[40]. Probably, the formation granuloma is a defensive mechanism of host to constraint TB bacilli and thereby restricting their multiplication and controlling disease progression[184].

It has been demonstrated from an animal experiment infected with MTB that, TNF-neutralization caused the disorganization of granuloma and subsequent increase in bacillary load and reactivation of TB[185] (Figure-3.16). In a similar kind of study in humans, administration of TNF-α antagonists for rheumatoid disease treatment resulted in the reactivation of TB indicating the determining role of TNF-α in granuloma formation in PTB[186].

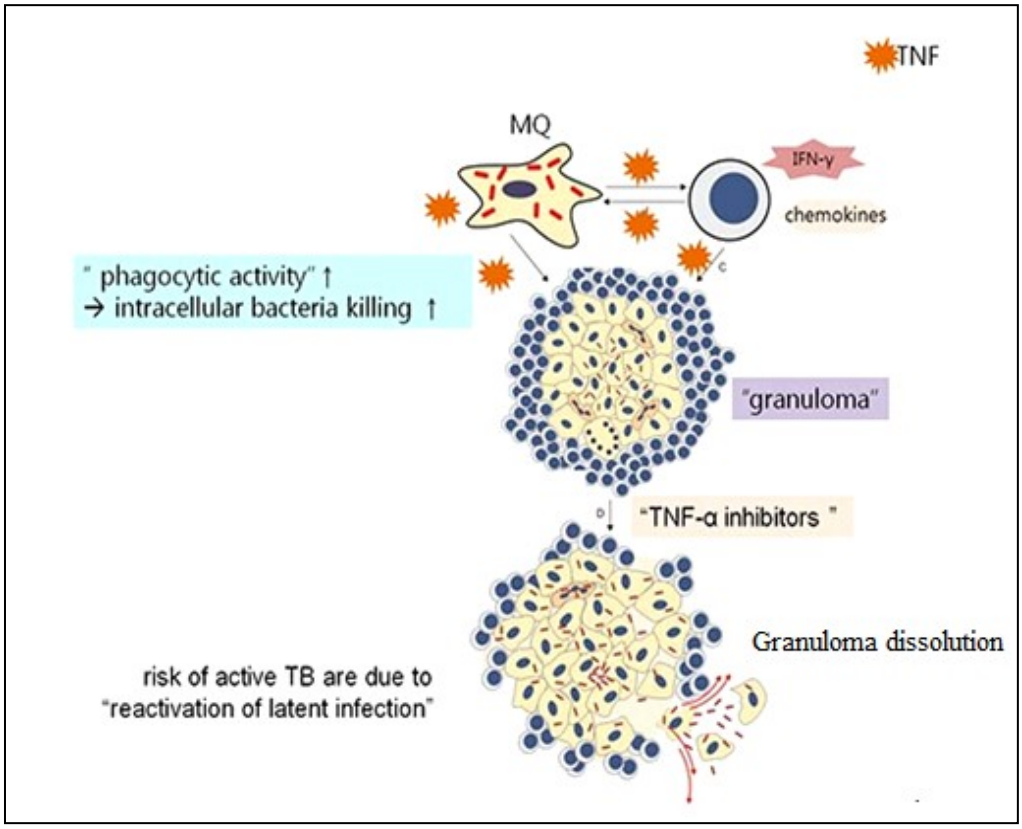



Figure-3.16: TNF-α in formation, granuloma dissolution and TB reactivation granuloma. (Source: *Eur Resp J* 2010; 36:1185-06)

There were reports from earlier studies that expression of TNF-α was also occurred in LTBI and further during the course of TB disease, TNF-α showed direct association with clinical severity[39]. Many studies have recorded increased levels of serum TNF-α in PTB patients and in continuation, they have also noted decreasing trend of serum TNF-α in patients after effective anti-tuberculous treatment (ATT) indicating its important role in disease progression and therapeutic response[187, 188].

The expression of TNF-α should be controlled because excess formation may lead to necrosis, increased inflammation and tissue damage. Probably, few typical symptoms of TB such as anorexia, weight loss and fever may be associated with immunopathological effect of over production of TNF-α[189, 190].

Thus, on one side it looks likeTNF-α is essential for host response against MTB, but on the other side causes tissue destruction, if produced in excess.

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# CHAPTER 4

# MATERIAL AND METHODS

**4.1 Study design:**

A laboratory based cross sectional study of symptomatic cases of PTB.

**4.2 Study duration:**

Two years (Dec 2015- Dec 2017)

**4.3 Source of data:**

The study included, clinically suspected PTB cases attending out-patient/in-patient Department of Pulmonary Medicine, Navodaya Medical College Hospital and Research Centre, Raichur, Karnataka.

**4.4 Sample size:**

197 newly diagnosed sputum positive PTB patients (age range 19-75 years) as cases and age and sex matched 100 apparently healthy individuals among hospital staff as control group were included in the present study

**4.5 Calculation of sample size:**

$$\mathbf{n=4pq/L^2}$$

Where, p= prevalence rate

q=100--p

L= permissible error in the estimate of p

$$\text{Prevalence} = \frac{\text{Persons with given health indicator (confirmed PTB) during the specified time period}}{\text{Population (suspected PTB) during same time period}}$$

In Raichur, clinically suspected PTB cases (in 2014) = 4837

Diagnosed cases (sputum positive cases) = 714

$$p = \frac{714 \times 100}{4837} = 14.76$$

p =14.76, q = 85.24

L= 10% of P, 10×14.76/100 = 1.476

So, n = 4×14.76×85.24/2.1785 = 2310

Approximately 2300 samples from clinically suspected cases of PTB were screened and clinically diagnosed by pulmonologist and PTB was confirmed by microscopic examination of sputum specimen for the detection of acid fast bacilli (AFB). Confirmed cases (197) were further evaluated.

**4.6 Ethical clearance:**

The present study protocol was approved by ethical committee of both the institutes (BLDE (DU) - IEC Ref No-121/2015-16 and NMC -IEC Ref No-59/2015-16). Before enrolment, written informed consent was taken from all the study participants after explaining in detail about study procedure. The study protocol was carried out in accordance with the principle of declaration of Helsinki.

**4.7 Inclusion and exclusion criteria:**

**4.7.1 Inclusion criteria:**

The subjects diagnosed as "new cases" of PTB, possessing at least two sputum smear test positive for AFB were included as cases and the healthy individuals with no previous history of any major diseases were included as controls.

**4.7.2 Exclusion criteria:**

The PTB patients with following conditions were excluded from the study.

- Age <19 years and >75 years
- Patients not willing to give consent
- The patients with extra pulmonary TB and/or patients requiring surgical intervention
- Patients with history of prior anti TB treatment
- Patients with other lung disorders
- Patients with HIV
- With organ transplantation
- Treatment with corticosteroids
- With chronic renal failure
- Diabetes mellitus
- Liver failure and patient with recent myocardial infarction.

**4.8 Study protocol:**

- All the study subjects were interviewed using a structured questionnaire format which was relevant to our study.
- Recording of clinical history and major health complaints was carried out with the help of Pulmonologist.
- Study subjects were asked to provide any predisposing factors related to PTB.
- BMI, WHR, blood pressure and pulse rate were recorded from cases and controls.
- Suspected PTB cases were screened for the detection of sputum AFB by microscopy.
- Blood samples were collected from sputum positive PTB cases and controls.
- Samples were further analyzed for hematological parameters and oxygen sensing molecular markers such as serum VEGF, EPO and TNF-α.

**4.9 Measurement of physical anthropometry parameters:**

**4.9.1 Measurement body mass index (BMI):**

All the study participants were weighed barefoot with minimum clothing using an electronic weighing machine. Body weight was recorded to the nearest of 0.1kg. By using standard measuring tape, height was noted to the nearest of 0.1cm. BMI was calculated by the formula, BMI=$kg/m^2$, where kg is weight in kilogram and $m^2$ is height in meter squared. The cases and controls were classified based on BMI ($kg/m^2$) as per WHO criteria[1].

**4.9.2 Measurement waist to hip ratio (WHR):**

It was calculated by measuring waist at narrowest point under lowest rib and hips at the widest portion of buttocks using a standard measuring tape and the ratio was obtained by dividing the waist circumference (in cm) by hip circumference (in cm).

## 4.10 AFB sputum smear preparation and microscopy[2, 3]:

**Specimen:**

Two sputum samples, one in the early morning without any food and another spot sample were collected in a properly labeled sterile container from the suspected PTB patients.

**Decontamination and concentration of sputum samples by Petroff's method:**

Sputum was incubated with an equal volume of 4% sodium hydroxide solution at 37°C for 20 minutes with continues shaking till it becomes clear followed by centrifugation for 20 minutes at 3000rpm and the sediments formed were neutralized with 0.1normal hydrochloric acid and used for smear preparation.

**Staining by Ziehl-Neelsen (Z-N) technique:**

Thick purulent part of the sputum was used for the preparation of smears which were air dried, heat fixed and stained by Z-N technique. The smear was run with strong carbol fuchsin and gently heated to steaming for 5-7 minutes (care should be taken not to boil and dry the stain). Then the slide was washed with water and decolourised with 20% sulfuric acid until no more stains comes off followed by 95% ethanol for two minutes. Later the smear was counterstained by using Loeffler's methylene blue and observed under oil immersion objective for the presence of bacilli. The smears were graded according to the RNTCP recommendations in India[4, 5] as shown in the Table-4.1.

Table-4.1: AFB sputum smear grading

| Number of bacilli | Result | Grading |
|---|---|---|
| >10/field | +ve | 4+ |
| 1-10/field | +ve | 3+ |
| 1-9/10 fields | +ve | 2+ |
| 10-99/100 fields | +ve | 1+ |
| 1-9/100fields | +ve | Scanty |
| No AFB in 100 fields | -ve | --- |

### 4.11 Collection of blood samples for the analysis of hematological and oxygen sensing molecular markers:

About 6ml of venous blood sample were drawn from median cubital vein under aseptic condition from both PTB cases and healthy controls included in the study. Out of 6 ml, one part of blood sample (2ml) was collected in an EDTA vial. The second part of blood sample collected in plain vial were allowed to clot for 30 minutes and then centrifuged at 3000 rpm for 10 minutes. The separated serum samples were stored at -80°C until assayed.

#### 4.11.1 Estimation of hematological parameters:

The blood sample collected in the EDTA vial were immediately processed for hematological parameters which includes complete blood picture counts with blood indices using automated cell counter (ABX Pentra 60, Horiba, Japan) by flow cytometry method. The erythrocyte sedimentation rate (ESR) was measured by Westergren method[6].

#### 4.11.2 Estimation of oxygen sensing molecular markers:

The separated serum samples were subjected to estimation of oxygen sensing molecular markers of the study such as VEGF, EPO and TNF-α in all the study participants by sandwich enzyme linked immunosorbent assay (ELISA) method using commercially available ELISA kits according to manufacturers' instructions.

The assay procedure was carried out by using the instruments such as ELISA reader (microplate reader) and automated ELISA washer (microplate washer). In the present study we used BIO RAD microplate washer (Model: PW 40, Figure-4.1) and BIO RAD microplate reader (Model: 680, Figure-4.2) to perform all the ELISA procedures.


Figure-4.1: Microplate washer (ELISA washer)


Figure-4.2: Microplate reader (ELISA reader)

### 4.11.2.1 Estimation serum VEGF:

Serum VEGF was estimated by commercially available RayBio human VEGF ELISA kit (RayBiotech, Norcross, GA) [7]. This kit is used for the quantitative estimation of human VEGF in serum, plasma and cell culture supernatants.

**Principle:**

Principle is based on the sandwich method. A polyclonal antibody specific for human VEGF has been coated onto the wells of the microtiter strips. Samples including standards and patient serum are pipetted in to these wells and the VEGF antigen present in the sample binds to the immobilized antibody. After washing, a biotinylated monoclonal antibody specific for VEGF is added. During the second incubation, this antibody binds to the immobilized VEGF captured during the first incubation.

After removal of excess second antibody, streptavidin–peroxidase is added which binds to the biotinylated antibody to complete the four member sandwich. The wells are again washed and TMB substrate solution is added, which reacts with bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of human VEGF in the sample, which is measured at 450nm.


Figure-4.3: VEGF ELISA kit and processed microplate

**VEGF kit components and reconstitution:**

All the reagents and microtiter strip plate were supplied as kit components along with VEGF ELISA kit. Out of these, few components were ready to use and few reagents require prior reconstitution before use. The reagents were reconstituted as per the instructions of user manual provided along with the kit. All the kit components and reconstitution procedure were described in the following table.

Table-4.2: VEGF ELISA kit components and reconstitution

| Components | Reconstitution |
|---|---|
| 96 wells microtiter strip plate | Ready to use (pre-coated). |
| VEGF standard | Reconstitution and preparation of serial standards were described below. |
| 20X Wash buffer | 25 ml made up to 500 ml with deionized water to get 1X working wash buffer. |
| Assay diluent A | Ready to use. |
| Assay diluent B | 15 ml made up to 75 ml with deionized water to get working assay diluent B. |
| Biotinylated detection antibody | Added 100 µl working assay diluent B to a vial containing detection antibody to get concentrated detection antibody which was further made up to 10 ml with working assay diluent B to produce working biotinylated detection antibody. |
| HRP-Streptavidin concentrate | 40 µl of HRP-Streptavidin concentrate was diluted to 12 ml with working assay diluent B to produce working HRP- streptavidin solution. |
| TMB substrate solution | Ready to use. |
| Stop solution (0.2 M $H_2SO_4$) | Ready to use. |

**Preparation of standards:**

640 µl of assay diluent A was added to a vial containing powdered form of human VEGF standard to get stock standard (50 ng/ml). From this, series of standards to be used in the preparation of standard curve were prepared as mentioned in the following table.

Table-4.3: Preparation of VEGF serial standards by dilution

| Standards | Prepared by adding | Concentration of standards |
|---|---|---|
| Standard-1 | 60 µl of concentrated standard + 440 µl assay diluent A | 6000 pg/ml |
| Standard-2 | 200 µl of standard-1 + 400 µl of assay diluent A | 2000 pg/ml |
| Standard-3 | 200 µl of standard-2 + 400 µl of assay diluent A | 666.7 pg/ml |
| Standard-4 | 200 µl of standard-3 + 400 µl of assay diluentsA | 222.2 pg/ml |
| Standard-5 | 200 µl of standard-4 + 400 µl of assay diluent A | 74.07 pg/ml |
| Standard-6 | 200 µl of standard-5 + 400 µl of assay diluent A | 24.69 pg/ml |
| Standard-7 | 200 µl of standard-6 + 400 µl of assay diluent A | 8.23 pg/ml |
| Zero- standard | 400 µl of assay diluent A | 0 pg/ml |

**Assay procedure:**

- Required number of strips was selected from microtitrer plate.
- 100 µl of standard and sample were added to appropriate wells.
- Wells were covered and incubated for two and half hours at room temperature.
- Washed the plate four times with wash buffer using auto washer.

- 100 µl of prepared biotinylated antibody was added to each well and incubated for one hour at room temperature.
- Washed the plate four times with wash buffer using auto washer.
- 100 µl of prepared streptavidin solution was added to each well and incubated for forty five minutes at room temperature.
- Washed the plate four times with wash buffer using auto washer.
- 100 µl of tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature in dark for thirty minutes.
- After adding 50 µl of stop solution optical density (OD) was recorded immediately at 450 nanometer (nm) using ELISA reader.
- From the OD values standard graph was plotted and the concentration of serum VEGF was calculated from the standard graph.

**4.11.2.2 Estimation serum EPO:**

Concentration of serum EPO was estimated by commercially available human EPO ELISA kit (Biomerica, USA) [8]. This kit is intended for the quantitative measurement of human serum EPO.

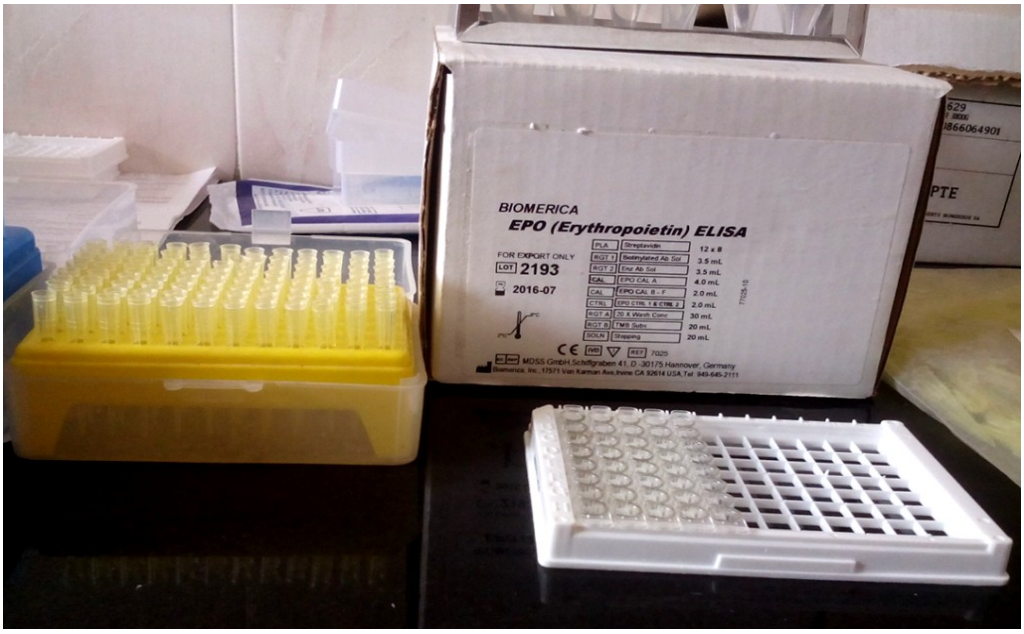


Figure-4.4: EPO ELISA kit

**Principle:**

The principle is based on the double antibody sandwich method. Here standards, controls and patients serum are simultaneously incubated with the biotin coupled mouse anti EPO monoclonal antibody and enzyme labeled mouse anti EPO monoclonal antibody in a streptavidin pre-coated micro plate wells. Excess conjugate is removed by washing. A chromogen is added to the wells and is oxidized by the enzyme reaction to form blue coloured complex. The reaction is stopped by the addition of stop solution, when the blue colour turns to yellow. The amount of colour generated is directly proportional to the amount of EPO in the sample. The absorbance is measured at 450 nm.

**EPO kit components and reconstitution:**

Kit components and reconstitution of provided reagents are explained in the following table.

Table-4.4: EPO ELISA kit components and reconstitution

| Components | Reconstitution |
|---|---|
| 96 wells microtiter strip plate | Ready to use (pre-coated with streptavidin) |
| EPO standard | Reconstitution and preparation were described below |
| EPO controls 1 and 2 | 2 ml deionized water to each |
| Biotinylated EPO antibody | Ready to use |
| Enzyme labeled EPO antibody | Ready to use |
| Concentrated wash buffer | 30 ml made up to 500 ml with deionized water to get working wash buffer |
| TMB substrate | Ready to use |
| Stop solution (1 N $H_2SO_4$) | Ready to use |

**Standard preparation:**

Table-4.5: Preparation of EPO standards

| Standards | Volume of deionized water | Concentration of standards |
|---|---|---|
| Standard-1 | 4 ml | 0 mIU/ml |
| Standard-2 | 2 ml | 10.1 mIU/ml |
| Standard-3 | 2 ml | 25.1 mIU/ml |
| Standard-4 | 2 ml | 51 mIU/ml |
| Standard-5 | 2 ml | 158 mIU/ml |
| Standard-6 | 2 ml | 456 mIU/ml |

The EPO calibration curves were prepared from the above standards.

**Assay procedure:**

- Required number of strips was selected from the microtitrer plate.
- 200 µl of standards, controls and samples were pipette into designated wells.
- Added 25 µl biotinylated antibody and followed by 25 µl of enzyme labeled antibody to each well.
- Wells were covered and kept on rotary shaker set at 170±10 rpm (Figure-4.5) for two hours at room temperature.
- Wells were washed five times with working wash buffer using ELISA washer.
- 150 µl of TMB substrate was dispensed to all the wells.
- Plates were coved and kept on shaker for thirty minutes.
- Reaction was stopped by adding 100 µl stop solution to all the wells

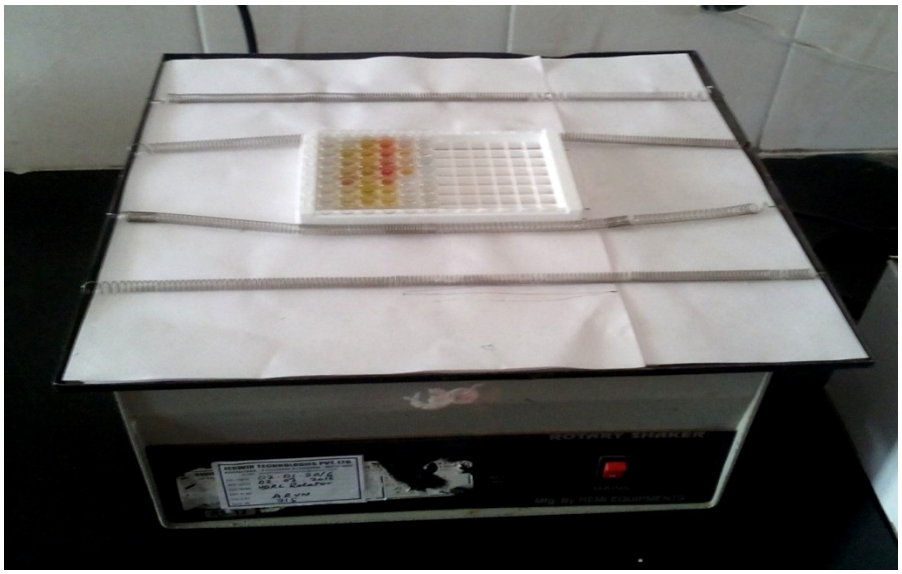

Figure-4.5: Processed EPO microplate incubation on Rotary shaker.

- OD values were recorded at 450 nm using ELISA reader and standard curve was generated by plotting absorbance versus standard concentration and the concentration of unknown specimen was calculated

**4.11.2.3 Estimation serum TNF-α:**

The commercially available human TNF-α ELISA kit (Diaclone, France) [9], was used to determine the concentration of serum TNF-α. This kit measures TNF-α in serum, plasma, cell supernatants or buffered solutions.

**Principle:**

Samples, standards and controls are captured by a solid bound specific antibody present in the precoated wells of microtiter plate. Biotinylated tracer antibody will bind to captured human TNF-α. Streptavidin-horseradish peroxidase conjugate will bind to the biotinylated tracer antibody. The conjugate will react with substrate, tetramethylbenzidine and forms blue colour and the colour intensity is directly proportional to the concentration of TNF-α in the specimen. The enzyme reaction is terminated by adding of stop solution. The OD value at 450nm is measured.

**TNF-α kit components and reconstitution:**

The reagents were reconstituted as per the instructions of user manual provided along with the kit as mentioned in the following table.

Table-4.6: TNF-α ELISA kit components and reconstitution

| Components | Reconstitution |
|---|---|
| 96 wells microtiter strip plate | Ready to use (pre-coated). |
| Standard diluent-serum | Ready to use |
| TNF-α standard | Reconstitution and preparation were described below. |
| TNF-α control | Reconstituted with 1 ml of standard diluent serum. |
| Biotinylated antibody diluent | Ready to use. |
| Biotinylated anti TNF-α antibody | 240 µl diluted to 6.6 ml with biotinylated antibody diluent to get working solution. |
| Concentrated wash buffer | 10 ml made up to 2000 ml with deionized water to get working wash buffer. |
| HRP diluent | Ready to use. |
| Streptavidin-HRP | 0.5 ml of HRP diluent was added to 5 µl of concentrated Streptavidin-HRP. From this 150 µl was further diluted to 10 ml with HRP diluent to get working Streptavidin-HRP solution. |
| TMB substrate | Ready to use. |
| Stop solution (1 N $H_2SO_4$) | Ready to use. |

**Preparation of standards:**

The concentrated standard provided (800 pg/ml) along with the kit were further diluted using standard diluent to get the serial standards as shown in the following table.

Table-4.7: Preparation of TNF-α serial standard by dilution

| **Standards** | **Prepared by adding** | **Concentration of standards** |
|---|---|---|
| Standard-1 | 1 ml of standard diluent to TNF-α standard vial | 800 pg/ml |
| Standard-2 | 100 µl of standard-1 + 100 µl standard diluent | 400 pg/ml |
| Standard-3 | 100 µl of standard-2 + 100 µl standard diluent | 200 pg/ml |
| Standard-4 | 100 µl of standard-3 + 100 µl standard diluent | 100 pg/ml |
| Standard-5 | 100 µl of standard-4 + 100 µl standard diluent | 50 pg/ml |
| Standard-6 | 100 µl of standard-5 + 100 µl standard diluent | 25 pg/ml |
| Zero standard | 100 µl standard diluent | 0 pg/ml |

The above serial standards were used for the preparation of standard curve and further calculation of TNF-α concentration in patient serum samples.


Figure-4.6: TNF-α ELISA kit and processed TNF-α microplate.

**Assay procedure:**

- Required number of strips was selected from the microtitrer plate.
- Pipetted 100µl serum, standard and control to pre assigned wells.
- Added 50 µl of biotinylated anti-TNF-α to all the wells.
- Covered the wells and incubated at room temperature for three hours.
- Wells were washed three times using automated ELISA washer.
- Dispensed 100 µl of Streptavidin- HRP conjugate into each well.
- Covered the wells and incubated at room temperature for thirty minutes.
- All the wells were washed three times using automated ELISA washer.
- Added 100 µl of TMB substrate to all the wells and incubated in dark at room temperature for fifteen minutes after covering the plates.
- Absorbance was recorded at 450 nm after adding 50 µl of stop solution to all the wells.
- A standard curve was prepared by plotting OD value versus the concentration of TNF-α standard. The serum TNF-α concentration was determined from the above curve.

**4.12 Statistical analysis:**

The statistical analysis was performed by using SPSS version 16.0 software. The results were analyzed with descriptive statistics, wherever appropriate and the *p* value of <0.05 was considered threshold of significance. The following tests were used to evaluate the statistical significance in the results.

- The student unpaired t test
- Chi square test
- Pearson's correlation coefficient test
- Spearman's rank correlation coefficient test
- Kruskal-Wallis test
- One way ANOVA with multiple comparison test (Bonferroni test).

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# CHAPTER 5

# RESULTS

**5.1 Anthropometric parameters:**

Table- 5.1**:** Demographic and Anthropometric characteristics of PTB cases and controls

| Characteristics | Controls (n=100) n (%) | Cases (n=197) n (%) | Chi square ($x^2$) / t value | *p* value |
|---|---|---|---|---|
| Age (years) mean±SD | 43.02±15.28 | 44.17±16.01 | t=0.7934 | 0.553* |
| Age groups(years)<br>15-24<br>25-34<br>35-44<br>45-54<br>55-64<br>≥65 | <br>13 (13)<br>15 (15)<br>21 (21)<br>18 (18)<br>20 (20)<br>13 (13) | <br>25 (12.69)<br>33 (16.75)<br>47 (23.85)<br>35 (17.77)<br>31 (15.74)<br>26 (13.20) | $x^2$= 5.634 | 0.405* |
| Sex<br>Male<br>Female | <br>54(54)<br>46(46) | <br>109(55.33)<br>88(44.67) | $x^2$= 3.169 | 0.117* |
| BMI (kg/$m^2$)<br>Severely under weight (<16)<br>Moderate under weight (16-16.9)<br>Mild under weight (17-18.49)<br>Normal (18.5-24.9)<br>Over weight( ≥25) | <br>00<br>00<br>2(2)<br>94(94)<br>4(4) | <br>54(27.41)<br>56(28.43)<br>71(36.04)<br>16(8.12)<br>00(00) | $x^2$= 115.3 | <0.001** |
| Waist hip ratio<br>Male ( mean ±SD)<br>Female (mean ±SD) | <br>0.91±0.03<br>0.86±0.04 | <br>0.82±0.05<br>0.78±0.03 | <br>t=6.9452<br>t=8.6684 | <br><0.001**<br><0.001** |

*$p$ >0.05 not significant, **$p$ <0.001 highly significant.

In the present study, 197 newly diagnosed PTB cases and 100 healthy controls were included. The Table-5.1 shows the demographic and anthropometric characteristics of all the study subjects.

In the present study mean age of PTB cases were 44.17±16.01 (age range 19-75 years) and mean age of controls were 43.02±15.28 (age range 19-72 years). Among 197 PTB cases, 109 (55.33%) cases were males and 88 (44.67%) cases were females and in

100 controls 54 (54%) were males and 46 (46%) were females as shown in Figure-5.1. There were no statistically significant differences between PTB cases and controls regarding mean age, sex and age groups as shown in Table-5.1. Out of 197 PTB cases, 115 (58.4%) cases were from age groups between 25 and 54 years. Among these115 cases, 47 (23.85%) cases constituted age group between 35 and 44 years as represented in Figure-5.2.

Figure-5.1: Percentage of male and female among PTB cases and healthy controls

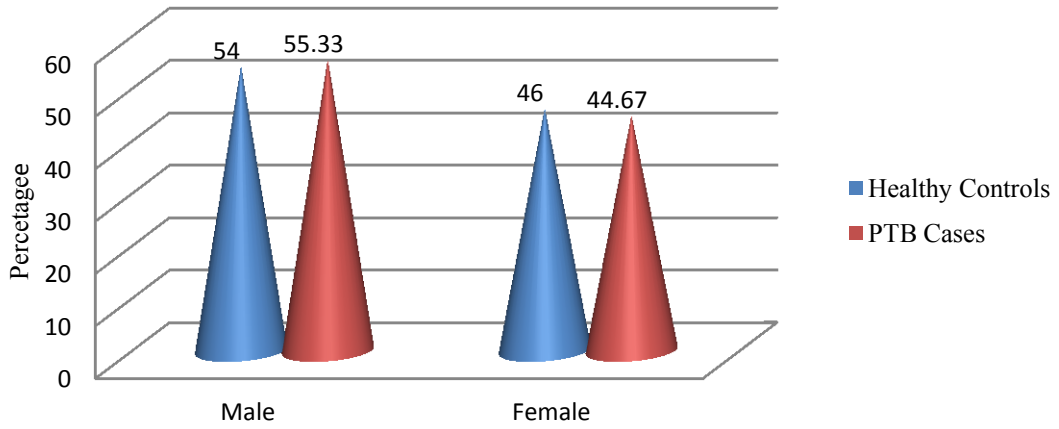
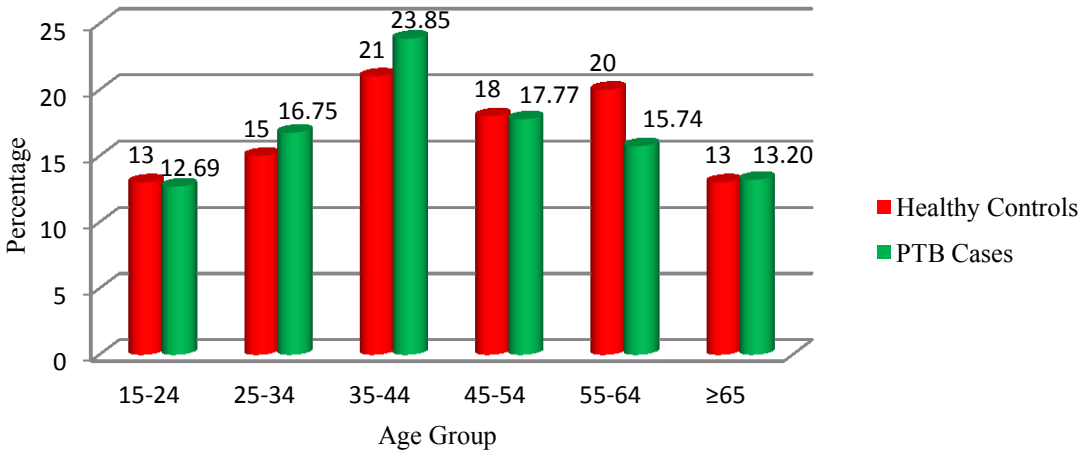



Figure-5.2: Percentage of age group in PTB cases and healthy control



We recorded statistically significant differences in BMI of PTB cases and healthy controls ($p$<0.001). Out of 197 PTB cases, BMI of 181 cases (91.87%) were lower (<18.5kg/$m^2$) than the controls (Table-5.1). Further it was observed that, from these 181 PTB cases, 54 cases (27.41%) were severely underweight (BMI <16.0 kg/$m^2$) indicating a poor nutritional status in PTB. Waist to hip ratio of PTB cases of both sexes was also found to be lower as compared to controls ($p$< 0.001) as displayed in Table-5.1.

**5.2 Hematological parameters:**

Table-5.2: Comparison between mean of different parameters in PTB cases and controls

| Parameters | Control (Mean±SD) n=100 | PTB Cases (Mean±SD) n=197 | $p$ value |
|---|---|---|---|
| VEGF (pg/ml) | 23.4±8.88 | 614.82±354.87 | < 0.001 |
| TNF-α (pg/ml) | 14.4±3.72 | 87.69±41.59 | < 0.001 |
| EPO (mIU/ml) | 17.3±6.60 | 36.08±7.11 | < 0.001 |
| Hb (g/dl) | 14.10±1.21 | 10.40±2.0 | < 0.001 |
| RBC (Millions/cumm) | 4.83±0.49 | 4.07±0.73 | < 0.001 |
| PCV (%) | 41.5±3.40 | 30.7±5.74 | < 0.001 |
| MCV (fL) | 85.5±7.47 | 75.54±9.71 | < 0.001 |
| MCH (pg/cell) | 29.2±2.01 | 25.53±3.89 | < 0.001 |
| MCHC (g/dl) | 34.1±1.21 | 33.50±2.34 | < 0.05 |
| WBC (Cells/cumm) | 6574±1420 | 10074±3770 | < 0.001 |
| Neutrophil (%) | 66.2±8.71 | 71.4±9.70 | < 0.001 |
| Lymphocytes (%) | 25.6±7.28 | 19.0±8.30 | < 0.001 |
| Monocyte (%) | 5.50±1.94 | 6.80±3.20 | < 0.001 |
| Eosinophils (%) | 2.70±1.35 | 2.80±1.60 | > 0.05 |
| Platelets (Lakhs/cumm) | 2.90±0.70 | 4.17±1.41 | < 0.001 |
| ESR (mm/hour) | 5.83±3.34 | 67.0±22.0 | < 0.001 |

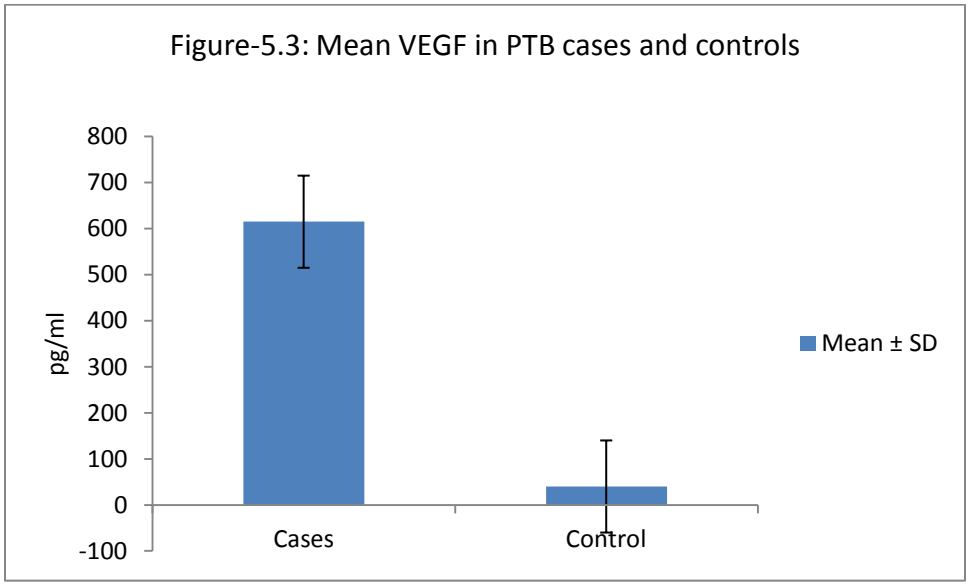
$p$ >0.05 not significant, $p$ <0.05 significant, $p$ <0.001 highly significant.

Table-5.2 shows the comparison between measured parameters in PTB cases and healthy controls and here we used unpaired t test to get the statistical significance. Except in the case of eosinophils count ($p$>0.05), all other parameters showed statistically significant differences between PTB cases and controls.

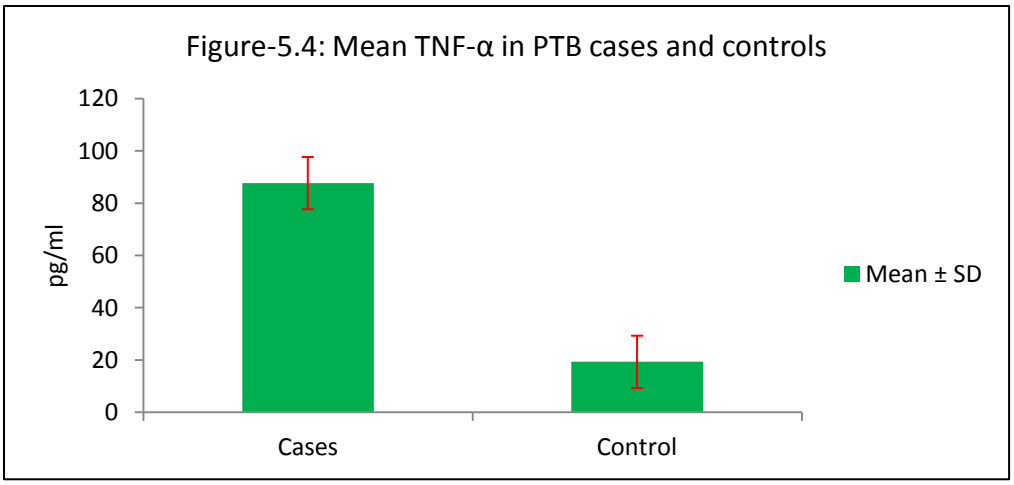
Our results indicated that the mean level of hemoglobin (Hb), red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were lower while white blood cells (WBC), neutrophils, lymphocytes, monocytes, platelets and erythrocyte sedimentation rate (ESR) were found to be higher in PTB cases compared to healthy controls ($p$< 0.05) as indicated in Table-5.2.

**5.3 Oxygen sensing molecular markers:**

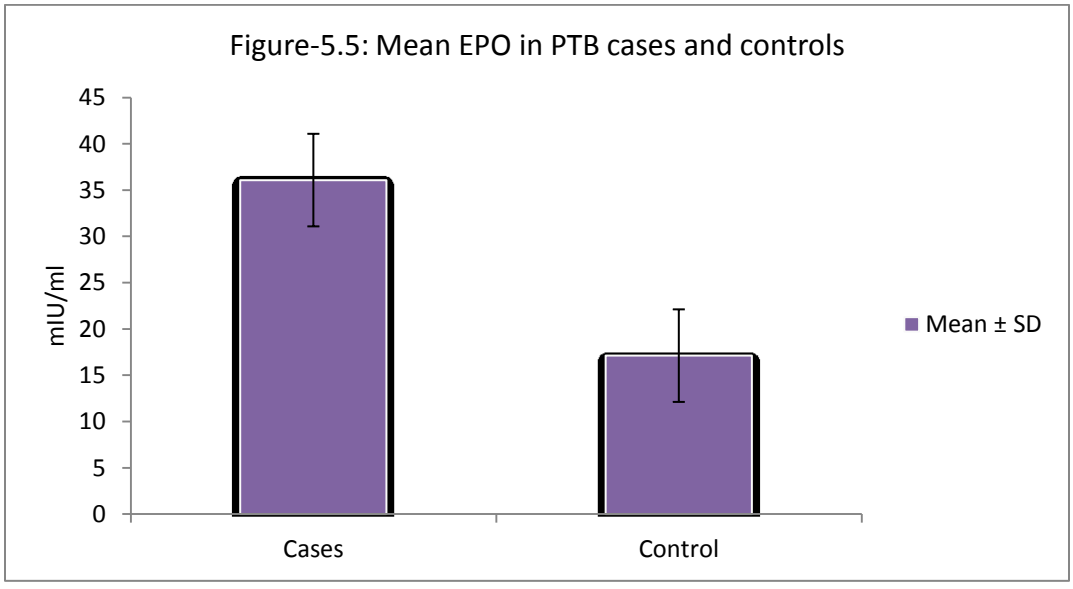
In the present study we measured oxygen sensing molecular markers such as serum VEGF, TNF-α and EPO in all the study participants. We noted huge differences in the values of serum VEGF between PTB cases and healthy controls as shown in Table-5.2 and Figure-5.3. The mean serum levels of VEGF (614.82±354.87 pg/ml) in PTB cases were higher than controls (23.4±8.88 pg/ml) and the difference was statistically significant ($p$< 0.001) indicated by unpaired t test.



Similar findings were observed in the serum levels of inflammatory marker like TNF-α between PTB cases and controls. The mean TNF-α level in PTB cases (87.69±41.59 pg/ml) and controls (14.4±3.72 pg/ml) showed statistically significant differences ($p< 0.001$) as indicated in Table-5.2 and Figure-5.4.




Our study reported slightly increased values of serum EPO in PTB cases compared to controls as illustrated in the Table-5.2 and Figure-5.5. The mean values of EPO in PTB cases and controls were 36.08±7.11 mIU/ml and 17.3±6.60 mIU/ml respectively and were found to be statistically significant ($p< 0.001$).



**5.4 Correlation between serum VEGF and TNF-α, serum VEGF and EPO and also between serum TNF-α and EPO in PTB patients:**

In the present study we evaluated correlation among oxygen sensing molecular markers in PTB. We applied Pearson's correlation coefficient analysis to evaluate the association between serum VEGF and TNF-α, serum VEGF and EPO and also between serum TNF-α and EPO in PTB patients. We found strong positive correlation between VEGF and TNF-α which was statistically significant (r = 0.813, $p< 0.001$) as shown in Figure-5.6. Between serum VEGF and EPO in PTB cases, the correlation observed (Figure-5.7) was not statistically significant (r = -0.115, $p> 0.05$). Figure-5.8 indicates statistically significant negative correlation between serum TNF-α and EPO (r = -0.294, $p< 0.001$) in PTB patients.

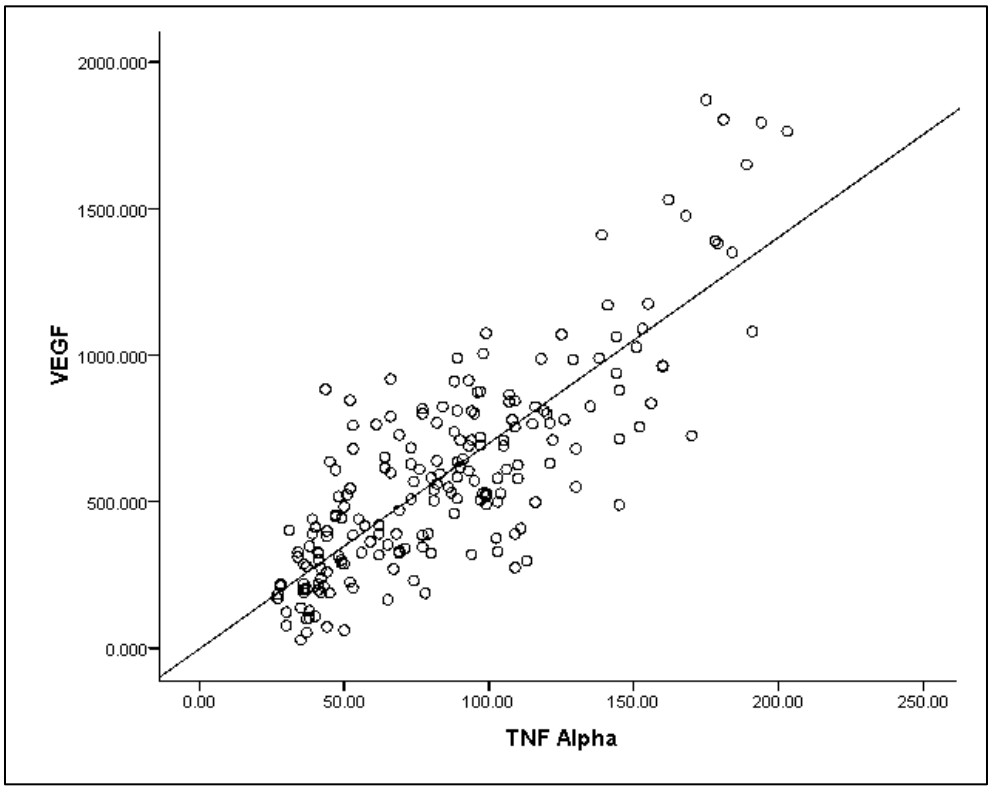



Figure-5.6: Correlation between serum VEGF (pg/ml) and TNF-α (pg/ml) in PTB patients (r = 0.813, $p< 0.001$)

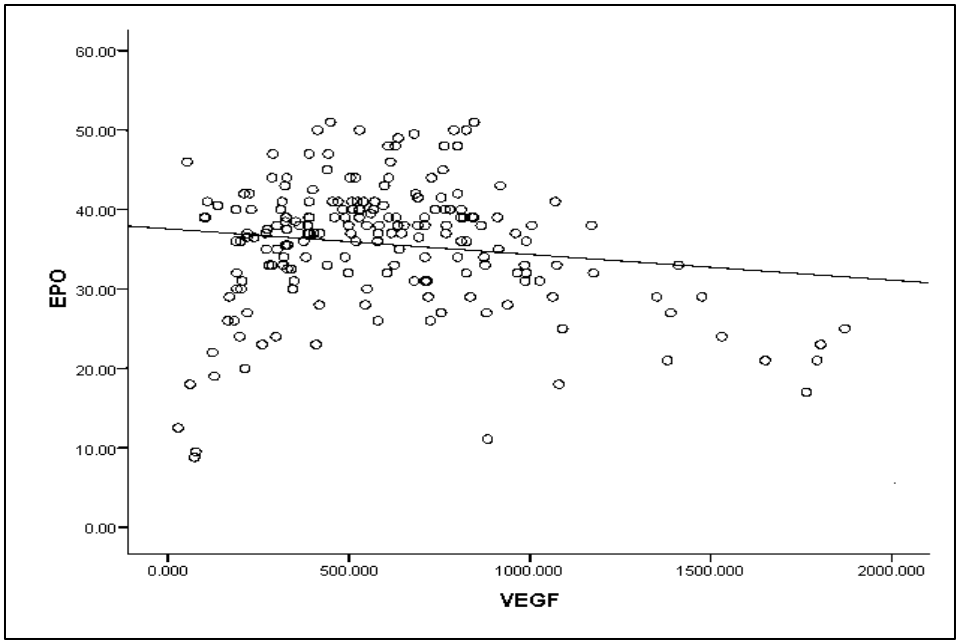



Figure-5.7: Correlation between serum VEGF (pg/ml) and EPO (mIU/ml) in PTB patients (r = -0.115, $p$> 0.05)

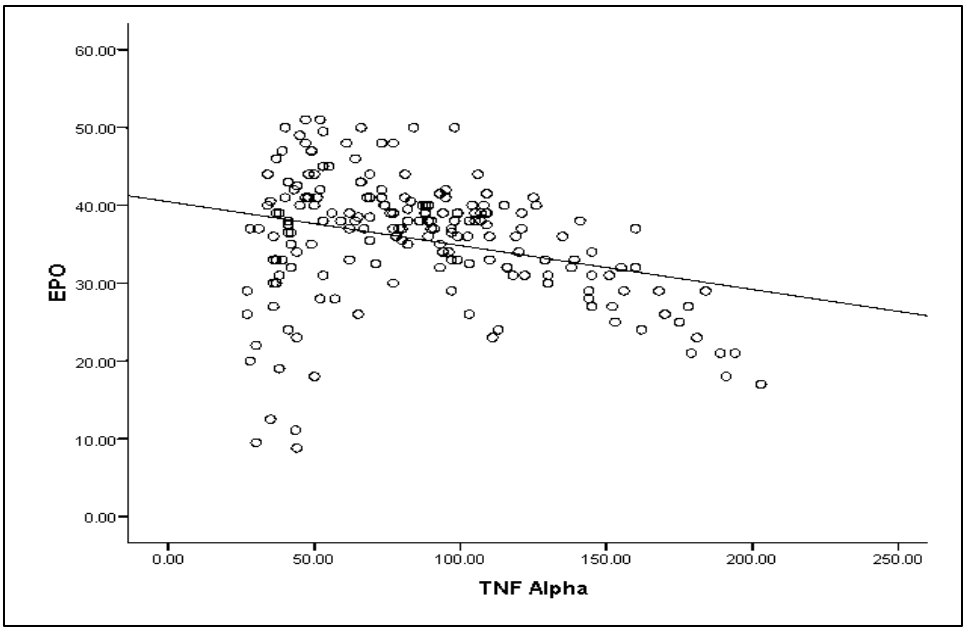



Figure-5.8: Correlation between serum TNF-α (pg/ml) and EPO (mIU/ml) in PTB patients (r = -0.294, $p$< 0.001)

**5.5 Distribution of PTB cases based on bacterial burden:**

In the present study, 2300 clinically suspected PTB patients were subjected to direct sputum smear microscopy for the detection of AFB and among them, 197 cases found to be positive for AFB and were distributed according to bacterial burden measured in terms of AFB grading. Out of 197 sputum positive PTB cases 8% were scanty, 25% were 1+ , 22% were 2+, 38% were 3+ and 7% were belongs to 4+ AFB grading as depicted in the Figure-5.9.

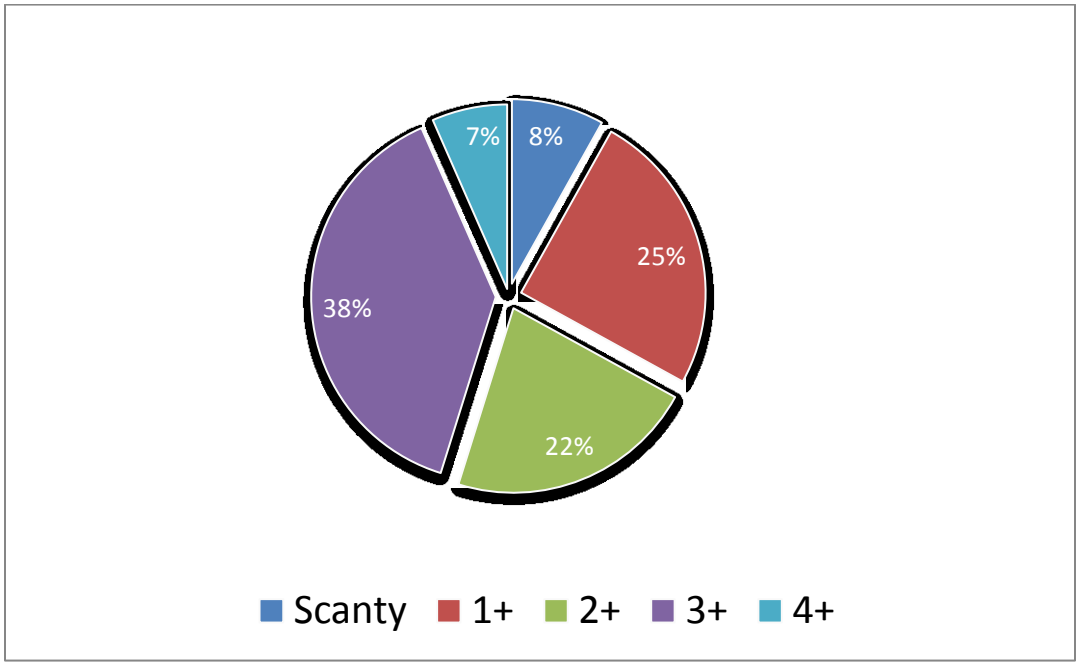



Figure-5.9: Distribution of PTB cases based on bacterial burden. Scanty, 1+, 2+, 3+ and 4+ are sputum smear AFB grading.

Among all the sputum positive PTB patients highest numbers of the patients were having 3+ AFB grading followed by 1+ AFB grading.

The following table (Table-5.3) demonstrates the mean±SD values of serum oxygen sensing molecular markers such as VEGF, TNF-α and EPO of 197 sputum positive PTB cases with different AFB grading. The mean serum VEGF and TNF-α level were very high in PTB patients with AFB grading 4+ followed by 3+ as indicated in the Figure-5.10 and 5.11 respectively. In contrast, we observed significantly decreased values

of mean serum EPO in PTB patients with AFB grading 4+ and 3+ as mentioned in the Figure-5.12

Table-5.3: Mean±SD values of oxygen sensing molecular markers in PTB cases with different AFB grading

| Parameters | AFB grading | N | Mean±SD |
|---|---|---|---|
| VEGF (pg/ml) | Scanty | 16 | 211.09±63.15 |
| | 1+ | 49 | 378.1±144-42 |
| | 2+ | 43 | 524.27±186.42 |
| | 3+ | 76 | 763.17±193.93 |
| | 4+ | 13 | 1538.54±234.70 |
| | Total | 197 | 614.82±354.87 |
| TNF-α (pg/ml) | Scanty | 16 | 34.00±4.97 |
| | 1+ | 49 | 53.62±15.95 |
| | 2+ | 43 | 80.12±19.19 |
| | 3+ | 76 | 111.39±28.92 |
| | 4+ | 13 | 174.0±20.10 |
| | Total | 197 | 87.69±41.59 |
| EPO (mIU/ml) | Scanty | 16 | 30.91±6.81 |
| | 1+ | 49 | 39.74±6.57 |
| | 2+ | 43 | 39.09±6.21 |
| | 3+ | 76 | 35.17±5.55 |
| | 4+ | 13 | 25.92±6.17 |
| | Total | 197 | 36.08±7.11 |

Figure-5.10: Quantitative expression of VEGF (by ELISA) in PTB with different Bacterial Burden

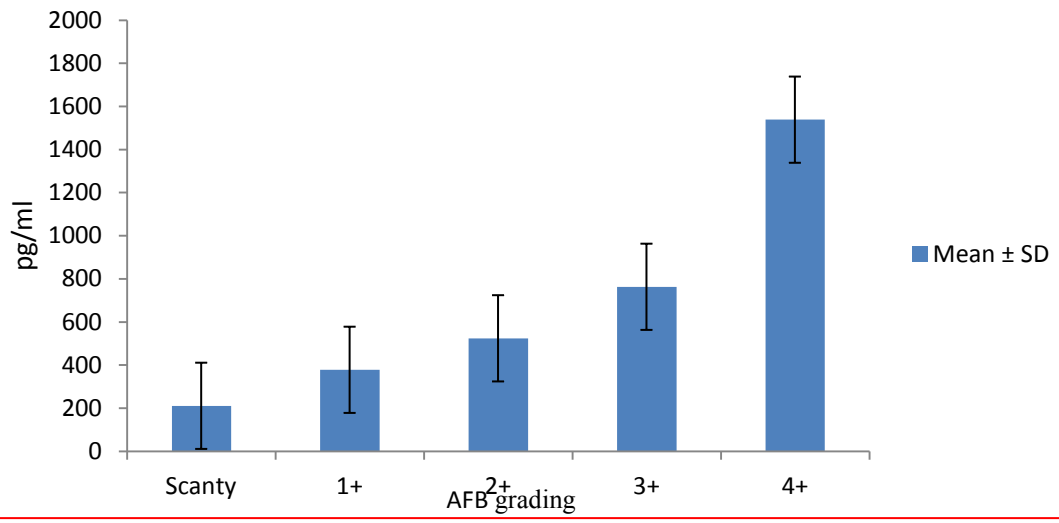



Figure-5.11: Quantitative expression of TNF-α (by ELISA) in PTB with different Bacterial Burden

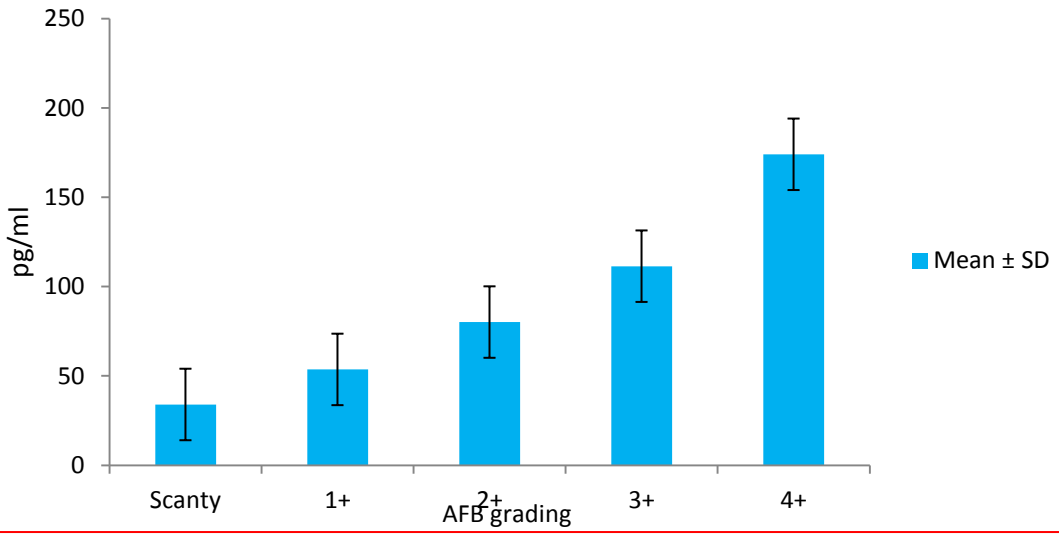
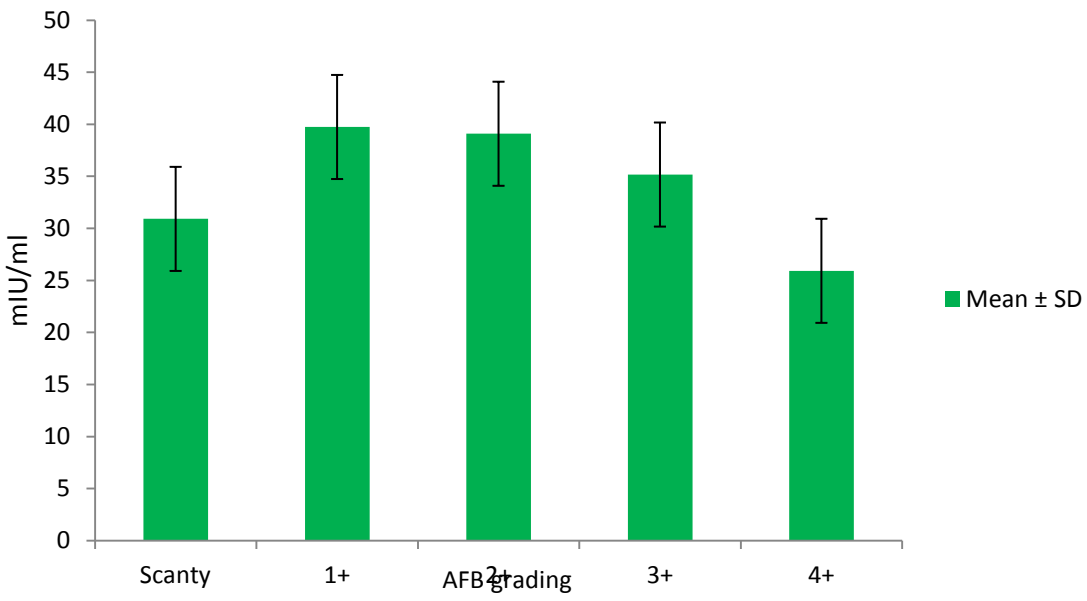


Figure-5.12: Quantitative expression of EPO (by ELISA) in PTB with different Bacterial Burden




**5.6 Association of circulating oxygen sensing molecular markers with bacterial burden in PTB patients:**

In the present study, we evaluated correlation of circulating oxygen sensing molecular markers like VEGF, TNF-α and EPO with bacterial burden in PTB patients by Spearman's rank correlation analysis. We observed positive correlation between serum VEGF and AFB grading (r= 0.807, $p< 0.001$), positive correlation between serum TNF-α and AFB grading (r= 0.849, $p< 0.001$) as shown in the Figure-5.13 and Figure-5.14. We recorded negative correlation between serum EPO and AFB grading (r= -0.257, $p< 0.001$) as illustrated in the Figure-5.15.

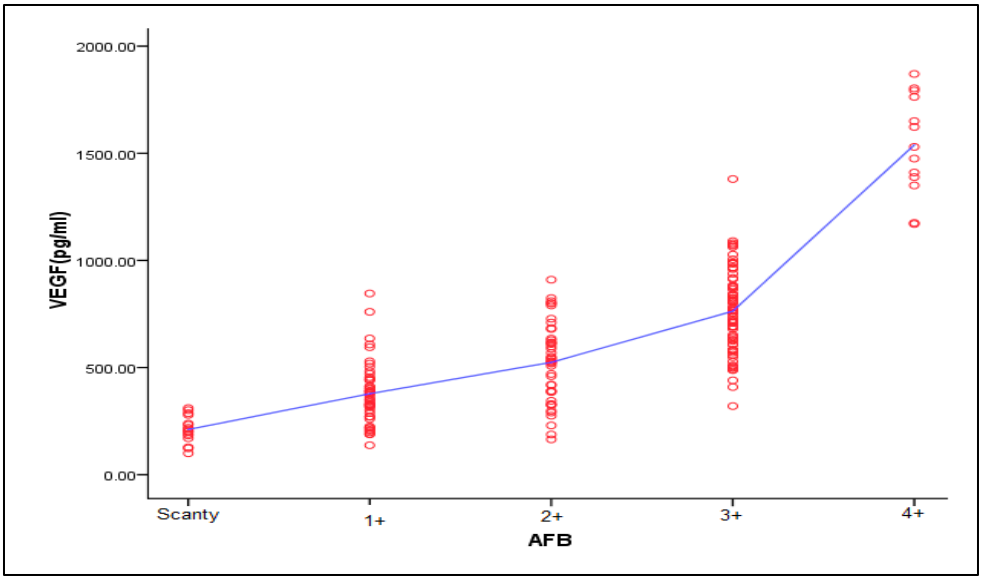



Figure-5.13: Correlation between serum VEGF and bacterial burden (r=0.807, $p< 0.001$)

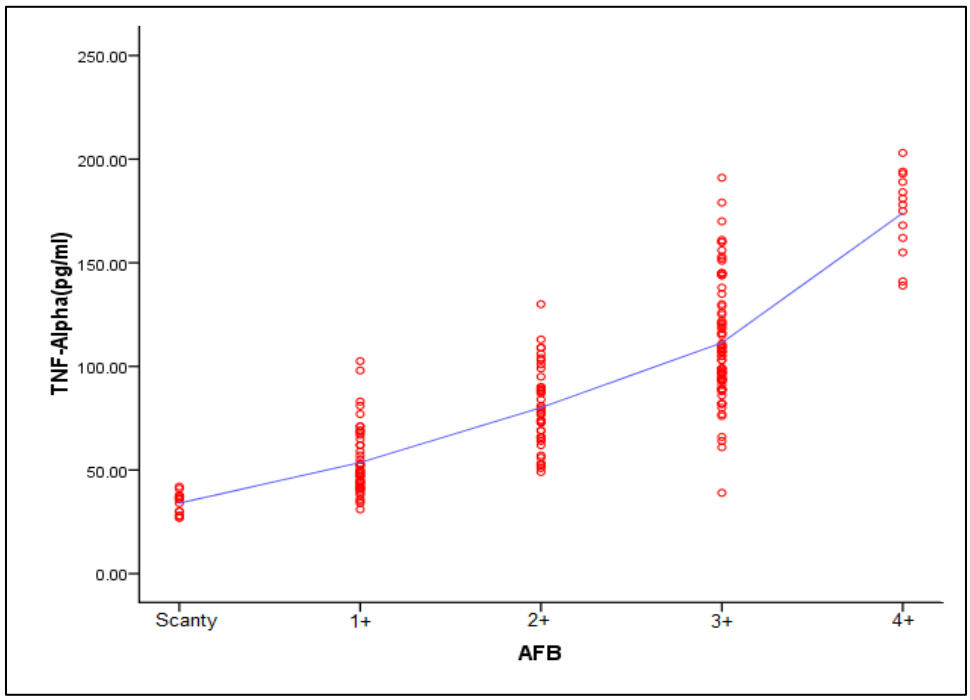



Figure-5.14: Correlation between serum TNF-α and bacterial burden (r=0.849, $p< 0.001$)

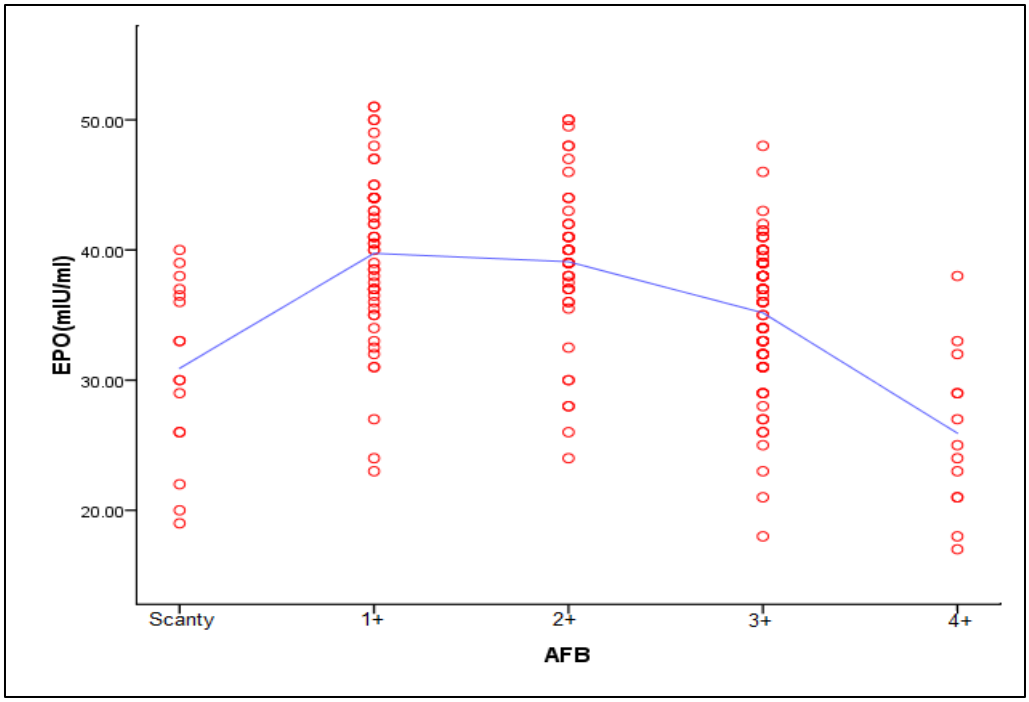



Figure-5.15: Correlation between serum EPO and bacterial burden (r=-0.257, $p< 0.001$)

**5.7 TNF-α influences EPO action on Hb in PTB patients:**

To evaluate whether TNF-α influences EPO action on Hb in PTB cases, we compared mean values of serum TNF-α, EPO and Hb with sputum AFB grading by Kruskal Wallis Test (Table-5.4) and found the significant associations ($p< 0.001$).

Among 197 PTB cases, 13 cases were belongs to 4+ AFB grading, where we observed very low mean Hb values (7.21±0.70 gm/dl) and the corresponding mean TNF-α and EPO values were 174.0±20.10 pg/ml and 25.92±6.17 mIU/ml respectively. This was followed by 76 PTB cases with 3+ AFB grading showed mean Hb values of 8.90±1.20 gm/dl, mean TNF-α 111.39±28.92 pg/ml and mean EPO values of 35.17±5.55 mIU/ml. In contrast we noted low levels of mean TNF-α (34.0±4.97 pg/ml) in 16 PTB cases with scanty AFB grading and the observed mean EPO and Hb levels in these patients were 30.91±6.81 pg/ml and 12.81±1.20 gm/dl respectively.

Table-5.4: Comparison of mean values of serum TNF-α, EPO and Hb with sputum AFB grading by Kruskal Wallis Test in PTB cases

| Sputum AFB grading | TNF-α (pg/ml) | EPO (mIU/ml) | Hb (gm/dl) |
|---|---|---|---|
| Scanty (n=16) | 34.0±4.97 | 30.91±6.81 | 12.81±1.20 |
| 1+ (n=49) | 53.62±15.95 | 39.74±6.57 | 10.97±1.55 |
| 2+ (n=43) | 80.12±19.19 | 39.09±6.21 | 9.84±1.40 |
| 3+ (n=76) | 111.39±28.92 | 35.17±5.55 | 8.90±1.20 |
| 4+ (n=13) | 174.0±20.10 | 25.92±6.17 | 7.21±0.70 |
| Chi-square value | 142.23 | 49.69 | 71.34 |
| *p* value | < 0.001 | < 0.001 | < 0.001 |

**5.8 Overall evaluation of oxygen sensing pathway in relation to VEGF, EPO and TNF-α in PTB patients:**

For overall evaluation we compared mean ± SD values oxygen sensing molecular markers like VEGF, TNF-α and EPO in PTB cases with different sputum AFB grading using one way ANOVA. The ANOVA test showed statistically significant *p* value ($p< 0.001$) for VEGF, TNF-α and EPO between and within groups as indicated in Table-5.5

Table-5.5: Comparison of mean values of serum oxygen sensing molecular markers in PTB patients with different sputum AFB grading by ANOVA test

| | | Sum of squares | Df | Mean square | F | *p* value |
|---|---|---|---|---|---|---|
| VEGF (pg/ml) | Between groups | 18462157 | 4 | 4615539 | 147.643 | < 0.001 |
| | Within groups | 6002203 | 192 | 31261.47 | | |
| | Total | 24464360 | 196 | | | |
| TNF-α (pg/ml) | Between groups | 244981 | 4 | 61245.26 | 122.9556 | < 0.001 |
| | Within groups | 95636.84 | 192 | 498.1086 | | |
| | Total | 340617.9 | 196 | | | |
| EPO (mIU/ml) | Between groups | 2877.469 | 4 | 719.3673 | 19.32133 | < 0.001 |
| | Within groups | 7148.498 | 192 | 37.23176 | | |
| | Total | 10025.97 | 196 | | | |

Furthermore, in the present study we compared serum levels of oxygen sensing molecular markers like VEGF, TNF-α and EPO with bacterial burden measured in terms of sputum smear AFB grading among each other by a descriptive statistics that is Bonferroni test. Here mean serum levels of VEGF, TNF-α and EPO from PTB patients with scanty AFB grading were compared with the same parameters of 1+, 2+, 3+ and 4+ AFB gradings and so on.

Table-5.6: Comparison of AFB grading among each other with respect to mean values of serum VEGF in PTB patients by descriptive statistics (Bonferroni test)

| Dependent Variable | AFB grading | | Mean Difference | Std. Error | *p* value |
|---|---|---|---|---|---|
| VEGF (pg/ml) | Scanty | 1+ | -167.006 | 50.91003 | <0.05 |
| | | 2+ | -313.174 | 51.77696 | <0.001 |
| | | 3+ | -552.073 | 48.63309 | <0.001 |
| | | 4+ | -1327.44 | 66.01947 | <0.001 |
| | 1+ | Scanty | -167.006 | 50.91003 | <0.05 |
| | | 2+ | -146.167 | 36.94591 | <0.05 |
| | | 3+ | -385.067 | 32.39328 | <0.001 |
| | | 4+ | -1160.44 | 55.16084 | <0.001 |
| | 2+ | Scanty | 313.174 | 51.77696 | <0.001 |
| | | 1+ | -146.167 | 36.94591 | <0.05 |
| | | 3+ | -238.899 | 33.73939 | <0.001 |
| | | 4+ | -1014.271 | 55.96195 | <0.001 |
| | 3+ | Scanty | -552.073 | 48.63309 | <0.001 |
| | | 1+ | -385.067 | 32.39328 | <0.001 |
| | | 2+ | -238.899 | 33.73939 | <0.001 |
| | | 4+ | -775.371 | 53.0666 | <0.001 |
| | 4+ | Scanty | -1327.445 | 66.01947 | <0.001 |
| | | 1+ | -1160.438 | 55.16084 | <0.001 |
| | | 2+ | -1014.271 | 55.96195 | <0.001 |
| | | 3+ | -775.371 | 53.0666 | <0.001 |

Table-5.6 illustrates the ccomparison of AFB grading among each other pertaining to mean values of serum VEGF in PTB patients by Bonferroni test. Here we

reported statistically significant *p* value. Further, the AFB grading scanty vs. 1+ and 1+ vs. 2+ showed significant *p* value ($p < 0.05$) elsewhere we found highly significant *p* value ($p< 0.001$)

Table-5.7: Comparison of AFB grading among each other with respect to mean values of serum TNF-α in PTB patients by descriptive statistics (Bonferroni test)

| Dependent Variable | AFB grading | | Mean Difference | Std. Error | *p* value |
|---|---|---|---|---|---|
| TNF-α (pg/ml) | Scanty | 1+ | -19.6224 | 6.426295 | <0.05 |
| | | 2+ | -46.1163 | 6.535725 | <0.001 |
| | | 3+ | -77.3947 | 6.13888 | <0.001 |
| | | 4+ | -140.00 | 8.333536 | <0.001 |
| | 1+ | Scanty | -19.6224 | 6.426295 | <0.05 |
| | | 2+ | -26.4938 | 4.663625 | <0.001 |
| | | 3+ | -57.7723 | 4.088953 | <0.001 |
| | | 4+ | -120.378 | 6.962867 | <0.001 |
| | 2+ | Scanty | -46.1163 | 6.535725 | <0.001 |
| | | 1+ | -26.4938 | 4.663625 | <0.001 |
| | | 3+ | -31.2785 | 4.258871 | <0.001 |
| | | 4+ | -93.8837 | 7.063991 | <0.001 |
| | 3+ | Scanty | -77.3947 | 6.13888 | <0.001 |
| | | 1+ | -57.7723 | 4.088953 | <0.001 |
| | | 2+ | 31.27846 | 4.258871 | <0.001 |
| | | 4+ | -62.6053 | 6.698515 | <0.001 |
| | 4+ | Scanty | -140.00 | 8.333536 | <0.001 |
| | | 1+ | -120.378 | 6.962867 | <0.001 |
| | | 2+ | -93.8837 | 7.063991 | <0.001 |
| | | 3+ | -62.6053 | 6.698515 | <0.001 |

The result of Bonferroni test on TNF-α suggested statistically highly significant *p* value ($p< 0.001$) whereas AFB grading scanty vs. 1+, we reported significant *p* value ($p< 0.05$) as indicated in Table-5.7.

Table-5.8: Comparison of AFB grading among each other with respect to mean values of serum EPO in PTB patients by descriptive statistics (Bonferroni test)

| Dependent Variable | AFB grading | | Mean Difference | Std. Error | *p* value |
|---|---|---|---|---|---|
| EPO (mIU/ml) | Scanty | 1+ | -8.83865 | 1.756934 | <0.001 |
| | | 2+ | -8.18677 | 1.786852 | <0.001 |
| | | 3+ | -4.2648 | 1.678355 | >0.05 |
| | | 4+ | -4.98317 | 2.278369 | >0.05 |
| | 1+ | Scanty | -8.83865 | 1.756934 | <0.001 |
| | | 2+ | -0.65187 | 1.275024 | >0.05 |
| | | 3+ | -4.57385 | 1.11791 | <0.05 |
| | | 4+ | -13.8218 | 1.903632 | <0.001 |
| | 2+ | Scanty | -8.18677 | 1.786852 | <0.001 |
| | | 1+ | -0.65187 | 1.275024 | >0.05 |
| | | 3+ | -3.92197 | 1.164365 | <0.05 |
| | | 4+ | -13.1699 | 1.931278 | <0.001 |
| | 3+ | Scanty | -4.2648 | 1.678355 | >0.05 |
| | | 1+ | -4.57385 | 1.11791 | <0.05 |
| | | 2+ | -3.92197 | 1.164365 | <0.05 |
| | | 4+ | -9.24798 | 1.831358 | <0.001 |
| | 4+ | Scanty | -4.98317 | 2.278369 | >0.05 |
| | | 1+ | -13.8218 | 1.903632 | <0.001 |
| | | 2+ | -13.1699 | 1.931278 | <0.001 |
| | | 3+ | -9.24798 | 1.831358 | <0.001 |

In case of serum EPO, the AFB grading scanty vs. 3+, scanty vs. 4+ and 1+ vs. 2+ not showed any significant differences ($p>0.05$). The AFB grading 1+ vs. 3+ and also 2+ vs. 3+ demonstrated significant differences ($p< 0.05$) and elsewhere we observed highly significant differences ($p< 0.001$) as mentioned in the Table-5.8.

**5.9 Prognostic applications of serum oxygen sensing molecular markers in PTB patients:**

Among 197 PTB cases, we did follow up of 10 cases (not included in the objective of our study) and compared mean values of hematological parameters and oxygen sensing molecular markers in PTB before and six months after the onset of ATT for evaluating prognostic applications of these parameters as illustrated in the Table-5.9. Though there was a significant difference in parameters measured among two groups, further extended studies have to be conducted with large sample size to prove the prognostic applications statistically.

Table-5.9: Comparison of measured parameters in PTB before and six months after the onset of ATT.

| Parameters | Before treatment (Mean±SD) | After treatment (Mean±SD) | *p* value |
|---|---|---|---|
| VEGF (pg/ml) | 604.40±221.36 | 209.40±99.73 | <0.001 |
| TNF-α (pg/ml) | 74.70±35.55 | 34.72±11.36 | <0.001 |
| EPO (mIU/ml) | 36.30±6.60 | 50.13±9.19 | <0.001 |
| Hb (g/dl) | 10.03±1.55 | 11.73±1.53 | <0.001 |
| RBC (Millions/cumm) | 3.94±0.60 | 4.32±0.52 | <0.05 |
| PCV (%) | 31.66±4.75 | 35.62±4.96 | <0.05 |
| MCV (fL) | 79.36±4.55 | 82.49±3.56 | <0.05 |
| MCH (pg/cell) | 24.9±2.09 | 26.70±1.62 | <0.05 |
| MCHC (g/dl) | 31.62±1.49 | 33.09±0.92 | <0.05 |
| WBC (Cells/cumm) | 11610±2996 | 7000±1178 | <0.001 |
| Platelets (Lakhs/cumm) | 4.04±0.79 | 3.38±0.87 | >0.05 |
| ESR (mm/hour) | 57.10±20.95 | 24.80±12.94 | <0.001 |

# CHAPTER 6
# DISCUSSION

**6.1 Anthropometry:**

In the present study, a total of 297 subjects were analyzed of which 197 were PTB cases and 100 were controls (apparently healthy individuals). Out of 197 PTB cases, 91.88% cases showed subnormal BMI and these results on BMI probably indicate poor nutritional status in PTB. The findings on BMI in our study were in accordance with earlier studies[1-5]. Further, BMI is also an important marker for the assessment of nutritional status in PTB patients[3, 6]. It has been suggested that reduced BMI is a significant risk factor for PTB due to the induced impairment of cellular immunity[3, 7] and probably this could be the reason behind the previous studies stressing about increased mortality rate in PTB patients with low BMI[8-11]. So it can be stated that reduced BMI is associated with increased mortality in PTB patients.

Even though many studies have related low BMI with TB, but it was not clear whether reduced BMI results in TB or TB induces low BMI[5]. This question was raised because, the victims of TB disease were mostly poor people with low socioeconomic background where BMI was obviously low and at the same time the increased production of inflammatory cytokines especially TNF-α with catabolic effect on body metabolism could reduce BMI in PTB[12-14].

The present study was conducted at one of the most backward area of Karnataka and in this study, most of the PTB patients were from poor background and further we have also noticed elevated levels of serum TNF-α in these patients. Thus considering both the factors, we could conclude that, the present study PTB cases were already with low BMI and was further reduced due to the induction of TNF-α in PTB. As the bacterial load increased TNF-α level were also increased resulting in the subsequent reduction of BMI in PTB patients as observed in our study.

The study results on BMI suggest the supplementation of essential macro and micro nutrients during ATT could confer better treatment outcome in PTB[15]. Apart from this, few studies conducted in young males with reduced BMI showed the congenital

development and enlargement of apical lung bullae which could be one of the easy target sites for TB reactivation[16, 17].

In addition, the extent of masculine distribution of adipose tissue can be indicated by waist to hip ratio (WHR) [18]. The muscle wasting and decrease in fat percentage in PTB patients may result in low WHR as noticed in our study and in agreement with the study conducted by Tungdim et al. who further showed increase in WHR in PTB patients after successful treatment with nutritional supplementation[19].

**6.2 Hematology:**

The abnormal hematological parameters in PTB cases observed in the present study were in accordance with previous studies[20-23]. Anemia is the most common hematological abnormalities in PTB patients as reported in our study which could be one of the significant risk factor associated with death due to TB[24]. The exact cause of anemia in PTB is not clear, but the prevalence of anemia is very high in active PTB[25] and probably iron deficiency and inflammatory cytokines could be regarded as causative factors in the development of anemia of chronic disease like PTB[26, 27].

The observed increase in WBC count in PTB patients could be considered as host defense strategy by increasing the production of WBC to fight against invading pathogens[23]. We reported increased platelet count in PTB patients compared to healthy controls. Baynes et al. demonstrated statistically significant correlation between extent of inflammatory condition and degree of thrombocytosis[28]. Another study suggested possible involvement of interieukin-6 (IL-6) in the establishment of thrombocytosis in PTB cases[29]. In contrary, Rohini et al. reported reduced platelet count in PTB cases compared to normal controls[23].

In fact it was obvious to record increased ESR in PTB cases just like other inflammatory conditions. Though ESR values were increased in PTB, it is not recommended as useful screening test[30]. However, estimation of ESR can be used as

routine test to know the progression of TB disease and also to have an idea about disease prognosis, since ESR value decreases after effective TB treatment[31].

The abnormal hematological findings in PTB patients will reverse back to normal provided successful treatment. Thus along with sputum microscopy, culture and chest x-ray, hematological parameters could be included as routine tests not only in supporting diagnosis but also to help clinicians to know the disease progression and monitoring the treatment outcome in PTB patients[32].

**6.3 Oxygen sensing molecular markers in PTB patients in comparison to healthy controls:**

The present study findings on oxygen sensing molecular markers such as serum VEGF, EPO and TNF-α in PTB cases showed increased expression when compared to healthy controls indicating the link between oxygen sensing mechanism and PTB. Among these, serum VEGF and TNF-α were more pronounced in PTB cases compared to serum EPO. Although we noted slight increase in EPO level in cases than controls, there was relative inadequacy of EPO in relation to degree of anemia in PTB patients.

Our findings on oxygen sensing major angiogenic factor VEGF were in parallel with other studies[33-37]. VEGF was found to be expressed in many parts of the lung where it plays important role as major angiogenic and vascular permeability factor. In addition, VEGF expression and subsequent angiogenesis in lungs has been linked with many respiratory disorders[38-41] but very few literatures mentioned about the role of oxygen sensitive VEGF in PTB.

Studies from Alatas et al. and Amin et al. found increased serum VEGF in PTB patients similar to our study and further, they stated estimation of VEGF levels in serum could be useful marker of active PTB indicating the association between increased expression of VEGF and disease activity in PTB cases[35, 36].

In another study conducted by Abe et al. proposed that the major protective role played by increased expression of VEGF was found to accelerate the supply of blood and oxygen to affected lung tissues by initiating angiogenesis to safe guard PTB patients from reduced oxygen sensing microenvironment which could further inhibit cavity formation in PTB patients[34].

According to Matsuyama et al. the major source of VEGF in PTB was alveolar macrophages and also pointed about probable link between increased serum VEGF and pathogenesis of PTB[33]. Since the follow up of PTB cases were not included in the objectives of our study, similar parameters were excluded. However, earlier literature indicated about the role of VEGF in monitoring the treatment response in PTB[37].

The increased serum TNF-α in the PTB patients of the present study was in corroboration with other studies[42-46]. Earlier workers mentioned that the inflammatory cytokine TNF-α increases early in PTB and initiates specific role in the host response to *Mycobacterium tuberculosis* (MTB) [47]. In PTB, the invading TB bacillus induces the synthesis of TNF-α along with other cytokines through the activation of monocytes and T- lymphocytes[46]. As a defense mechanism, TNF-α promotes the migration of immune cells to the site of infection and along with IFN-γ and IL-2 contributes in granuloma formation and thus helps in the process of infection control and further reduces the progression of disease[47, 48]. Thus in the present study, the high level of TNF-α in PTB patients indicate the important role played by this cytokine against MTB. Moreover, inhibition of TNF-α production resulted in the disorganization of TB granuloma and reactivation of TB as reported by animal and human experiments[49, 50].

The diagnosis of PTB is primarily based on the initial clinical suspicion and x-ray outcome followed by bacteriological confirmation by AFB sputum microscopy and culture. Variable sensitivity in sputum microscopy, low specificity of x-ray findings and time factor for culture reporting has necessitated in search of new and simple approaches in the early diagnosis of PTB in resource restricted areas where molecular methods are not available. While determination of serum adenosine deaminase (ADA) activity which

fulfills above criteria could be considered as simple test for early diagnosis of PTB but it is noteworthy to mention that, ADA has no role in measuring bacterial burden and disease severity in PTB[51]. Thus we propose the determination of serum VEGF and TNF-α in suspected cases of PTB could serve as better tool not only in early diagnosis but also in measuring bacterial burden, disease severity and in predicting active disease in PTB in any patient presenting at any health care facility.

In our study, the resultant blunted responses of serum EPO with respect to hemoglobin (Hb) concentration in chronic disease like PTB was in conjunction with the study conducted by Ebrahim et al.[52] and Kulkarni et al.[53]. Indeed, previous literature survey emphasized the fact that, extensive research activities have been carried out in cancer and chronic kidney disease patients with anemia and reported inappropriate response of EPO to the degree of anemia indicating the involvement of inflammatory cytokine in the down regulation of EPO in chronic diseases[54-58]. But the literatures indicating the relationship between PTB and EPO were limited and hence to explore the link between EPO and PTB, we included oxygen sensing EPO in our study and further studies are suggested to confirm the findings.

### 6.4 Hypoxia and oxygen sensing molecular markers in PTB:

Study by previous researcher reported that, during inflammation associated with bacterial infection, occurrence of hypoxia is a common micro environmental feature[59]. Numerous factors can induce hypoxia during bacterial infections like PTB, however, increased oxygen demand by inflamed resident cells, infiltrating inflammatory cells, vascular pathology or multiplying pathogen itself are some of the significant factors implicated in the development of hypoxia[60, 61]. It may be noted that, during bacterial infection, it is difficult to predict from the host perceptive that, the occurrence of hypoxia is protective or destructive and probably it depends largely on the nature and virulence factors of invading pathogens[62].

In human beings, the expression of major oxygen sensing transcription factor HIF-1α increases in tissue hypoxia observed during bacterial infection such as PTB. HIF-1α was shown to be involved in host response to hypoxia in inflammatory and bacterial diseases[63]. Previous studies have mentioned that, during hypoxia seen in bacterial infection, the HIF-1α activates anaerobic mechanism to full fill the energy requirement of immune cells so that these cells can exert effective immune response against invading microbes during hypoxic conditions[64]. Apart from its immunomodulating function, earlier researchers have also implicated the role of HIF-1α in the activation of B and T lymphocytes, macrophages, neutrophils and dendritic cells during infectious and inflammatory conditions[65].

Furthermore, the activated HIF-1α during hypoxia induced by bacterial infection can bind with the target gene promoter sequence in the host cell and induces the transcription of target genes involved in the regulation of angiogenesis, apoptosis, cellular stress, immunity and metabolism. VEGF, EPO, basic fibroblast growth factor, glycolytic enzymes, glucose transporters are few examples of target gene translated protein products induced by HIF-1α pathway in response to hypoxia as observed during bacterial infection like PTB[66].

Studies have also highlighted about the fact that, during bacterial infection, hypoxia can stimulate the production of inflammatory cytokine TNF-α through the mediation of HIF-1α[67]. Thus in the present study, elevated levels especially oxygen sensing serum VEGF and TNF-α in PTB patients compared to healthy controls could be attributed to inflammatory and hypoxic microenvironment induced by MTB. However, hypoxia stimulated increase in oxygen sensing serum EPO was not promising to maintain Hb level in PTB patients because of under lying cytokine mediated inhibitory mechanism.

The TNF-α induced formation of TB granuloma is a typical and most prominent feature of TB disease and studies have demonstrated nature of these complex structures as hypoxic around its central necrotic core[68, 69]. Study findings showed the presence of

extensive vascularisation in TB granuloma due to the transcriptional induction of major angiogenic factor VEGF by hypoxia stimulated HIF-1α[69, 70]. Moreover, increased angiogenesis seen in TB granulomas mediated by VEGF could be beneficial to control the progression of disease by transporting immune cells within the structure[69]. Conversely, results from animal experiments indicated granuloma associated angiogenesis mediated by VEGF could benefit mycobacterial growth and further spread of infection to other tissue sites of the host resulting in the development of miliary TB or disseminated forms of TB[71, 72].

**6.5 Correlation among oxygen sensing molecular markers (VEGF vs. TNF-α vs. EPO) in PTB:**

In the present study we witnessed statistically significant positive correlation between oxygen sensing serum VEGF and TNF-α in PTB patients indicating their direct association with active PTB and also explains their important pathophysiological role in PTB. Among the two molecular markers, serum VEGF was more pronounced than serum TNF-α.[73]. We noticed an increasing tendency of serum VEGF and TNF-α in relation to disease severity suggesting these parameters could be used as marker to indicate disease severity in PTB and thus helpful in monitoring the progression of the disease.

Moreover, serum VEGF and TNF-α could be useful markers in differentiating PTB from extra pulmonary TB (EPTB) [74]. In addition, Momi et al proposed the combined measurement of pleural effusion VEGF and TNF-α could help in the differential diagnosis between malignant pleural effusion and tuberculous pleurisy[75]. Yet another study reported higher levels of serum VEGF and TNF-α in pleural TB compared to PTB[76]. Thus it becomes important to mention that along with PTB, VEGF and TNF-α were also associated with EPTB.

Although the expression of VEGF and EPO were directly associated with degree of hypoxia but in our study we have not observed any correlation between serum VEGF and EPO. Watts et al. observed hypoxic condition and elevated levels of serum VEGF

and EPO in cystic fibrosis patients and further he demonstrated significant positive correlation between serum VEGF and EPO[77].

In PTB patients of our study, the prevailing hypoxic condition could not up regulate oxygen sensing serum EPO even when most of the patients were anemic indicating the involvement of certain EPO suppressing mechanism in PTB patients. Evidence from previous studies suggested the involvement of TNF-α in inhibiting EPO production and interfering with its action in anemia of chronic disease[78, 79]. Since PTB is one of the chronic infectious disease and we also observed increased TNF-α in PTB patients and so combining both the facts we can state that, increased levels of TNF-α in PTB patients showed negative impact on EPO production and functioning as observed in our study leading to EPO resistance and occurrence of anemia in PTB patients.

The above findings of present study were further strengthened by our reported negative correlation between TNF-α and EPO in PTB cases. As the expression of TNF-α increased, we observed reduction in the levels of EPO indicating blunted EPO response in the presence of increased TNF-α level in PTB patients. Our findings were supported by previous studies that in kidney increased TNF-α in chronic disease decreases the number of EPO producing peritubular cells and interferes with EPO production and action[56]. Moreover, TNF-α decreases the EPO receptor protein leading to EPO resistance and along with IFN-γ, TNF-α induces the production of oxygen and nitrogen free radicals which further interfere with expression of specific transcription factors involved in erythrocyte differentiation control[80, 81]. In addition, TNF-α along with other cytokine alters body's iron metabolism and inhibits the action of erythroid progenitors[57]. All these factors could contribute in the poor response of EPO to prevailing low Hb concentration and further development of anemia in PTB patients as observed in our study[82].

Our findings on EPO in PTB patients suggests the inclusion of human recombinant EPO in ATT panel which could reduce anemia related mortality in TB and may improves the treatment outcome and probably help the TB victims to lead quality life.

Earlier studies conducted on PTB patients showed increased serum levels of VEGF, TNF-α and relative insufficiency of serum EPO. But so far we have not came across any study like ours to evaluate correlation and also to demonstrate the association among oxygen sensing molecular markers (VEGF vs. TNF-α vs. EPO) in PTB patients. However, in a study by Kulkarni et al, correlated TNF-α with EPO similar to our study but with small sample size[53]. Thus our study may be considered as one of its kind in demonstrating association of these oxygen sensing molecular markers with PTB.

**6.6 Oxygen sensing circulating molecular markers and bacterial burden in PTB patients:**

In the present study we measured bacterial burden in PTB patients in terms of sputum smear AFB grading[73] and distributed the patients according to AFB grading (Figure-5.9). We studied association of oxygen sensing molecular markers such as VEGF, TNF-α and EPO in PTB cases by correlating them with different AFB grading in PTB. Among three circulating oxygen sensitive parameters included in our study, serum VEGF and TNF-α showed significant positive correlation with bacterial burden.

It was understood that increased bacillary load in PTB induces the increased expression of inflammatory cytokine TNF-α as a body's inbuilt defense mechanism against invading MTB[46]. Increased bacterial burden in turn increases inflammatory and hypoxic condition and this could be the reason for increased serum VEGF production through the mediation of hypoxia-induced HIF-1α in PTB cases as observed in our study. In addition, the inflammatory and hypoxic condition seen during increased bacterial burden in PTB may further up regulate TNF-α as in the case of our study. Hence, as the bacterial load increased the expression of VEGF and TNF-α were also increased indicating their direct association with bacterial burden in PTB. This clearly shows the influence of VEGF in PTB and its association with TNF-α in PTB and further increased levels of serum VEGF and TNF-α could be useful in the early diagnosis and early prediction of active PTB. Thus, so far in the present study we demonstrated the important roles for both VEGF and TNF-α in the pathogenesis of mycobacterial infection.

During the initial stage of the disease we reported increased serum EPO in PTB cases but as the bacterial burden increased we observed decreasing trend of EPO concentration indicating a negative but not linear correlation between EPO and bacterial burden in PTB. The down fall of EPO and EPO resistance in the present study might be related to the increased inflammatory condition due to elevated bacterial burden and subsequent induction of TNF-α in PTB which has been discussed earlier in detail. Thus in the present study we reported inverse association of serum EPO with bacterial burden and subsequent development of anemia in PTB cases.

Similar to our study, Kumar et al. performed correlation study between serum VEGF and bacterial burden in PTB and found the same result and in continuation the study encouraged the use of serum VEGF estimation as a marker in differentiating PTB from latent TB infection (LTBI) and no TB (NTB) cases[83].

However, in the present study along with VEGF we have also correlated TNF-α and EPO with bacterial burden and our study is one of the latest to systemically explore the association of oxygen sensing molecular markers such as VEGF, TNF-α and EPO with bacterial burden in PTB.

**6.7 TNF-α influences EPO action on Hb level in PTB patients:**

In the present study we made an attempt to evaluate whether TNF-α influences EPO action on Hb in PTB patients with different bacterial load and probably our study may be one of the earliest to report about this fact. Here we compared mean values of serum TNF-α, EPO and Hb with different sputum AFB grading of PTB patients and further we concluded that inspite of increased serum EPO in PTB cases compared to healthy controls, the present study results firmly indicated the relative and functional deficiency of endogenous EPO with respect to Hb concentration. Thus we were able to show a link between Hb concentration and serum levels of both TNF-α and EPO in PTB patients with different bacterial burden.

The recorded above results probably explained on the basis that, during initial phases of the disease in PTB, the degree of increase in serum TNF-α was not up to the mark to inhibit EPO production and action and hence we noted slight up hill in serum EPO level. But as the disease progressed and bacterial load increased and despite of the fact that Hb levels are subnormal, instead of increasing the serum EPO level started declining in PTB patients with simultaneous increase in serum TNF-α concentration and thus proving the fact that increased TNF-α inhibits EPO production and interferes with its activity leading to EPO resistance in PTB patients. This mechanism explains why most of the PTB patients are anemic in the present study. Nevertheless, we were aware about the fact that, most of the PTB patients of our study were from socioeconomically deprived population where deficiency of essential nutrition was common. Hence, both inflammatory cytokine mediated anemia and iron deficiency anemia may occur together in PTB patients[27]. So, we recommend further extended study to arrive at firm conclusion with regard to cause of anemia in PTB.

**6.8 Overall evaluation of oxygen sensing pathway in relation to molecular markers in PTB patients:**

For overall evaluation of oxygen sensing pathway in relation to molecular markers like VEGF, TNF-α and EPO in PTB we adapted ANOVA test followed by descriptive statistics that is Bonferroni test. Here we compared mean values of VEGF, TNF-α and EPO in PTB cases with different sputum AFB grading to see the association. ANOVA test showed significant association of serum VEGF, TNF-α and EPO between and within groups with respect to different AFB grading in PTB patients (Table-5.5).

Further, we applied Bonferroni test, where oxygen sensing parameters such as VEGF, TNF-α and EPO in PTB patients were compared in different AFB grading. Here, individual parameters in different AFB gradings were compared among each other. The results indicated significant differences in the values of serum VEGF and TNF-α among different AFB grading indicating the increased bacterial burden (measured in terms of sputum AFB grading) induces the inflammatory and hypoxic condition in PTB which in turn stimulate increased expression of oxygen sensing hypoxic marker (VEGF) and

inflammatory marker (TNF-α). In normal subject, hypoxia can stimulate EPO production[84]. In PTB, the observed hypoxia due to increased bacterial burden as in the case of our study could not stimulate EPO synthesis indicating inhibitory effect of increased TNF-α on EPO.

The findings of our study on oxygen sensing circulating VEGF and supported by other studies indicated the role and influence of VEGF in PTB wherein VEGF mediated angiogenesis increases the supply of blood to affected tissues and the resultant increased delivery of oxygen and nutrients could enhance and further stabilizes the immune mechanism of the host against MTB or by providing favorable condition may support bacterial growth and further dissemination of infection to other body parts.

The direct correlation of VEGF and TNF-α in PTB implicate their important pathophysiological role in the progression of the disease. In addition TNF-α has a role in muscle wasting and tissue necrosis when present in excess. So, when these two molecular markers are present in desirable amounts may show protective effects in PTB or else in excess may deteriorate the patient's condition in PTB. On the other hand absence of expected positive correlation between VEGF and EPO even during hypoxic condition caused by invading MTB, probably resulted in the development of anemia and further poor prognosis in PTB.

Thus, the findings of our study clearly demonstrated the role of oxygen sensing molecular markers such as VEGF, TNF-α and EPO and their correlation among each other and with bacterial burden in PTB. Therefore, from these findings, finally we accept our hypothesis stating VEGF protein expression (measured quantitatively by ELISA) not only influences PTB but also it is correlated with serum EPO and TNF-α concentration in PTB.

**6.9 Prognostic applications of oxygen sensing molecular markers in PTB patients:**

Among 197 PTB cases, we did follow up of 10 cases (not included in the objectives of our study) and compared mean values of molecular markers in PTB patients

before the onset and six months after the onset of ATT for evaluating prognostic applications of these parameters (follow up). After successful treatment, we recorded decreased serum levels of VEGF and TNF-α and increased serum EPO and also all the hematological parameters reverted to normal level. While sample size was less to apply any statistical tests but findings indicated that these parameters could prove useful as biomarker in monitoring prognosis and therapeutic response in PTB. Further study will be planned to evaluate oxygen sensing biomarkers in prognostic and therapeutic response.

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# CHAPTER 7

# SUMMARY AND CONCLUSION

**7.1 Summary:**

- ❖ Our study findings on circulating oxygen sensing molecular markers such as VEGF, EPO and TNF-α indicated that,
- ❖ In PTB, increased serum VEGF and TNF-α and relative deficiency of serum EPO were associated with disease severity and bacterial burden.
- ❖ Increased bacterial burden induces hypoxic and inflammatory condition which in turn stimulates increased expression of oxygen sensing molecular markers like VEGF and TNF-α.
- ❖ The increased inflammatory cytokine TNF-α in turn play an important role by inhibiting oxygen sensing EPO production and action and thus resulting in the pathophysiology of anemia in PTB patients.

The summary can be illustrated in the following graphical abstract (Figure-7.1).

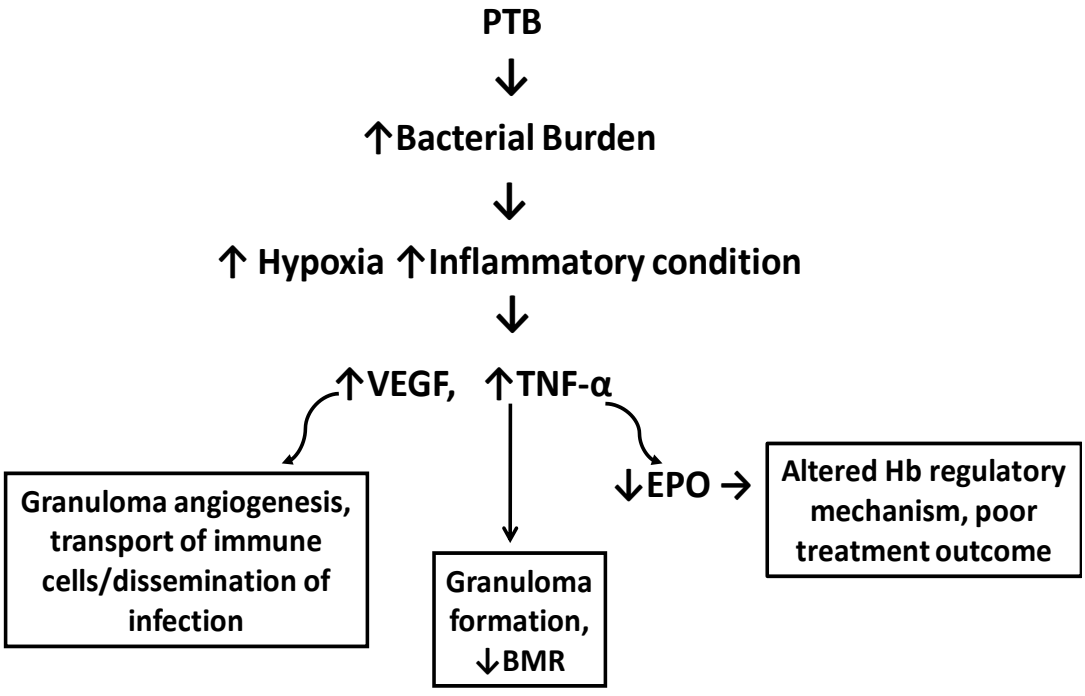


Figure-7.1: Graphic abstract

## 7.2 Conclusion:

- The present study concludes that bacterial burden increases concurrently with induction of hypoxic and inflammatory conditions resulting in the increased expression of oxygen sensing molecular markers such as VEGF and TNF-α.

- It may be further concluded that positive sputum smear for AFB, reduced BMI along with elevated serum levels of oxygen sensing molecular markers like VEGF and TNF-α proved to be useful tool in the early diagnosis and prediction of active PTB and also it could be used as marker of early detection of tissue damage in PTB.

- This would enable physician to initiate isolation and treatment of PTB patients without delay, thus reducing the risk of TB exposure to others.

- Our Study highlighted on the fact that increased bacterial burden was associated with increased serum TNF-α levels and relative deficiency of serum EPO levels in proportion to Hb concentration which played a vital role in the pathophysiology of anemia in PTB.

- Thus during the course of treatment, to reduce the negative prognostic impact of anemia associated with reduced survival and increased morbidity, we suggest therapeutic administration of human recombinant EPO during anti tuberculoses therapy (ATT) could be beneficial to overcome anemia in PTB.

- The increased serum levels of VEGF and TNF-α were directly correlated with sputum AFB grading in PTB patients, probably reflecting their role as reliable marker to establish the disease severity and bacterial burden in PTB.

- In addition serum VEGF, TNF-α and EPO prove to be a predictive biomarker to know the extent of disease, prognosis and monitoring the clinical effect of ATT in PTB patients.

- Finally, determination of serum oxygen sensing molecular markers such as VEGF, TNF-α and EPO along with hematological parameters seems to be better option in PTB patients for further implementation of supportive care and other treatment modalities to enhance the treatment outcome and follow up.

**7.3 Social relevance:**

- The reason for low BMI is multifactorial, either low socioeconomic status or increased TNF-α activity which contribute to the deficiencies of macro and micronutrients in PTB patients.
- This clearly shows that, treatment with nutritional supplementation can be recommended for the better prognosis in PTB patients.

- Presently, sophisticated molecular diagnostic methods requiring complex laboratory facilities are available for the precise diagnosis of PTB and are available only in few district government hospitals under RNTCP.
- Patients from remote areas find it difficult to approach and utilize such facility and again transport of samples is not an easy task (if samples are collected at patient's native place).
- However, if such facilities are available in private set up, then the affordability of these tests by economically deprived patients of the backward area is questionable.
- On the other hand sputum culture takes minimum 6- 8 weeks to confirm the diagnosis even though it is a gold standard test. Whereas, sensitivity and specificity of sputum smear and chest radiography are variable.
- Hence, we recommend the use of circulating oxygen sensing molecular markers like VEGF and TNF-α screening test which could facilitate physician and health care workers with a simple and fast tool to assess and predict the risk of active tuberculosis and to know the extent of tissue damage in any patient presenting at any health care unit with limited diagnostic facilities.

**7.4 Study limitations**:

Since there was constraint regarding budget as this study was self funded;

- In the present study we have not done follow up of all the PTB patients included in this study.
- We did not do any molecular techniques. Further molecular study pertaining to gene expression can be carried out to confirm expression of genes related to oxygen sensing molecular mechanism.

**7.5 Future perspective:**

- It has been reported in animal models infected with MTB that anti angiogenic factors could be beneficial as a host-targeting TB therapy which improves treatment outcome in PTB. So further elaborated research should be carried out in human PTB patients to examine the role of VEGF mediated angiogenesis in dissemination of MTB infection and also to explore the probability of improvement in treatment outcome in PTB patients after the administration of anti angiogenic agent regimens with regular ATT.
- Future study is required to verify the diagnostic role of oxygen sensing VEGF, TNF-α and EPO in clinically suspected sputum negative PTB patients.
- Further extended studies should be conducted to investigate whether administration of human recombinant EPO along with regular ATT could be beneficial to reduce negative prognostic impact of anemia in PTB patients or not.

# APPENDIX

# INFORMATION SHEET

I, Mr. Harish Bhat K have undertaken research work on **"Influence of oxygen sensitive Vascular Endothelial Growth Factor (VEGF) gene expression in Pulmonary Tuberculosis and its correlation with Erythropoietin and Tumor Necrosis Factor- alpha (TNF-α)"**. The main objective of the study is to explore the influence of oxygen on MTB in vivo by evaluating oxygen sensing pathway in relation to VEGF, Erythropoietin and TNF-α in pulmonary tuberculosis. This study will be useful academically as well as clinically.

The Blood sample given by you will be processed for research purpose. If needed, you will be asked to give another blood sample.

I would like to interview you to learn about the signs, symptoms duration of illness or any other predisposing factors/relevant information required for the present study.

Your participation in the study will be completely voluntary and you may refuse to participate at any point of time.

**Name & Signature of Researcher**

## CONSENT FORM FOR PARTICIPATION IN RESEARCH

I ….....

being over the age of 19 years hereby consent to participate as requested for the research project on **"Influence of oxygen sensitive Vascular Endothelial Growth Factor (VEGF) gene expression in Pulmonary Tuberculosis and its correlation with Erythropoietin and Tumor Necrosis Factor- alpha (TNF-α)"**

1. I have read the information provided.
2. Details of procedures and any risks have been explained to my satisfaction.
3. I agree to audio/video recording of my information and participation.
4. I am aware that I should retain a copy of the Information Sheet and Consent Form for future reference.
5. I understand that:
    - I may not directly benefit from taking part in this research.
    - I am free to withdraw from the project at any time and am free to decline to answer particular questions.
    - While the information gained in this study will be published as explained, I will not be identified, and individual information will remain confidential.
    - Whether I participate or not, or withdraw after participating, will have no effect on any treatment or service that is being provided to me.
    - I may ask that the recording/observation be stopped at any time, and that I may withdraw at any time from the session or the research without disadvantage.
6. I do not agree to the transcript being made available to other researchers who are not members of this research team, but who are judged by the research team to be doing related research, on condition that my identity is not revealed.
7. I have had the opportunity to discuss taking part in this research with a family member or friend.

**Participant's signature……Date……...**

I certify that I have explained the study to the patient/ volunteer and consider that she/he understands what is involved and freely consents to participation.

**Researcher's name…….…….....**

**Researcher's signature……..Date…….**

*NB: Two signed copies should be obtained. The copy retained by the researcher may then be used for authorisation of Items 8 and 9, as appropriate.*

8. I, the participant whose signature appears below, have read a transcript of my participation and agree to its use by the researcher as explained.

**Participant's signature……Date……...**

9. I, the participant whose signature appears below, have read the researcher's report and agree to the publication of my information as reported.

**Participant's signature……Date……...**

# PROFORMA FOR COLLECTION OF SAMPLE

Date:

1. Name of the patient :
2. Age. :
3. Sex. :
4. Address. :

5. IP/ OP No. :
6. Clinical history :

7. Height (m) :
8. Weight (kg) :
9. BMI :
10. WHR :
11. Blood pressure (mm of Hg):
12. Pulse rate (beats/min) :
13. Complaints :
    - Cough for more than 3 weeks
    - Chest pain
    - Breathlessness
    - Fever
    - Weight loss
    - Loss of appetite
14. Predisposing factors :
    (Smoking/ HIV/ Immuno-compromised/Any other):
15. Clinical diagnosis :
16. Radio diagnosis :
    (PA chest x ray)

17. LABORATORY DIAGNOSIS

a) Hematological Investigations:

Hb :

TC :

DC :

ESR :

Platelet count :

RBC count :

PCV :

MCV :

MCH :

MCHC :

b) Biochemical Investigations (Molecular markers):

VEGF :

EPO :

TNF-α :

c) Microbiological Investigation:

Sputum for AFB :

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The Ethical Committee of this University met on 16th March 2015 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

**Title** "Infulence of oxygen sensitive Vascular Endothelial Growth Factor (VEGF) gene expression in Pulmonary Tuberculosis and its correlation with Erythropoietin and Tumor Necrosis Factor-alpha (TNF-α)."

**Name of Ph.D./ P. G. / U. G. Student / Faculty member**. Mr. Harish Bhat. K Department of Biochemistry.

**Name of Guide** : Dr.J.G.Ambekar.Professor Department of Biochemistry.

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- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant documents.

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**CERTIFICATE**

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**Title: Influence of oxygen sensitive Vascular Endothelial Growth Factor (VEGF) gene expression in Pulmonary Tubeculosis and its correlation with Erythropoietin and Tumor Necrosis Factor-alpha.**

Name of P.G / U.G Student's / Faculty Member's : Mr. Harish Bhat K, Dept of Biochemistry

Name of the Guide : Dr. J G Ambekar, Dept of Biochemistry

Sd/-
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**Research articles published:**

1. Bhat H, Ambekar JG, Harwalkar AK, Dongre N, Das KK. Role of TNF-α on the function of erythropoietin and haematological profile in pulmonary tuberculosis. *J Clin Diag Res* 2018; 12(8):BC01-04. **(Scopus)**

2. Bhat H, Ambekar JG, Harwalkar AK, Dongre N, Das KK. Serum VEGF and TNF-α Correlate Bacterial Burden in Pulmonary Tuberculosis. *Indian J Public Health Res Dev* 2019; 10(1):189-94. **(Scopus)**

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Original Article

Biochemistry Section

# Role of TNF-α on the Function of Erythropoietin and Haematological Profile in Pulmonary Tuberculosis Patients

HARISH BHAT[1], JEEVAN G AMBEKAR[2], ANAND KUMAR HARWALKAR[3], NILIMA DONGRE[4], KUSAL K DAS[5]

## ABSTRACT

**Introduction:** Pulmonary Tuberculosis (PTB) is one of the major causes for morbidity and mortality to the mankind and India accounts for over one fourth of global Tuberculosis (TB) burden. In PTB, the expression of inflammatory cytokine Tumour Necrosis Factor-Alpha (TNF-α) increases with increase in bacterial burden, which in turn interfere with Erythropoietin (EPO) action and play a vital role in pathophysiology of anaemia.

**Aim:** To study the role of TNF-α on EPO function and its correlation with haematological profile in different grades of PTB.

**Materials and Methods:** The present cross-sectional study was conducted at Navodaya Medical College Hospital and Research Centre, Raichur, Karnataka, India from January 2016 to January 2017. The study included 180 newly diagnosed sputum positive PTB cases and 100 healthy controls. The PTB was confirmed by microscopic examination of sputum specimen of the cases for the detection of Acid Fast Bacilli (AFB). Both cases and controls were subjected to haematological analysis by automated cell counter and serum TNF-α and EPO by ELISA method. The statistical analysis was performed by using SPSS version 16.0 software. The results were analysed with descriptive statistics, wherever appropriate.

**Results:** The mean haematological parameters between PTB cases and controls showed statistically significant differences ($p<0.05$). We reported statistically significant differences in serum levels of TNF-α and EPO among PTB cases and controls ($p<0.001$ and $p<0.001$ respectively). We compared mean values for serum TNF-α, EPO and Haemoglobin (Hb) levels in PTB patients with different sputum AFB grading and noted statistically significant association between AFB grading and TNF-α, AFB grading and EPO and also AFB grading and Hb ($p<0.001$ respectively) in PTB. We observed inverse correlation between TNF-α and EPO ($r=-0.49$, $p<0.001$) and TNF-α and Hb ($r=-0.58$, $p<0.001$) in PTB cases. Further, we found no correlation between EPO and Hb ($r=-0.101$, $p>0.05$) in PTB cases.

**Conclusion:** Increased bacterial burden was associated with increased TNF-α and relative deficiency of EPO. Increased TNF-α and proportionately decreased EPO values were accordant with Hb level and further anaemia in PTB. Hence, during the treatment, therapeutic administration of recombinant human EPO could be useful to combat anaemia and also for better prognostic impact in PTB.

**Keywords:** Anaemia, Bacterial burden, Erythroid progenitors, Haemoglobin

## INTRODUCTION

Pulmonary tuberculosis is one of the major chronic infectious disease and major cause for morbidity and mortality to the mankind. Globally, about 6.3 million new cases of TB were reported in 2016 and worldwide it is the 9th leading cause of death [1]. In India, TB is one of the major public health burden and accounts for one fourth of global TB burden [2].

The inflammation is an immunological defence mechanism of the body when it meets challenges like injury, infection and allergy which results in the significant immigration of White Blood Cells (WBCs) and release of cytokines [3]. The TNF-α is one of the major inflammatory cytokine secreted from macrophages, dendritic cells and T cells during infectious disease like PTB which is essential for host defense against *Mycobacterium tuberculosis* and also promotes granuloma formation [4]. Perhaps TNF-α is an indispensible inflammatory cytokine with multiple roles in both immunological and pathophysiological responses in TB [5].

EPO is an endogenous glycoprotein hormone produced mainly by kidney and is crucial for controlling Red Blood Cell (RBC) production. It stimulates erythropoiesis by acting on the bone marrow where it promotes terminal differentiation of progenitor cells into erythrocytes [6]. Indeed the potent stimulus for increased production of EPO is diminished arterial oxygen content along with anaemia or hypoxia [7].

In chronic diseases like PTB, anaemia is the most common haematological disorder with multifactorial aetiology, however inflammatory cytokine and iron deficiency have been indicated in the pathophysiology of anaemia in PTB [8,9]. In the presence of increased levels of TNF-α, the production of EPO is impaired and the biological activity is reduced [10].

Probably, to know the disease severity, extent of disease, prognosis and monitoring the clinical effect of Anti Tuberculoses Therapy (ATT), estimation of immunological and pathological parameters seems to be the better predictive factors in PTB. From this background, the present study was carried out with the hypothesis that increased bacterial burden increases TNF-α level which in turn influences EPO action on pathophysiology of anaemia during chronic infection like PTB.

## MATERIALS AND METHODS

### Study Design and Population

The present cross-sectional study was conducted at Navodaya Medical College Hospital and Research Centre, Raichur, Karnataka, India from January 2016 to January 2017. The study included 180 newly diagnosed sputum positive PTB patients as cases. The clinical diagnosis of cases was done by pulmonologist and PTB was confirmed by microscopic examination of sputum specimen for the detection of AFB. The age and sex matched 100 healthy individuals were included as controls. The study protocol was approved by Institutional Human Ethics Committee (IEC Ref No.-59/2015-16). Before enrolment, written informed consent was taken from all the study participants after explaining in detail about study procedure.

**Inclusion criteria:** The subjects diagnosed as "new cases" of PTB, possessing at least two sputum smear test positive for AFB were included as cases and the healthy individuals with no previous history of any major diseases were included as controls in the present study.

**Exclusion criteria:** The patients with extrapulmonary TB and/or patients requiring surgical intervention, patients with history of prior anti TB treatment, patients with other lung disorders, patients with HIV, with organ transplantation, treatment with corticosteroids, with chronic renal failure, diabetes mellitus, liver failure and patient with recent myocardial infarction were excluded from the study.

### Haematological and Biochemical Parameters

About 6 mL of venous blood sample was drawn from median cubital vein under aseptic condition from each study subjects. Out of which, one part of blood sample (2 mL) was collected in an EDTA vial and immediately processed for haematological parameters using automated cell counter (ABX Pentra 60, Horiba, Japan) by flow cytometry method.

The second part of blood sample collected in plain vial were allowed to clot for 30 minutes and then centrifuged at 3000 rpm for 10 minutes. The separated serum samples were stored at -80°C until assayed. Serum levels of TNF-α and EPO in all the study participants were measured by sandwich ELISA method. Serum levels of TNF-α were measured by using commercially available TNF-α ELISA kit (Diaclone, France) according to manufacturers' instructions [11]. Serum EPO was estimated by using commercially available EPO ELISA kit (Biomerica, USA) according to manufacturers' instructions [12].

## STATISTICAL ANALYSIS

The statistical analysis was performed by using SPSS software version 16.0. The results were analysed with descriptive statistics, wherever appropriate. The Student unpaired t-test, and Pearson's correlation coefficient test were used to evaluate the statistical significance in the results. We compared mean values for serum TNF-α, EPO and Hb levels in PTB patients with different sputum AFB grading using Kruskal-Wallis test [13]. The p-value of <0.05 was considered as threshold of significance.

## RESULTS

In the present study, 180 newly diagnosed sputum positive PTB cases with mean age of 44.1±16.2 years (age range 19-75 years) and 100 healthy controls with mean age of 43.49±15.6 years (age range 19-72 years) were included. Among 180 PTB cases 101 were males and 79 were females and in 100 controls 54 were males and 46 were females. There was no difference between cases and controls with respect to age and sex.

The mean level of Hb, RBC, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were lower while WBC, platelets, Erythrocyte Sedimentation Rate (ESR), TNF-α and EPO were found to be higher in PTB cases compared to healthy controls (p<0.05) as illustrated in [Table/Fig-1].

We noted increased serum levels of TNF-α and EPO in PTB cases when compared to controls (p<0.001 and p<0.001 respectively) as shown in [Table/Fig-1]. Further, we compared mean values for TNF-α, EPO and Hb levels in PTB cases with different sputum AFB gradings. Chi-square test showed statistically significant associations between TNF-α, EPO and Hb with bacterial burden (p<0.001) measured in terms of sputum AFB gradings in PTB [Table/Fig-2].

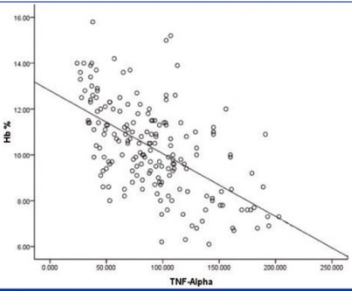
We observed statistically significant inverse correlation between serum TNF-α and Hb level (r=-0.58, p<0.001) in PTB cases [Table/Fig-3] and also between serum levels of TNF-α and EPO (r=-0.49, p<0.001) in PTB cases [Table/Fig-4]. Furthermore, we found statistically insignificant correlation between serum EPO and Hb level (r=-0.101, p>0.05) in PTB cases [Table/Fig-5].

| Parameters | Healthy control (Mean±SD) | PTB cases (Mean±SD) | p-value |
|---|---|---|---|
| Hb (gm/dlL) | 14.10±1.21 | 10.30±2.0 | <0.001* |
| RBC (Millions/cumm) | 4.96±0.57 | 4.05±0.75 | <0.001* |
| PCV (%) | 41.50±3.40 | 30.53±5.79 | <0.001* |
| MCV (fL) | 85.50±7.47 | 75.45±9.73 | <0.001* |
| MCH (pg/cell) | 29.20±2.01 | 25.51±3.90 | <0.001* |
| MCHC (gm/dL) | 34.10±1.21 | 33.65±2.49 | <0.05* |
| WBC (Cells/cumm) | 6574±1360 | 10227±3645 | <0.001* |
| Platelets (Lakhs/cumm) | 2.96±0.70 | 4.24±1.42 | <0.001* |
| ESR (mm/hour) | 5.83±3.34 | 67.40±22.10 | <0.001* |
| TNF-α (pg/mL) | 14.40±3.72 | 90.68±31.69 | <0.001* |
| EPO (mIU/mL) | 17.30±6.60 | 35.87±7.82 | <0.001* |

**[Table/Fig-1]:** Comparison between mean of different parameters in PTB cases and healthy controls.
*p<0.05 is significant
PTB: Pulmonary tuberculosis; Hb: Haemoglobin; RBC: Red blood cell; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White blood cell; ESR: Erythrocyte sedimentation rate; TNF-α: Tumour necrosis factor-alpha; EPO: Erythropoietin

| Sputum AFB grading | TNF-α (pg/mL) | EPO (mIU/mL) | Hb (gm/dL) |
|---|---|---|---|
| Scanty (n=14) | 33.18±5.73 | 32.81±6.18 | 12.81±1.2 |
| 1+ (n=41) | 52.74±16.24 | 40.53±7.15 | 10.97±1.55 |
| 2+ (n=41) | 84.14±20.56 | 37.40±7.0 | 9.84±1.4 |
| 3+ (n=73) | 112.45±28.46 | 33.73±6.20 | 8.90±1.2 |
| 4+ (n=11) | 177.0±17.94 | 24.45±6.30 | 7.21±0.7 |
| Chi square value | 126.46 | 51.06 | 71.34 |
| p-value | <0.001* | <0.001* | <0.001* |

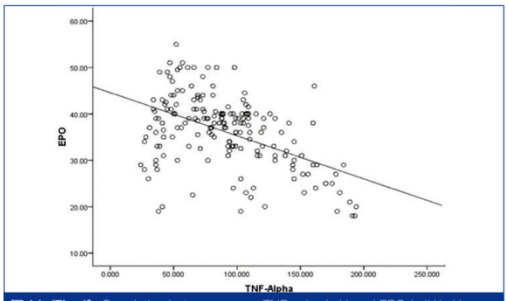
**[Table/Fig-2]:** Comparison between mean±sd values for TNF-α, EPO and Hb in PTB cases with different sputum AFB grading.
*p<0.05 is significant by Kruskal-Wallis test
AFB: Acid fast bacilli; n: Number of pulmonary tuberculosis cases; TNF-α: Tumour necrosis factor-alpha; EPO: Erythropoietin; Hb: Haemoglobin; PTB: Pulmonary tuberculosis



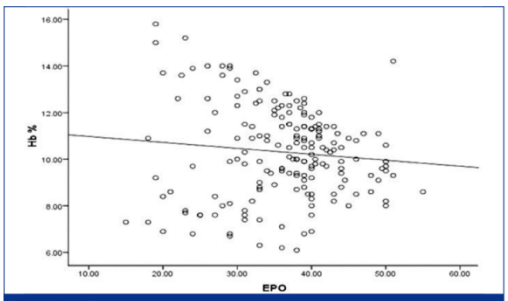

**[Table/Fig-3]:** Correlation between serum TNF-α (pg/mL) and Hb (gm%) level in PTB patients (r=-0.58, p<0.001).
p<0.05 is significant
TNF-α: Tumour necrosis factor-alpha; Hb: Haemoglobin; PTB: Pulmonary tuberculosis

## DISCUSSION

In the present study, we aimed to estimate the role of TNF-α on EPO function and its correlation with haematological profile in different grades of PTB. The abnormal haematological parameters in PTB patients observed in the present study were in accordance with previous studies [12,14-18]. Present study result showed statistically significant differences in serum levels of TNF-α and EPO between PTB cases and controls. We observed inverse correlation between TNF-α and EPO and between TNF-α and Hb in PTB. Further, we also noticed statistically insignificant correlation between EPO and Hb in PTB cases.




**[Table/Fig-4]:** Correlation between serum TNF-α (pg/mL) and EPO (mIU/mL) levels in PTB patients(r=-0.49, p<0.001).
p<0.05 is significant
TNF-α: Tumour necrosis factor-alpha, EPO: Erythropoietin; PTB: Pulmonary tuberculosis




**[Table/Fig-5]:** Correlation between serum EPO (mIU/mL) and Hb (gm%) level in PTB patients (r=-0.101, p>0.05).
p>0.05 is not significant
EPO: Erythropoietin; Hb: Haemoglobin; PTB: Pulmonary tuberculosis

In PTB the invading bacteria induces the synthesis of TNF-α along with other inflammatory cytokines through the activation of monocytes and T-lymphocytes. There was a significant positive correlation between clinical severity and serum TNF-α level. Further to monitor the disease severity, TNF-α seems to be a useful marker in PTB patients [19]. Similarly in the present study, we have found increased serum TNF-α level in PTB patients with increase in bacterial load. The subnormal Hb associated with severity of PTB as observed in present study was considered to be multifactorial and might be related to the underlying inflammatory mechanism.

Few studies have reported blunted EPO response in the presence of increased TNF-α level in chronic disorders [12,20-23]. The documentary evidence of these studies throws more light on the fact that in kidney TNF-α inhibits EPO production and interferes with EPO mediated expression of specific transcription factors involved in erythrocyte differentiation control [24,25]. Further, the TNF-α directly inhibits the proliferation and differentiation of erythroid progenitor cells. In addition, TNF-α along with IL-1, IL-6 and IL-10 as a defence against invading microbial pathogens to effectively withhold the iron from microbes, induces the expression of ferritin and stimulates iron storage and retention within the macrophage. The resulted low circulatory iron fortuitously decreases the iron supply for erythroid cells establishing hypoferremia. Thus, ineffective erythropoiesis together with relative inadequacy of EPO production contributed in low Hb and subsequent development of anaemia of chronic disease [20,24,26].

The results of present study probably explained on the basis of above findings, wherein we have reported, commensurate increase in serum TNF-α and decrease in Hb level with increased bacterial burden in PTB patients. Despite of increased serum EPO in PTB cases compared to controls, present study results strongly indicated a relative lack of endogenous EPO in proportion to the Hb concentration. The EPO down regulation in present study might be due to the burst of inflammation that took place during increased bacterial burden with concomitant increase of TNF-α which might have ultimately contributed in the development of anaemia in PTB.

Few studies have highlighted on increased levels of serum EPO in both anaemia of chronic disease like PTB and iron deficiency anaemia however, found more significant levels of EPO in iron deficient subjects indicating a role of inflammatory cytokines on EPO response in chronic diseases [18,21]. Yet another study demonstrated comparatively low levels of serum EPO in patients with chronic infection and malignancy than in patients with iron deficiency anaemia and primary haematopoietic disease with same degree of anaemia [27]. The TNF-α mediated reduction of both EPO as well as erythropoietin receptor protein advancing to ineffective erythropoiesis in β-Thalassaemia/haemoglobin E patients has been also reported by another researcher [28].

Kulkarni RA et al., compared the levels of TNF-α, EPO and Hb in PTB patients and found inverse correlation between TNF-α and Hb and between TNF-α and EPO. Further in the same study, they found no inverse correlation between EPO and Hb however, blunted response of EPO to the degree of anaemia was reported [11]. However, a similar type of study conducted recently on COPD patients reported an inverse correlation between serum EPO level and both Hb and haematocrit levels [23].

Concurrent findings were observed in present study with significant inverse correlation between TNF-α and Hb levels and also between TNF-α and EPO levels in PTB cases. Furthermore, the expected inverse correlation between serum EPO and Hb levels was absent in PTB cases. These findings showed that TNF-α along with other cytokines might inhibit EPO production and also induce the formation of nitric oxide and oxygen free radicals which directly inhibit the expression of EPO and damage EPO producing cells [29]. In addition, these cytokines alters iron metabolism, inhibits proliferation of erythrocyte progenitor cells and also reduces EPO receptor proteins leading to EPO resistance and resultant under maintenance of Hb level [28,30]. Further in continuation to this, we have grouped the PTB patients according to sputum AFB gradings to show the disease severity and compared the mean values for serum TNF-α, EPO and Hb levels which were not done previously. The [Table/Fig-2] clearly showed that, increased bacterial burden measured in terms of sputum AFB grade resulted in increased activity of TNF-α in PTB cases. This shows that there was a direct association between bacterial burden and serum TNF-α. There was a decreasing trend among serum EPO and Hb levels with agreement with severity of PTB. Initially, when the disease severity were less, we found raised serum EPO level in PTB cases which could be due to little increase in serum TNF-α at that time. However, as the bacterial burden increased, we observed down fall of both serum EPO and Hb levels, which might be due to the inhibitory effect of increased TNF-α in PTB cases. The PTB is most commonly seen in people with low socioeconomic and poor nutritional status and hence anaemia of inflammation as well as iron deficiency anaemia may occur simultaneously in PTB patients. Probably in the present study, absence of coordination between EPO and Hb due to increased expression of inflammatory cytokine TNF-α might have progressed towards the establishment of anaemia in PTB cases.

One of the stimulus for increased production of EPO is low Hb level, however in the present study, none of the healthy controls showed low levels of Hb and hence there was no increase in serum EPO in controls [31]. However, many PTB cases of the present study showed low levels of Hb. This might have stimulated EPO production in the initial stages, when disease severity was less as evident from

sputum AFB gradings and little increase in serum TNF-α. Therefore, there was no significant inhibitory effect of TNF-α on EPO. Probably this was the reason behind higher EPO in PTB cases than controls as observed in present study. Further it is stated that, as the disease severity was increased, despite of low Hb, the level of EPO could not increase which might be due to the suppression of EPO by increased levels of TNF-α and further anaemia in PTB. Thus, in the present study, so far we were able to show the link between Hb concentration and serum levels of both TNF-α and EPO in PTB patients with different sputum AFB gradings.

## LIMITATION

Further extended large scale study would be required to attain firm conclusion. In the present study, we have not done follow-up of PTB patients which could have been more informative in evaluating prognostic applications of inflammatory marker and EPO.

## CONCLUSION

The present study highlighted on the fact that increased bacterial burden was associated with increased serum TNF-α level. The relative deficiency of serum EPO levels in proportion to Hb concentration which played a vital role in the pathophysiology of anaemia was due to the increased TNF-α in PTB. Thus, we concluded that during treatment, to reduce the negative prognostic impact of anaemia associated with reduced survival and increased morbidity, we suggest therapeutic administration of recombinant human EPO could be helpful to overcome anaemia in PTB.

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# Serum VEGF and TNF-α Correlate Bacterial Burden in Pulmonary Tuberculosis

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## ABSTRACT

**Background:** Pulmonary tuberculosis (PTB) is one of the predominant causes of death worldwide. The tissue hypoxic condition seen in PTB induces the increased expression of vascular endothelial growth factor (VEGF) and the inflammatory cytokine tumor necrosis factor-alpha (TNF-α). In the present study we aimed to evaluate the role of serum VEGF, TNF-α and their correlation with bacterial burden measured in terms of sputum acid fast bacilli (AFB) grading in PTB.

**Method:** The study included 120 newly diagnosed PTB cases and 60 healthy controls. Sputum samples from cases were subjected to microscopy for the detection of AFB. Demographic and anthropometric characteristics were recorded. Serum VEGF and TNF-α were estimated by ELISA method.

**Results:** Serum levels of VEGF and TNF-α in PTB patients were significantly higher compared to controls ($p<0.001$ & $p<0.001$ respectively). BMI of PTB cases was lower than controls ($p<0.001$).We observed significant positive correlation between serum VEGF and sputum AFB grade ($r=0.773$, $p<0.001$) and serum TNF-α and sputum AFB grade ($r=0.662$, $p<0.001$). We also noted a significant positive correlation between serum VEGF and serum TNF-α ($r=0.763$, $p<0.001$) in PTB patients.

**Conclusions:** The increased serum levels of VEGF and TNF-α were associated with bacterial burden in PTB. Hence, positive sputum smear for AFB, low BMI and increased serum VEGF and TNF-α could be early diagnostic markers and may help immediate treatment regimen for PTB.

***Keywords:*** *Angiogenic biomarker; Body mass index; Hypoxia; Inflammatory cytokine; Pulmonary tuberculosis; Sputum acid fast bacilli grading.*

## INTRODUCTION

Pulmonary tuberculosis (PTB) is one of the predominant causes of death worldwide. Among the estimated global incidence of 10.4 million new tuberculosis (TB) cases, India alone accounted for approximately 2.2 million cases[1].

Several socioeconomic factors contribute towards the occurrence of TB like poverty, poor nutrition, illiteracy, poor housing, overcrowding, immigration and poor access to the health[2, 3]. The increased risk of progression from TB infection to active disease is associated with deficiencies of essential macro and micronutrients leading to a negative impact on cell mediated immunity[4]. A low body mass index (BMI) is another individual risk factor for the development of active TB[5].

The microenvironment is a characteristic feature of tissue hypoxia during inflammation and is associated with bacterial infection such as PTB. The increased

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oxygen demand and its decreased supply may result in hypoxia, which is observed during bacterial infection[6]. In mammalian cells response to hypoxia is mediated by hypoxia inducible factor-1α (HIF-1α), a transcription factor which is found to be a potent stimulant to express vascular endothelial growth factor (VEGF), erythropoietin, basic fibroblast growth factor, glycolytic enzymes and glucose transporters synthesizing genes involved in the regulation of metabolism, angiogenesis, apoptosis and cellular stress[7, 8].

VEGF is also considered as one of the major mediators of angiogenesis and vascular permeability. VEGF has multiple roles in lung development and is expressed in many parts of lungs and pleura[9]. Low oxygen microenvironment which can alter expression of VEGF and inflammatory marker like tumor necrosis factor-alpha (TNF-α) has been interest of research in TB. It has also been observed that stimulation of HIF-1α by hypoxia and bacterial exposure can also induce the production of TNF-α[8, 10].

The objective of the study is to evaluate the role of oxygen sensing cellular biomarkers like VEGF and TNF-α in the progression of PTB in a semi-urban backward area of North Karnataka, India.

## MATERIALS AND METHOD

**Study design and population:** 120 newly diagnosed sputum positive PTB patients were enrolled prospectively in this cross sectional study as cases. The study was conducted at Navodaya Medical College Hospital and Research Centre Raichur, Karnataka, India from Jan 2016 to Jan 2017. All the cases were clinically diagnosed by pulmonologist and PTB was confirmed by microscopic examination of sputum specimen for the detection of acid fast bacilli (AFB). Age and sex matched 60 healthy individuals were included in the study as controls. Ethical clearance from the Institutional Ethics Committee was obtained. The participants enrolled as cases and controls in this study were explained in detail about the study procedure. The written informed consent was taken from all the study participants

**Inclusion criteria:** Cases diagnosed as "new case" of TB, possessing at least two sputum smear test positive for AFB were included as cases in the study. Healthy individuals with no previous history of any major diseases were included as control.

**Exclusion criteria:** Patients with extra pulmonary TB and/or patients requiring surgical intervention, chronic PTB (receiving at least two courses of anti TB treatment for more than six months), patients with other lung disorders, such as chronic obstructive pulmonary disease asthma, bronchitis and lung cancer, HIV, with organ transplantation, treatment with corticosteroids, chronic renal disease, liver failure and recent myocardial infarction were excluded from the study.

**Physical anthropometry:** Body mass index (BMI): All the study participants were weighed barefoot with minimum clothing using an electronic weighing machine. Body weight was recorded to the nearest of 0.1kg. Height was measured to the nearest of 0.1cm using standard measuring tape. BMI was calculated using the formula BMI=Weight (kg)/Height$^2$ (m). The cases and controls were classified based on BMI (kg/m$^2$) as per WHO criteria[11].

**Waist to hip ratio:** Was obtained by dividing the waist circumference by hip circumference

**Molecular markers:** From all the study subjects about 5 ml of venous blood samples were collected in plain vial from median cubital vein under aseptic condition which were allowed to clot for 30 minutes and then centrifuged at 3000 rpm for 10 minutes. Separated serum samples were stored at -80°C until assayed. Serum VEGF levels were measured by using commercially available VEGF ELISA kit (RayBiotech, Norcross, GA) and serum levels of TNF-α were measured by using commercially available TNF-α ELISA kit (Diaclone, France) according to manufacturers' instructions.

## STATISTICAL ANALYSIS

The results were analyzed with descriptive statistics, wherever appropriate. The student unpaired "t" test, Spearman's rank correlation coefficient test, chi-square test and Pearson's correlation coefficient test were used to evaluate the statistical significance in the results. The *p value* of <0.05 were considered statistically significant. Statistical analysis was performed by using SPSS version 16.0 software.

## RESULTS

In the present study, demographic and anthropometric characteristics of PTB cases and controls were shown in Table-1. About 81 (67.5%) PTB cases were from age groups between 25 and 54 years. Among these 81 cases, 33 (27%) cases constituted age group between 35 and 44 years.

Among 120 PTB cases, BMI of 110 PTB cases (91.67%) were lower (<18.5kg/m²) than the controls. Further it was observed that, out of 110 PTB cases, 32 PTB cases (26.67%) were having <16.0kg/m² BMI. Waist to hip ratio of PTB cases was also found to be lower as compared to controls ($p$<0.001).

**Table 1: Demographic and Anthropometric characteristics of PTB cases and controls**

| Characteristics | Controls n (%) | PTB Cases n (%) | Chi square ($x^2$)/t value | $p$ value |
|---|---|---|---|---|
| **Age (years) mean ± SD** | **44.8 ± 16.9** | **42.5 ± 15.3** | **t = 0.7704** | **0.451*** |
| **Age groups (years)** | | | | |
| 15-24 | 7(11.67) | 14(11.66) | $x^2$=5.534 | 0.345* |
| 25-34 | 8(13.33) | 19(15.83) | | |
| 35-44 | 10(16.67) | 33(27.50) | | |
| 45-54 | 14(23.33) | 29(15.83) | | |
| 56-64 | 13(21.67) | 18(15) | | |
| ≥65 | 8(13.33) | 17(14.16) | | |
| **Sex** | | | | |
| Male | 32(53.33) | 75(62.50) | $x^2$=3.069 | 0.080* |
| Female | 28(46.67) | 45(37.50) | | |
| **BMI (Kg/m²)** | | | | |
| Severely under weight (<16) | 00 | 32(26.67) | $x^2$=115.3 | <0.001** |
| Moderate under weight (16-16.9) | 00 | 34(28.33) | | |
| Mild under weight (17-18.49) | 00 | 44(36.67) | | |
| Normal (18.5-24.9) | 57(95) | 10(8.33) | | |
| Over weight (≥25) | 03(05) | 00(00) | | |
| **Waist hip ratio** | | | | |
| Male (mean ± SD) | 0.91 ± 0.03 | 0.82 ± 0.05 | t=6.9452 | <0.001** |
| Female (mean ± SD) | 0.86 ± 0.04 | 0.78 ± 0.03 | t=8.6684 | <0.001** |

**p* value (>0.05) not significant. ***p* value (<0.001) highly significant.

Serum levels of VEGF and TNF-α in PTB patients were significantly higher compared to controls ($p$<0.001) as shown in Table-2.

**Table 2: Statistical comparison between PTB patients and control regarding serum VEGF (pg/ml) and TNF-α (pg/ml)**

| Parameters | Control | PTB Cases | t value | p value |
|---|---|---|---|---|
| VEGF (Mean ± SD) | 26.36 ± 10.59 | 604.89 ± 323.43 | 10.557 | <0.001** |
| TNF-α (Mean ± SD) | 14.01 ± 3.59 | 92.79 ± 38.57 | 12.045 | <0.001** |

***p* value (<0.001) highly significant

Distributions of PTB cases based on bacterial load were illustrated in Fig.1.

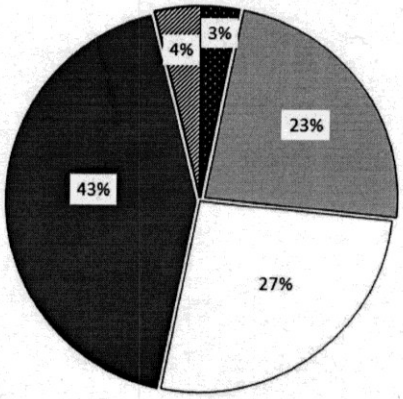


**Fig. 1: Distribution of Pulmonary Tuberculosis cases based on bacterial load.**
**Scanty, 1+, 2+, 3+ and 4+ are AFB grading.**

We observed significant positive correlation between serum VEGF and sputum AFB grade (r=0.773, $p$<0.001) in PTB (Fig. 2) and also between serum TNF-α and sputum AFB grade (r=0.662, $p$<0.001) in PTB (Fig. 3). Serum VEGF levels were more pronounced than serum TNF-α levels. Further, we have also found a significant positive correlation between serum VEGF and serum TNF-α (r=0.763, $p$<0.001) in PTB cases (Fig. 4).

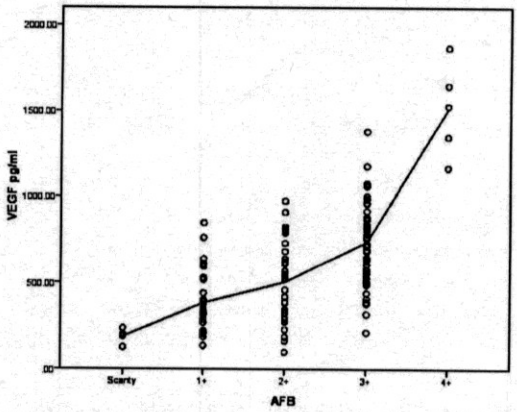


**Fig. 2: Correlation between VEGF and sputum AFB grading in PTB cases, (r=0.773, p<0.001).**

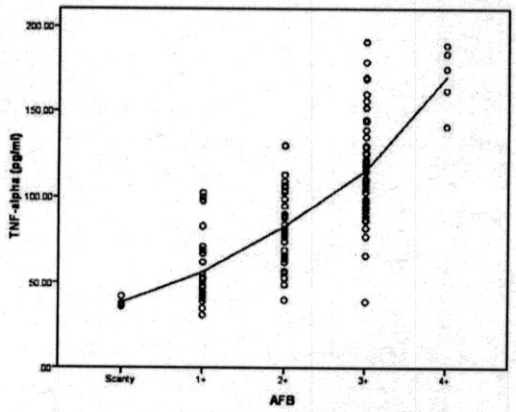


**Fig. 3: Correlation between TNF-α and sputum AFB grading in PTB cases, (r=0.662, p<0.001).**

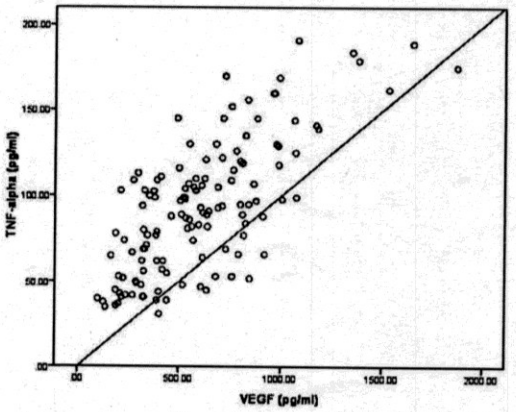


**Fig. 4: Correlation between VEGF and TNF-α in PTB cases, (r=0.763, p<0.001).**

## DISCUSSION

Results from our study on BMI indicate a poor nutritional status in PTB. Previous studies showed that reduced BMI is a significant risk factor for PTB due to the induced impairment of cellular immunity[4, 12]. Further BMI was an important marker for the assessment of nutritional status in PTB patients[13]. In the present study waist to hip ratio of PTB patients was also found to be low when compared to control, which was in agreement with the study conducted by Tungdim *et al*[14].

Our study showed that serum levels of VEGF and TNF-α in PTB patient were significantly higher compared to controls. There was an increasing trend of hypoxia markers (VEGF and TNF-α) in agreement with severity

of PTB. As the bacterial burden increased the expression of VEGF and TNF-α were also increased. Tissue Hypoxia is a common feature in bacterial infection which induces increased expression of HIF-1α transcription factor which in turn increases the expression of VEGF gene by up regulating NF-κB[15]. Several factors regulate VEGF gene expression; among these hypoxia plays a key role in PTB. The activated alveolar macrophages are the main cells which secrets VEGF in PTB[16]. Few studies have indicated higher levels serum VEGF in PTB patients [17-20]. Our findings on serum VEGF corroborate with Alatas *et al*, who concluded increased activities of serum VEGF in PTB patients may be an indicator of active PTB[17]. VEGF might associate with pathogenesis of PTB and measurement of serum VEGF may be a useful screening marker of active PTB and also useful for the prognosis and monitoring the clinical effect of anti tuberculoses therapy[18]. VEGF could be used to indicate bacterial burden in addition to know the disease severity, extent of disease and therapeutic monitoring. Our study was in accordance with previous studies by researchers wherein we have showed, increased bacterial burden measured in terms of sputum AFB grade resulted in increased activity of VEGF in PTB cases[19]. It can be further stated that increased expression of VEGF probably increased the supply of blood and oxygen to affected lung tissues and develop angiogenesis to protect PTB patients from low oxygen sensing microenvironment[20]. It has been reported that circulating levels of angiogenic biomarker VEGF in individual with PTB, latent TB (LTB) or no TB infection (NTB) confirmed that VEGF was an important biomarker to distinguish PTB from LTB and NTB[19].

In the present study along with VEGF, the serum levels of inflammatory cytokine TNF-α were also increased in proportion with bacterial load suggesting a possible pathophysiological role of both VEGF and TNF-α in PTB. The low BMI observed in our study was probably due to the increased production of TNF-α. In PTB, TNF-α induces muscle and fat tissue catabolism resulting in weight loss[21]. This suggests that increased bacterial load increases TNF-α which may further lower BMI in PTB patients. The TNF-α increases early in TB and performs complex role in the host response to *Mycobacterium tuberculosis* by exhibiting antimycobacterial activity and promoting the formation of granuloma in PTB patients[22]. The high levels of TNF-α observed in PTB patients in our study were in agreement with other studies[23-26]. Few studies have indicated hypoxia can also induce TNF-α production in diseases[8, 10]. A significant positive correlation between VEGF and TNF-α in the present study indicate their association with bacterial burden and also shows their important pathophysiological role in PTB.

## CONCLUSIONS

The present study concludes that bacterial burden increases concurrently with induction of hypoxia resulting in the increased expression of both VEGF and TNF-α. However among low oxygen sensing microenvironment markers, serum VEGF found to be relatively more pronounced when compared to serum TNF-α in PTB. It may be further concluded that positive sputum smear for AFB, low BMI along with increased serum levels of VEGF and TNF-α could be useful in the early diagnosis of active PTB and also it could be used as marker of early detection of tissue damage in PTB which could help in the immediate initiation of treatment regimen and propagation of PTB.

**Conflict of Interest:** The authors have none to declare.

**Source of Funding:** Self funded study.

**Ethical Approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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**Conferences attended:**

- ✓ Attended 3rd Annual Conference of Association of Physiologists of India, ASSOPICON 2016 at BLDE(DU)'s Shri B. M. Patil Medical College Hospital and Research Centre, Vijayapura held from 14th to 17th September 2016 and presented poster entitled "**Correlation between Expression of Hypoxia Induced Factors and Bacterial Burden in Pulmonary Tuberculosis"** and awarded as **2nd Best Paper**.
- ✓
- ✓ Attended South Zone Conference of Association of Clinical Biochemist of India, ACBICON-2018 held from 7th to 8th December 2018 at Kasturba Medical College, MAHE, Manipal and presented a paper entitled "**Role of Circulating Predictive Markers in Measuring Bacterial Burden in Pulmonary Tuberculosis Patients".**

# 3rd Annual Conference of Association of Physiologists of India

**ASSOPICON 2016**

**14th-17th September 2016**

*Theme : Physiology Decodes Novelty of Vascular Science*

## CERTIFICATE

This is to certify that

Dr/Mr/Miss Harish Bhat K

Bearing Reg No. - - has partcipated as Delegate/Chairperson/Jury/Resource person/Presented Oral /Poster

Topic entitled Correlation betn expression of ... in pulmonary tuberculosis.

In ASSOPICON-2016 held from 15th-17th September 2016,

Organized by Department of Physiology,

BLDE University's Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapur, Karnataka.

Karnataka Medical Council has granted 4 credit hours for Delegates/Faculty

vide letter No.: K.M.C./C.M.E./701/2016

| Dr. Manjunatha Aithala | Zonal Chairman | Dr. S. P. Guggarigoudar | Dr. G. K.Pal |
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3rd Annual Conference of Association of Physiologists of India

# 3rd Annual Conference of Association of Physiologists of India

**ASSOPICON 2016**

**14th-17th September 2016**

*Theme : Physiology Decodes Novelty of Vascular Science*

## Best Paper Award

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Dr/Mr/Miss Harish Bhat K

Bearing Reg No. —— has been confered with the **Best Paper Award (Oral/Poster)** ✓

Topic entitled "Correlation between Expression of Hypoxia Induced Factors and Bacterial Burden in PTB"

In ASSOPICON-2016 held from 15th-17th September 2016,

Organized by Department of Physiology,

BLDE University's Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapur, Karnataka.

**Dr. Manjunatha Aithala**
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**Association of Clinical Biochemists of India - South Zone Conference 2018**

Theme: Recent trends in Biochemistry Education, Diagnostics & Research

# *Certificate of Participation*

This is to certify that

Harish Bhat K

**has presented a Scientific Paper (Oral / Poster)**

titled "Role of Circulating Predictive Markers in Measuring Bacterial Burden in PTB patients."

in "Association of Clinical Biochemists of India - South Zone Conference"

organized by Department of Biochemistry, Kasturba Medical College, Manipal, MAHE held between 7 & 8 December 2018

Karnataka Medical Council has granted **FOUR credit hours** for delegates. Vide letter no KMC/CME/517/2018 Dated: 12-11-2018

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Professor & Head, Department of Biochemistry, KMC Manipal

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