

**FERROXIDASE/ALBUMIN RATIO-
DIAGNOSTIC AND PROGNOSTIC MARKER
OF PULMONARY TUBERCULOSIS**

Dissertation
**M.D. (BRANCH XIII)
BIOCHEMISTRY**



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INTRODUCTION

Tuberculosis is a curable infectious disease causing significant morbidity and preventable deaths worldwide. This disease usually affects the Lungs , although other organs are involved in up to one-third of cases.

India , China , Indonesia , South Africa and Nigeria rank first to fifth Countries respectively in terms of absolute number of cases.

By recent survey in India both pulmonary and extra-pulmonary TB cases are endemic in Indian subcontinent . India has the highest TB burden in the world in terms of absolute number of incident Cases . To make the situation worse , TB has formed a lethal partnership with HIV.Untreated HIV infection is the greatest risk factor for progression from Latent Tuberculosis infection to TB disease . Hence timely screening for TB infection and treatment in HIV-infected people is necessary to increase the chances of survival and reduce the transmission of TB in the community.

In India broad- spectrum antibiotics are being prescribed inappropriately leading on to MDR-TB. Studies have reported that MDR-TB is significantly higher among treatment failures.This can be prevented by early referral for culture. Nowadays, Radiometry using BACTEC instrument is used to reduce the diagnostic time in cultured specimens. But still, using this technique, organisms can be detected only after 7 to 8 days in smear positive patients and 16 to20 days in smear negative patients¹.

While the treatment of TB is considered one of the most cost-effective interventions in DOTS programme, we are still without a fast and simple diagnostic test that would be applicable in high-burden but resource-poor settings.

The present study was conducted to find an application of serum Ferroxidase/albumin ratio as a marker to assist in diagnosis and monitoring the prognosis of PTB patients.

AIM AND OBJECTIVE

The aim of this study is to incorporate Serum Ferroxidase/albumin ratio as a surrogate marker to assist in diagnosis and prognosis of Pulmonary tuberculosis patients.

The Objective of this study-

- To measure and document the serum Ferroxidase/ albumin ratio in freshly diagnosed and completely treated cases with PTB.
- To compare the serum ferroxidase /albumin ratio between freshly diagnosed cases of PTB and apparently healthy controls.
- To compare the serum ferroxidase/ albumin ratio between completely treated cases of PTB and apparently healthy controls.

REVIEW OF LITERATURE

Once thought to be under control, TB is now the number one cause of infection related death world-wide². One third of the global population is infected with TB, of which 95% of the incidence is in developing countries³. At present, ~2 billion people are infected with TB and the number is expected to increase by 2020⁴. WHO estimated that 1.5 million people died from TB in 2006⁵.

The resurgence of TB due to rise in drug-resistant strains made WHO to declare TB as global health emergency in 1993⁶.

WHO-THE GLOBAL PLAN TO STOP TB 2006-2015

Achievement of the millennium development goal:" to have halted by 2015 and begun to reverse the incidence "of TB⁷.

EPIDEMIOLOGY

According to US center for disease control and prevention,

Clinical case definition⁸

A case that meets all of the following criteria:

*A positive tuberculin skin test

*Other signs and symptoms compatible with TB, such as abnormal chest radiograph or clinical evidence of current disease.

*Treatment with two or more ant tuberculosis medications

Laboratory criteria for diagnosis⁸

*Isolation of MTB from a clinical specimen.

*Demonstration of MTB from a clinical specimen by NAA test (Nucleic Acid Amplification Test)

*Demonstration of AFB in a clinical specimen when a culture has not been or cannot be obtained.

It is this last definition that satisfies the only criteria available in a resource-poor setting where only microscopy is available.

EPIDEMIOLOGICAL INDICES IN INDIA (2006)¹⁰

Population (thousands)	:1 151 751
Global rank (based on incident cases)	:Rank1
Incidence (all cases/100 000 population/year)	:168
Incidence (sputum smear positive/100 000 pop/yr)	:75

Prevalence (all cases /100 000 pop)	: 299
Mortality (deaths/100 000 pop/yr)	: 28
*of new TB cases, % HIV positive	: 1.2
*of new TB cases, % MDR-TB	: 2.8

INTERESTING FACTS ABOUT TB

TB has been present in humans since ancient times. The earliest detection of MTB is in the remains of bison dated 17,000 years before the present¹¹. Skeletal remains show prehistoric humans (3500-2650 BC) had TB¹² and tubercular decay has been found in the spines of mummies^{13, 14}.

OTHER NAMES OF TB

It includes Phthisis (Greek for consumption) ; wasting disease ; white plague because sufferers appear markedly pale ; King's evil because it was believed that a King's touch would heal the disease; Koch's disease, after the discovery of TB by Robert Koch¹⁵.

WORLD TB DAY

It falls on 24 March each year. It is designed to build public awareness about TB¹⁶.

ETIOLOGY OF TB

Mycobacterium belong to the family mycobacteriaceae and the order actinomycetales. MTB is the most common and important agent of human disease. MTB, which is a rod-shaped, aerobic bacterium resists decolorization by acid alcohol on gram's staining. Hence called as Acid-fast bacilli¹⁷.

CELL WALL OF MTB

High content of mycolic acid and long chain cross-linked fatty acids, mono and multiacylated trehalose glycolipids, diacylated long-chain polyketol diols and acylated phosphatidyl – myo - inositol- mannosides present in the cell wall of MTB contributes acid-fastness to the bacilli¹⁸.

HOST FACTORS

- 1 .Age: TB affects all ages¹⁹.
2. Sex: TB is more prevalent in males than in females²⁰.
3. Nutrition: Malnutrition is important risk factor for acquiring TB²¹.

SOCIAL FACTORS

Poor quality of life, homelessness²², lack of education, lack of awareness

of TB, tobacco smoking²³ are some of the known social factors contributing to the risk of TB infection.

MODE OF TRANSMISSION

MTB from infectious PTB patients spread to others by **droplet nuclei**, which are aerosolized by coughing, sneezing or speaking.

RISK FACTORS FOR DEVELOPING ACTIVE TB

1. Risk of active TB in adult-house hold contacts of smear positive PTB is high²⁴.

2. Risk of active TB among persons who have been infected with tubercle bacilli includes-

- * Recent infection (<1 yr)
- * Fibrotic lesions
- * Comorbidity-HIVinfection
- * Silicosis
- * Chronic renal failure
- * Diabetes
- * Intravenous drug use
- * Immunosuppressive treatment²⁵

CLINICAL MANIFESTATIONS OF PTB

It includes

1. Chest pain,
2. Coughing up blood, and
3. A productive, prolonged cough for more than 3 weeks.

Systemic symptoms includes

1. Fever,
2. Chills,
3. Night sweats,
4. Appetite loss,
5. Weight loss,
6. Pallor, and
7. Easy fatigability.

PATHOGENESIS OF PTB

PTB infection begins with inhalation of mycobacterium from infectious patients. Inhaled bacilli reaches the alveoli. There, alveolar macrophages phagocytize the bacilli and the phagocytosis is enhanced by complement activation²⁶. After a phagosome forms, the survival of MTB depends on virulence of tubercle bacilli.

VIRULENCE OF TUBERCLE BACILLI

Lipo- arabinomannan, a glycolipid in the bacterial cell wall inhibits the intra - cellular increase of calcium. This impairs calcium / calmodulin dependent fusion of phagosome – lysosome thereby it facilitates the survival of bacilli within the phagosome²⁷.

IMMUNOLOGIC RESPONSE TO MTB

Once MTB antigens, such as early secretory antigenic target6-KDa (ESAT-6) are internalized²⁸, the macrophages digest them into smaller fragments. These fragments(epitopes) binds to infected macrophages and other antigen presenting cells like monocytes and dendritic cells. Toll – like receptors (TLRs) play an essential role in the recognition of MTB fragments by macrophages and dendritic cells. This in turn transports the epitopes to the

macrophage surface for presentation to T lymphocytes^{29, 30}.

The result is the activation and proliferation of CD4+ T lymphocytes which contribute to host defense by macrophage-activating response and tissue-damaging response³¹.

MACROPHAGE-ACTIVATING RESPONSE

Activated CD4+ T cells produce cytokines such as IFN- γ , an activator of Macrophages and monocytes, IL-2, IL-5, IL-10, IL-13, IL-1 and TNF- α . These cytokines induce the generation of reactive nitrogen intermediates which has anti mycobacterium activity. In addition, macrophages undergo apoptosis in containing the growth of the bacilli^{32, 33}.

TISSUE DAMAGING RESPONSE

It is a delayed-type hypersensitivity reaction to various bacillary antigens. It destroys inactivated macrophages that contain bacilli but also causes caseous necrosis in the central part of lesion³⁴.

Although both these responses can inhibit mycobacterium growth, it is the balance between the two that determines the form of TB that will develop subsequently³⁵.

PATHOLOGICAL CLASSIFICATION OF PTB³⁶

PRIMARY TB

It occurs soon after the initial infection with tubercle bacilli accompanied by hilar or paratracheal lymphadenopathy. In majority, the lesion heal spontaneously evident as Gohn's lesion.

At the site of primary lesion, the activated macrophages secrete various cytokines resulting in the formation of granuloma. Mycobacteria are contained within granuloma and thus prevented from spreading all over the body.

PROGRESSIVE PRIMARY TB³⁶

In children and in persons with impaired immunity (eg, those with malnutrition or HIV infection), primary PTB may progress rapidly to clinical illness. The primary site rapidly enlarges, its central portion undergoes necrosis and cavitation.

POST PRIMARY TB³⁶

Also called adult-type or secondary TB, post primary TB results from

endogenous reactivation of latent infection. The characteristic pathological feature is the tuberculous cavity.

Granulomas of secondary TB fail to contain the spread of the TB infection and is the major cause of tissue damage in TB.

CERULOPLASMIN

Cp is an α_2 -globulin that contains ~95% of the total copper found in serum³⁷. Each molecule of Cp contains six to eight copper atoms, most of which are tightly bound.

BIOCHEMISTRY

Cp has a single polypeptide chain with 1046 amino acids and three Glucosamine - linked oligosaccharide. The peptide chain and carbohydrate together result in a mean molecular mass of 132 KDa. Cp is very susceptible to proteolysis by many proteases including trypsin, plasmin, leukocyte elastase, and a plasma metalloproteinase³⁸.

SITE OF SYNTHESIS

Cp is synthesized primarily by the hepatic parenchymal cells, with small amounts synthesized by macrophages and lymphocytes.

Recent studies have identified the lung as another site of Cp synthesis and support Cp critical role in host defense against oxidative damage and infection in the lung³⁹.

CLINICAL SIGNIFICANCE^{40, 41}

Increased plasma levels

1. Primary (Genetic) elevation.
2. Secondary elevation : Synthesis of Cp is increased in acute phase reaction. Synthesis is stimulated markedly by oestrogen. Larger increase occurs during pregnancy.

Decreased plasma levels

1. Primary (Genetic) deficiency.
2. Secondary deficiency due to dietary Copper insufficiency (including malabsorption), Menke's, Wilson's disease.

FUNCTIONS OF Cp

1. Cp is an important extracellular antioxidant and free radical scavenger⁴².
2. Cp is a plasma protein that functions as a copper transporter⁴³.
3. Studies in atherosclerosis have reported that Cp oxidatively modifies Low Density Lipoprotein. The pro-oxidant activity of Cp

requires an intact structure, and a single copper atom at the surface of the protein, near His⁴²⁶ is required for LDL oxidation⁴⁴.

ROLE OF Cp DURING INFLAMMATION

Elevation of serum Cp level during inflammation is one of the body's inbuilt defensive mechanism⁴⁵.

During early phase of inflammation there is increased polymorphonuclear leukocyte (PMN) adhesion to the endothelial cells of the blood vessels for subsequent PMN migration between the endothelial cells into the connective tissue and towards the focus of injury. The adhesion event is influenced by inflammatory mediators, such as Leucotriene -B₄ and IL-1. Cp reduces the adhesion and scavenges superoxide during the interaction of activated PMN leukocytes with endothelial cells⁴⁶.

Ferroxidase activity of Cp

Acting as a ferroxidase, Cp is vitally important in catalyzing the enzymatic oxidation of ferrous iron to ferric iron⁴⁷. This ferroxidase activity is important for iron fluxing in and out of cells because iron is stored in its storage molecule (ferritin) and transported in the blood in the ferric form, bound to transferrin⁴⁸.

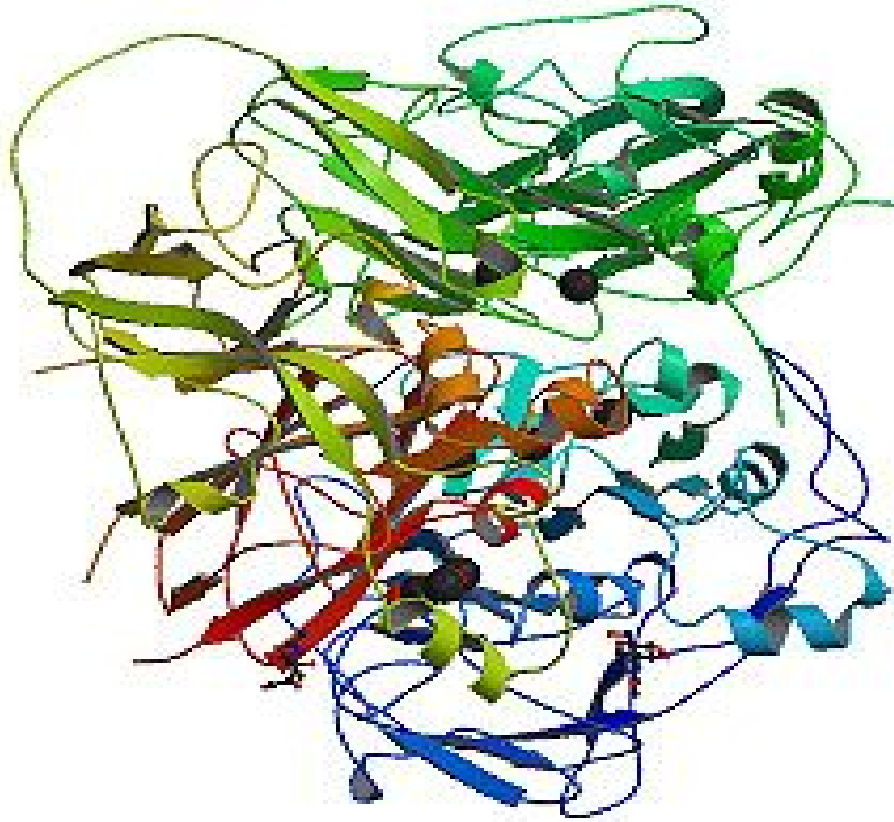
Cp thus permits incorporation of ferric iron into transferrin. It prevents the formation of toxic iron products by inhibiting the fenton reaction.

The Battle for Iron between MTB and its host

MTB relies primarily on Iron chelating compounds called Siderophores for acquisition of iron from its human host. Siderophores chelate extracellular Fe^{3+} to form Fe^{3+} -Siderophore complexes that are captured by the bacteria for subsequent metabolism⁴⁹.

Cp by its ferroxidase activity oxidizes ferrous iron, facilitates its binding with transferrin and thereby inhibits the iron uptake by MTB.

STRUCTURE OF CERULOPLASMIN



REPRODUCED FROM WEBSITE:<http://en.wikipedia.org/wiki/Ceruloplasmin>

IRON

Body Iron is distributed in different compartments including Hemoglobin, storage iron and Myoglobin.

CLINICAL SIGNIFICANCE⁵⁰

Iron deficiency and Iron overload are the major disorders of Iron metabolism.

REFERENCE VALUE: Male: 60 to 150 µg/dl, Female: 50 to 130 µg/dl

COPPER

Copper is an important trace element. 90% of copper exported from liver into peripheral blood is in the form of ceruloplasmin.

CLINICAL SIGNIFICANCE

Copper deficiency: Seen in malnourished infants, premature infants, Menke's syndrome.⁵¹

Copper toxicity: Seen in Wilson's disease⁵¹.

REFERENCE VALUE: 70 to 150 µg/dl

ALBUMIN

Albumin is the most abundant plasma protein with molecular mass of 663KDa⁵².

BIOCHEMISTRY

Albumin has a single polypeptide chain of 580 amino acids with no carbohydrate side chains⁵². It is a very stable protein with a high net negative charge at physiological pH. The plasma half-life is 15 to 19 days.

SITE OF SYNTHESIS

Albumin is synthesized primarily by the hepatic parenchymal cells except in early fetal life, when it is synthesized largely by the yolk sac.

CLINICAL SIGNIFICANCE

Relative increase in plasma levels

It is seen in acute dehydration. Chronic Vomiting and diarrhea may impair intestinal absorption of amino acids, so reducing plasma albumin formation⁵³.

Decreased plasma levels: Occurs with

- (1) An impairment in synthesis , as in malnutrition or liver disease
- (2) An increase in catabolism, as in the acute phase reaction,
- (3) Loss of protein through urine or faeces or
- (4) Change in distribution between the intravascular and extra vascular compartments⁵⁴ .

FUNCTIONS⁵⁴

1. The primary function of albumin is maintenance of colloidal osmotic pressure in both the vascular and extra vascular spaces.
2. Albumin binds and transport a large number of compounds including free fatty acids, phospholipids, cholesterol, metallic ions, amino acids, drugs, hormones, and bilirubin, among many others.
3. Albumin binds many hormones such as Triiodothyronine or T₃, but with lower affinity.
4. Albumin also functions as an amino acid source for peripheral tissue.
5. Albumin is an important component of plasma antioxidant activity.

6. Albumin acts as a buffer, especially in non physiological conditions.
7. Albumin inhibits leukotriene and prostaglandin production, thus reducing the inflammatory response of platelets and neutrophils.
8. Albumin binds copper and transports it to the liver where copper is incorporated into cuproenzymes.

ROLE OF ALBUMIN DURING INFLAMMATION⁵⁴

Albumin level is decreased during inflammation because of following factors.

- (1) Hemodilution.
- (2) Loss into the extra vascular space caused by increased vascular permeability.
- (3) Increased consumption by cells locally.
- (4) Decreased synthesis as the result of direct inhibition by cytokines
- (5) Increased colloidal osmotic pressure (COP).

STRUCTURE OF ALBUMIN



REPRODUCED FROM WEBSITE:<http://en.wikipedia.org/wiki/Albumin>

ACUTE PHASE RESPONSE

It is a non specific response induced by bacterial infection. After infection with MTB, alveolar macrophages, neutrophils and granulocytes secrete pro-inflammatory cytokines, such as TNF- α and IL-1 (IL-1 α or IL-1 β) into the blood stream. The liver responds to these cytokines release by producing acute phase proteins⁵⁵.

MAJOR ASPECTS OF THE ACUTE PHASE RESPONSE⁵⁶

Metabolic aspects

1. Protein catabolism.

Inflammatory cytokines induces muscle protein degradation and thereby provides amino acids for acute phase protein synthesis.

2. Osteoporosis.

Increased resorption of bone occurs due to increase in pro inflammatory cytokines levels.

3. Increased hepatic lipogenesis.

Cytokines increases the synthesis of fatty acids. Tubercle bacilli inhibits the Lipoprotein lipase leading to decrease in clearance of very low

density lipoprotein.

Neuroendocrine changes

1.Fever.

2.Somnolence.

3.Increased Corticotrophin-releasing hormone , Corticotrophin , and Cortisol.

Cytokines stimulates hypothalamic, pituitary and adrenal glands. Cytokines induce the production of corticotrophin-releasing factor. It generates Adreno corticotrophin hormone in the pituitary and then in turn corticosteroid in adrenal cortex.

4.Increased secretion of catecholamine's due to stimulation by cytokines.

5.Decreased insulin-like growth factor 1(ILGF-1).

Immunostimulatory hormones such as Growth hormone and ILGF-1 are suppressed. The decrease in concentration of growth hormone and ILGF-1 are the reason for the protein imbalance that leads to catabolism of muscle protein.

Micronutrient changes

1.Reduced serum Zinc due to increased production of metallothionein in

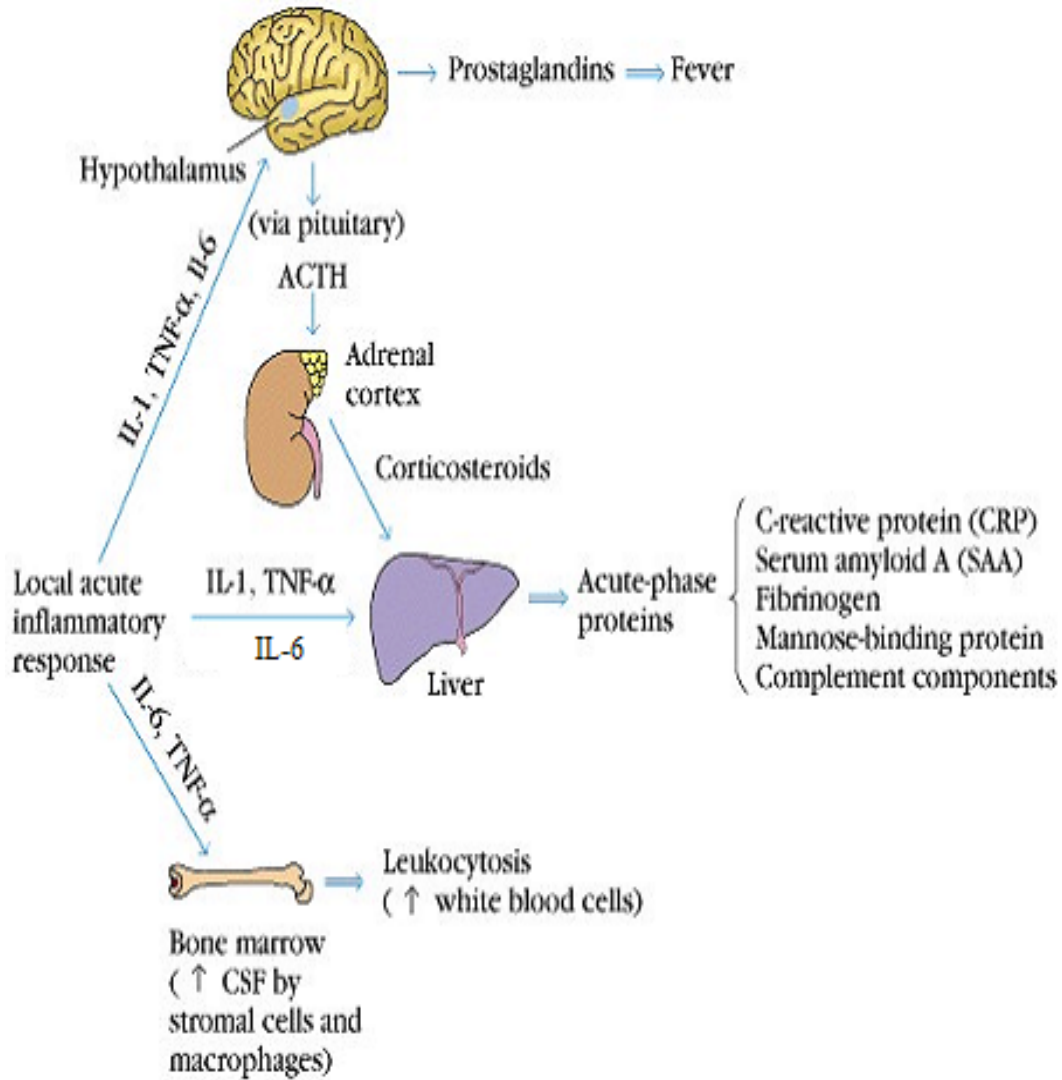
liver.

2.Reduced plasma Vitamin A because of reduced levels of carrier protein.

Positive acute phase proteins⁵⁷ are those whose plasma concentration increase during inflammation. Examples are – α_1 Antitrypsin, α_1 - Antichymotrypsin, Fibrinogen, Prothrombin, Factor VIII, Complement proteins, transport proteins such as Cp, Haemopexin, and Haptoglobin.

Negative acute phase proteins⁵⁷ are those plasma concentrations decrease during inflammation. Examples are Albumin, Transthyretin, Transferrin, Retinol binding protein.

ACUTE PHASE REACTION



REPRODUCED FROM WEBSITE:<http://wiki.healthhaven.com/Acute-phase-reaction>

MATERIALS AND METHODS

The present study was conducted after getting the approval from the ethical committee of Stanley medical college.

It's an age and sex matched comparative study. Eighty cases of PTB were taken for the study of which, forty were freshly diagnosed, sputum for AFB +ve (Group I), and forty were completely treated for 6 months with ATT and had completely recovered (Group II).

Forty healthy subjects without any history of PTB infection were also included in the study as controls.

The study subjects were selected from those attending the TB- Tambaram sanatorium from January 09 to April 09. The study subjects were clearly informed of the nature of the study and the blood samples were collected after getting written informed consent.

The Control subjects were volunteers with good health as evidenced by medical history, complete physical examination and routine laboratory tests performed before the commencement of the study.

SAMPLE COLLECTION:

5ml of venous blood drawn from the subjects. Individuals fasted for 12 hour prior to sample collection. After collection, blood samples were allowed to clot and then centrifuged to separate serum. Serum samples were stored at 4°C for 1 week.

The samples were analyzed for serum Ferroxidase, serum Albumin, serum total Protein, serum total Bilirubin, serum ALT, serum AST, serum Alkaline phosphatase, blood Urea, serum Creatinine and the results were analyzed based on the data collected.

INCLUSION CRITERIA

1. PTB patients include both males and females between 20 to 60yrs.
2. PTB patients who were freshly diagnosed with sputum for AFB+ve.
3. PTB patients who had completed their treatment and had completely recovered from the infection.
4. Apparently healthy individuals of both sexes in the same age group.

EXCLUSION CRITERIA

1. PTB patients with any other active medical conditions like Pleural effusions (extra Pulmonary TB), HIV infection , Nephrotic syndrome, Bronchial asthma etc.
2. Children with Primary pulmonary TB.
3. Pregnant PTB patients.
4. PTB patients with hepatocellular damage.
5. PTB patients with renal damage.
6. PTB patients with malignancies such as leukemia, lymphoma, breast carcinoma.
7. Patients not willing to give written informed consent were excluded from the study.

QUANTITATIVE ESTIMATION OF SERUM FERROXIDASE⁵⁸

Serum Ferroxidase activity of Ceruloplasmin was determined by end point measurement method using ferrous ions.

PRINCIPLE:

Serum was incubated with known amount of ferrous ion in acetate buffer. Ferroxidase activity of Cp oxidizes ferrous ions to ferric ions. At the end of the incubation period, chromogen was added. It forms a highly colored Fe^{2+} complex with non oxidized ferrous ions, blue color intensity was then measured photo metrically at 600nm. The difference in the Fe^{2+} ion concentration before and after the enzymatic reaction indicated the amount of oxidized Fe^{2+} ions. The amount of enzyme that converted $1\mu\text{mol}$ of substrate into product per minute was defined as one unit.

REAGENTS:

Reagent1: Sodium acetate buffer, 0.45mol/l

36.91gm of sodium acetate was dissolved in 1000ml of deionized water. 25.87 ml of reagent grade glacial acetic acid was diluted to 1000ml with deionized water. 940ml of sodium acetate solution was mixed with 60ml of acetic acid solution. pH adjusted to 5.8. 1ml of chloroform was added. The buffer solution is stable for at least 6 months at room temperature.

Reagent2: Substrate solution, $367\mu\text{mol/l}$

9.9gm of thiourea was dissolved in 1000 ml of deionized water and then

0.1440 gm of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ was dissolved in 1000ml of this 130mmol/l thiourea solution. 1 ml of chloroform was added. The substrate solution is stable for at least 6months at 4°C.

Reagent3: Chromogen solution-18mmol/l

0.8899gm of 3-(2-pyridyl)-5, 6-bis [2-(5-furyl sulfonic acid)]-1, 2, 4-triazine was dissolved in 100ml of buffer solution. The chromogen solution is stable for at least 6months at 4°C.

CALIBRATION:

The calibrator used was reagent grade deionized water. Six different concentrations of Fe^{2+} ions were prepared. They were 60 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$, 40 $\mu\text{mol/l}$, 30 $\mu\text{mol/l}$, 20 $\mu\text{mol/l}$, 10 $\mu\text{mol/l}$.

The absorbance of the calibrators is plotted against the concentration of Fe^{2+} ions on a graph paper.

TABLE

Blank Absorbance: 0.02

Standard concentration($\mu\text{mol/l}$)	Corrected Absorbance(std-Blank)
60	1.20
50	0.98
40	0.85
30	0.60
20	0.41
10	0.18

PROCEDURE

	Blank	Standard	Test
Distilled water	78 μl	3 μl	-
Serum	-	-	3 μl
Buffer	350 μl	350 μl	350 μl
After 1min, substrate added			
Substrate	-	75 μl	75 μl
Incubated for 3.8min at 37 $^{\circ}\text{c}$, then added			
Chromogen	30 μl	30 μl	30 μl

3 μl of serum or calibrator was added to 350 μl of reagent1. Then, 75 μl of reagent2 was added after 1min, and the mixture was incubated for 3.8min at 37 $^{\circ}\text{c}$. At the end of incubation period, 30 μl of reagent3 was added and the absorbance of the colored complex at 600nm was recorded.

CALCULATION:

$$\text{Unknown Fe}^{2+} (\mu\text{mol/l}) = \frac{\text{Test-Blank absorbance}}{\text{Std-Blank absorbance}} \times \text{concentration of std}$$

$$\text{Enzyme activity} = (C_1 - C_2) \times t^{-1} \times d_f$$

$$= \Delta C_s \times t^{-1} \times d_f, \text{ where}$$

C_1 is the substrate concentration at the beginning of the enzymatic reaction.

C_2 is the substrate concentration at the end of the enzymatic reaction.

t is the incubation time of the enzyme with the substrate.

d_f is the dilution rate of the sample (total volume / sample volume).

$$\text{Enzyme activity} = \Delta C_s \times (1/3.8) \times (458/3)$$

$$= \Delta C_s \times 40 \text{ U/L}$$

REFERENCE VALUE: Adult-500 to 1110 U/L

QUANTITATIVE ESTIMATION OF SERUM ALBUMIN

(by Bromocresolgreen, dye binding method)

PRINCIPLE : Albumin in a buffered solution reacts with the anionic bromocresol green, a dye binding reaction gives a proportionate green

color which was measured at 628nm (600-650nm).

REAGENTS :(Bromocresol green)

Succinic acid-94 mmol/l

NaOH-10.2 mmol/l

BCG-0.149 mmol/l

PROCEDURE:

1ml of reagent was added to 10 µl of sample , incubated for 1min at room temperature, mixed and read.

System Parameters

Reaction type	End point
Reaction slope	Increasing
Wavelength	628nm
Flow cell temperature	30°c
Incubation	1 min at room temperature
Sample volume	10 µl
Reagent volume	1ml
Standard concentration	5g/dl
Zero setting with	Reagent blank

REFERENCE VALUE: Adult: 3.5 to 5 g/dl

QUANTITATIVE ESTIMATION OF SERUM TOTAL PROTEIN

(by Biuret method.)

PRINCIPLE:

Proteins in serum react with biuret reagent to form a purple colored complex which is measured colorimetrically. The intensity of the color developed was directly proportional to the amount of proteins present in the sample.

REAGENTS:

1. Biuret diluent: 5gm KI in 0.25mol NaOH/L
2. Stock Biuret reagent: 15gm of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 70 ml of water. Prepared a solution of 45 gm Rochelle salt (potassium sodium tartrate, tetrahydrate) in 600 ml of biuret diluent and then added CuSO_4 solution. Biuret diluent is then added to 1000ml.
3. Working Biuret: The stock biuret was diluted 5-fold with biuret diluent.

PROCEDURE:

10 μl of serum was added to 500 μl of reagent. Mixed well, incubated for 10min at room temperature and read at 540nm.

REFERENCE VALUE: Adult: 6.4 to 8.3 g/dl

QUANTITATIVE ESTIMATION OF SERUM TOTAL BILIRUBIN

(by Jendrassik and Grof method)

PRINCIPLE:

Serum was added to a solution of sodium acetate and caffeine-sodium benzoate. The sodium acetate buffers the pH of the diazotization reaction, while the caffeine-sodium benzoate accelerates the coupling of bilirubin with diazotized sulfanilic acid to form azobilirubin and the intensity of the color was read at 620nm.

REAGENTS:

1. Hydrochloric acid, 0.05mol/l
2. Caffeine reagent (50g of caffeine, 75g of sodium benzoate and 125g of sodium acetate was added to 1L of distilled water.
3. ReagentA (5g of sulfanilic acid added to 15ml of concentrated HCL and diluted to 1L with distilled water).
4. ReagentB (500mg of sodium nitrite was dissolved in 100 ml of distilled

water).

5. Diazo reagent (10ml of reagent A+0.25ml of reagent B).

6. Ascorbic acid solution (4g/100ml).

7. Tartrate solution (100g of NaOH and 350g of sodium tartrate was added to 1L of distilled water).

PROCEDURE:

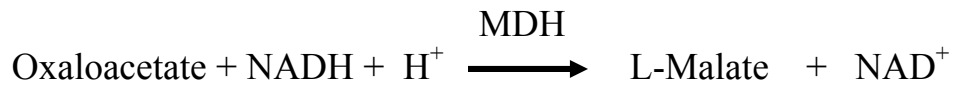
500 µl caffeine benzoate + 125 µl of Diazo reagent + 50 µl of sample were added , incubated for 10min at room temperature. Then 25 µl of ascorbic acid solution , 375 µl of alkaline tartrate solution, 250 µl of 0.05N HCL were added and read at 620nm.

REFERENCE VALUE: Adult: 0 to 2.0 mg/dl.

QUANTITATIVE ESTIMATION OF SERUM AST

(Aspartate aminotransferase) by UV-Kinetic method

PRINCIPLE:



AST-Aspartate amino transferase

MDH-Malate dehydrogenase

There was decrease in absorbance at 340nm as NADH got converted to NAD⁺. The rate of decrease in absorbance was measured.

REAGENTS:

MDH ≥ 800 U/L

LDH ≤ 4000 U/L

NADH > 0.20 mmol/l

αKetoglutarate > 13 mmol/l

Tris buffer, pH 7, 80-88 mmol/l

L-Aspartate-264 mmol/l

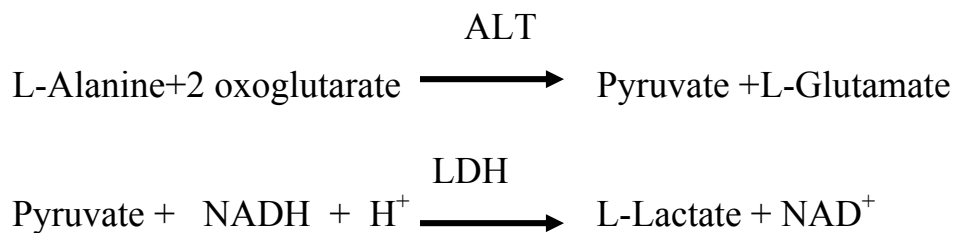
PROCEDURE:

1ml of reagent was added to 100 µl of sample at 37°C, mixed and read at 340nm.

REFERENCE VALUE: Adult :< 40 IU/L (37°C)

QUANTITATIVE ESTIMATION OF SERUM ALT

(Alanine aminotransferase) by UV kinetic method

PRINCIPLE:

ALT: Alanine aminotransferase

LDH: Lactate dehydrogenase

There was decrease in absorbance at 340nm as NADH got converted to NAD. The rate of decrease in absorbance was measured.

REAGENTS:

L-Alanine-500mmol/l

NADH-0.18mmol/l

LDH \geq 1820IU/L

2-oxoglutarate-12mmol/l

Tris buffer, pH7.5 – 80mmol/l

PROCEDURE:

1ml of reagent was added to 100 μ l of sample at 37 $^{\circ}$ c. Mixed and read at 340nm.

REFERENCE VALUE: Females :< 31 IU/L at 37 $^{\circ}$ c

Males :< 40 IU/L at 37 $^{\circ}$ c

QUANTITATIVE ESTIMATION OF SERUM

ALKALINEPHOSPHATASE by PNPP (p-Nitrophenyl phosphate) method

PRINCIPLE:

Alkaline phosphatase hydrolyses PNPP into p-Nitrophenol and phosphate. At the alkaline pH of the buffered medium, p-Nitrophenol was yellow. The color developed by hydrolysis was measured at 405nm.

REAGENTS:

PNPP-10mmol/l

Diethanolamine-1mol/l

Magnesium chloride-0.5mmol/l

PROCEDURE:

1ml of reagent was added to 30 µl of sample at 37°C, mixed and read at 405nm.

REFERENCE VALUE: Adult: 60 to 170 U/L (25°C)

QUANTITATIVE ESTIMATION OF BLOOD UREA NITROGEN

(by UV Kinetic Method)

PRINCIPLE:

Urea was hydrolyzed in the presence of water and urease to produce ammonia and CO₂. The ammonia produced combines with α Ketoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD. The amount of urea nitrogen may be calculated by determining the absorbance decrease per minute relative to urea nitrogen standard at 340nm.

REAGENTS:

Tris buffer, pH7.55 – 75mmol/l

GLDH \geq 1000U/L

Urease \geq 30,000 U/L

NADH-0.32mmol/l

α Ketoglutarate-9mmol/l

PROCEDURE:

1ml of reagent was added to 10 μ l of sample at 37 $^{\circ}$ c, mixed and read at 340nm.

REFERENCE VALUE: BUN: 6-20 mg/dl

QUANTITATIVE ESTIMATION OF SERUM CREATININE

(by modified Jaffe's Method)

PRINCIPLE:

Creatinine present in the sample reacted with picric acid in alkaline medium and formed creatinine picrate (red colored complex) which was measured at 490nm.

REAGENTS:

Saturated picric acid-10ml

0.75N NaOH-10ml

Distilled water-20ml

PROCEDURE:

1ml of reagent was added to 100 μ l of sample at 37 $^{\circ}$ c, mixed and read at 490nm.

REFERENCE VALUE: Adult: 0.8 to 1.2 mg/dl.

RESULTS AND STATISTICAL ANALYSIS

The Present study was conducted on the subjects attending the TB-Tambaram sanatorium hospital. The total number of subjects included for the study was 120. Out of 120, 40 were freshly diagnosed PTB cases, 40 were completely treated cases and 40 were controls.

Mean and Standard deviation of variables - serum Ferroxidase , serum Albumin, serum F/A ratio, serum total Protein, serum total Bilirubin, serum AST , serum ALT , serum Alkaline phosphatase , Blood Urea , serum Creatinine were estimated for subjects in GroupI, GroupII and Controls (as shown in Table no.1).

ANOVA was employed to find out the level of significance between three groups. Student independent t test was used to find the P value between study group and Control [as shown in table no.7 ,8, 9 and 10(age wise comparison) ,3,4,5 and 6 (sexwise comparison)].

STATISTICAL RESULTS:

1.Serum Ferroxidase level ranges between 550 – 1012 U/L in control, 820 – 2102 U/L in GroupI, 719 – 1380 U/L in GroupII (estimated by end point method using Ferrous ions).

- 2) Serum Albumin level ranges between 35 – 47g/l in control, 24 - 40 g/l in GroupI, 31 - 45 g/l in GroupII (estimated by Bromocresol green dye binding method).
- 3) Serum F/A ratio level ranges between 13 – 28.8 in control, 24.1 – 82.9 in GroupI, 15.2 – 43.1 in GroupII.
- 4) Serum total Protein level ranges between 6.4 – 8.0g/dl in control, 7.4 – 9.1 g/dl in GroupI, 6.6 – 8.2 g/dl in GroupII (estimated by Biuret method).
- 5) Serum total Bilirubin level ranges between 0.2 -1.2mg/dl in control,0.3 – 1.2 mg/dl in Group I, 0.2 – 1.1 mg/dl in Group II(estimated by Jendrassik and grof method).
- 6) Blood Urea level ranges between 15 – 37mg/dl in control , 15 - 39 mg/dl in GroupI , 16 - 38 mg / dl in GroupII (estimated by UV Kinetic method).
- 7) Serum Creatinine level ranges between 0.8 – 1.2 mg/dl in control, 0.7 – 1.2 mg / dl in Group I ,0.7 – 1.1 mg / dl in GroupII (estimated by modified jaffe's method).
- 8) Serum AST level ranges between 13 – 40IU/L in control, 15 - 40 IU/L in Group I , 16 – 36 IU/L in Group II (estimated by UV Kinetic method).

9) Serum ALT level ranges between 11 – 40 IU/L in control, 10 - 38 IU/L in Group I, 10 – 40 IU/L in Group II (estimated by UV Kinetic method).

10) Serum Alkaline phosphatase ranges between 60 – 125 U/L in control, 51 - 132 U / L in Group I , 66 - 143 U / L in Group II (estimated by p-Nitrophenyl phosphate method).

DISCUSSION

Pulmonary tuberculosis is a global disease affecting about 1 /3rd of the world's population with its attendant mortality and morbidity. PTB can be diagnosed by a thorough evaluation of history, clinical symptoms and signs, bacteriological and radiological features. But still, in quite a number of cases it becomes difficult to predict the activity of a tubercular lesion where precise information is not available particularly in developing countries like India.

LIMITATIONS OF EXISTING DIAGNOSTIC INVESTIGATIONS

1. The diagnosis of PTB is based primarily on the rapid and inexpensive, microscopic examination of sputum for AFB but it is limited by its poor sensitivity (40-60%)⁵⁹.
2. Mycobacterium culture is able to detect as few as 10 organisms per milliliter of sputum and overcomes many of the limitations of AFB staining but even with the use of broth-based culture systems, confirming the presence of MTB from the time of specimen collection takes at least a week⁵⁹.
3. Current rapid nucleic acid amplification techniques with sensitivity of about 96 % and specificity of 100 % for AFB smear positive samples are

commercially available. But their performance in AFB smear - negative specimens is less impressive with sensitivities ranging from 48% to 53%. Moreover the accuracy of this testing may be reduced by the concurrent use of ATT and inhibitors in the patient's sputum which may give false-negative result^{59, 60}.

With these factors taken into account, the present study was undertaken to evaluate a new biochemical parameter for diagnosis of active PTB infection and prognosis of PTB patients under treatment. In the present study, the PTB cases were compared with apparently healthy controls. Also, the nutritional status of the PTB cases and control were assessed by estimating the serum Albumin and total Protein level. Liver function was also analyzed in the PTB cases and control using the enzymes- AST, ALT, Alkaline phosphatase. Renal parameters - Urea and Creatinine was also analyzed in these groups.

In the present study, age wise comparison of Group I, Group II with controls was made (as shown in table no. 7, 8, 9, and 10). Based on the age, subjects were divided into two age groups - upto 40 yrs and above 40 yrs. Following results obtained:

1. In both age groups there was significant P value on comparison of the mean levels of serum Ferrroxidase (P=0.001), serum Albumin (P=0.001) and

serum F/A ratio ($P=0.001$) between Group I and Control.

2. In both age groups there was significant P value on comparison of the mean levels of serum Ferrroxidase ($P=0.001$), serum Albumin ($P=0.001$) and serum F/A ratio ($P=0.001$) between Group II and Control.

In the present study, equal number of Male and Female subjects were selected in Group I, Group II and controls to make sexwise comparison. (as shown in table no.3,4,5 and 6). Following results obtained:

1. In both males and females, there was significant P value on comparison of the mean levels of serum Ferrroxidase ($P=0.001$), serum Albumin ($P=0.001$) and serum F/A ratio ($P=0.001$) between Group I and Control.

2. In both males and females, there was significant P value on comparison of the mean levels of serum Ferrroxidase ($P=0.001$), serum Albumin ($P=0.001$) and serum F/A ratio ($P=0.001$) between Group II and Control.

In the present study, the mean levels of serum Ferrroxidase, serum Albumin, serum F/A ratio, serum total Protein, serum total Bilirubin, serum AST, serum ALT, serum Alkaline phosphatase, Blood Urea, serum Creatinine was found to be normal in the control subjects.

In the present study , Group I i.e., freshly diagnosed sputum for AFB+ve subjects showed significant increase in the mean level of serum Ferroxidase as compared to controls . These findings suggest that levels of serum Ferroxidase are related with the activity of tubercular process⁶¹ . Serum Ferroxidase , an acute phase reactant got increased due to tissue destruction, increase in severity of the lesion and persistence of fever⁶².

The present study in Group I showed significant decrease in the mean level of serum albumin as compared to controls. Albumin being an acute phase reactant, its level in the serum decrease with increase in severity⁶³. This is because of two reasons.

1. Hemodynamic changes that occur in response to T cell reaction.
2. Decreased synthesis as a result of direct inhibition by cytokines.

Decrease in the Albumin level supports the induction of malnutrition and loss of weight by MTB infection⁶³.

In the present study, the mean level of total serum Protein was increased in Group I as compared to controls. This has been solely due to increase in the globulin fractions as the albumin fraction has been decreased in these patients. Serum ferroxidase is one of the globulin fraction that contributes to

the increase in total Protein level⁶⁴.

Regarding Albumin-Globulin ratio, the ratio decreases in direct proportion to the severity of the lesion⁶⁵.

In the present study, Group I showed significant elevation of serum F/A ratio as compared to controls. Serum F/A ratio is statistically easier to compare the PTB patients with the controls rather than the individual parameters.

In the present study, Group II patients showed that the mean levels of serum Ferroxidase decreased considerably after 6 months of chemotherapy compared to Group I. The fall of serum Ferroxidase might be due to the caseation or diminution in tissue destruction, healing of the lesions and subsidence of fever as a result of ATT.

The present study showed that the mean level of serum Albumin was increased to near normal in completely treated Group II cases compared to freshly diagnosed PTB patient i.e., Group I. The elevation of serum Albumin level in completely treated cases suggest the cessation of acute hemodynamic changes that occurred previously in PTB freshly diagnosed cases in response to tissue damage.

Also study on Group II showed that the mean level of serum total Protein

was decreased to near normal as compared to Group I. It might be due to increased synthesis of Albumin and reduction in globulin level (i.e., serum ferroxidase- α 2 globulin)⁶⁶.

In Group II treated cases, the mean F/A ratio was found significantly reverting back near to the control levels after complete treatment for 6 months. These findings substantiate the relationship of serum F/A ratio with the activity of the tubercular lesion.

In the present study, there was no significant change in the mean levels of other parameters in Group I and Group II as compared to controls suggesting that liver function is not entirely affected and renal function is normal.

SUMMARY AND CONCLUSION

Pulmonary Tuberculosis is a communicable disease of global importance. The present study on PTB patients (both freshly diagnosed and treated cases) showed that there was highly significant increase in the serum Ferroxidase / Albumin ratio level in freshly diagnosed PTB cases which on ATT treatment falls back to near normal level on comparison with apparently healthy individuals.

The serum F/A ratio level is useful in predicting the course of PTB infection. It shows the degree of tuberculosis activity and thus it may serve as a guide in the management of PTB disease.

A favorable course will always be associated with a tendency towards the near normalization of serum F / A ratio and the patient is not considered healed unless the serum F/A ratio is near normal.

The method employed to estimate serum Ferroxidase (by end point measurement method using ferrous ions) and serum Albumin (by Bromocresolgreen dye binding method) level are simple, rapid and cost-effective . Hence serum F / A ratio can feasibly be incorporated as a biochemical marker to assist in diagnosis and prognosis of PTB.

SCOPE FOR FURTHER STUDY

Studies can be conducted to assess the levels of serum Ferroxidase /Albumin ratio in

1. TB Patients with HIV infection.
2. Children with primary pulmonary tuberculosis.
3. Extra pulmonary TB patients.
4. MDR-TB patients.
5. Other Mycobacterium infection.

STANDARDISATION OF Fe^{2+} IONS

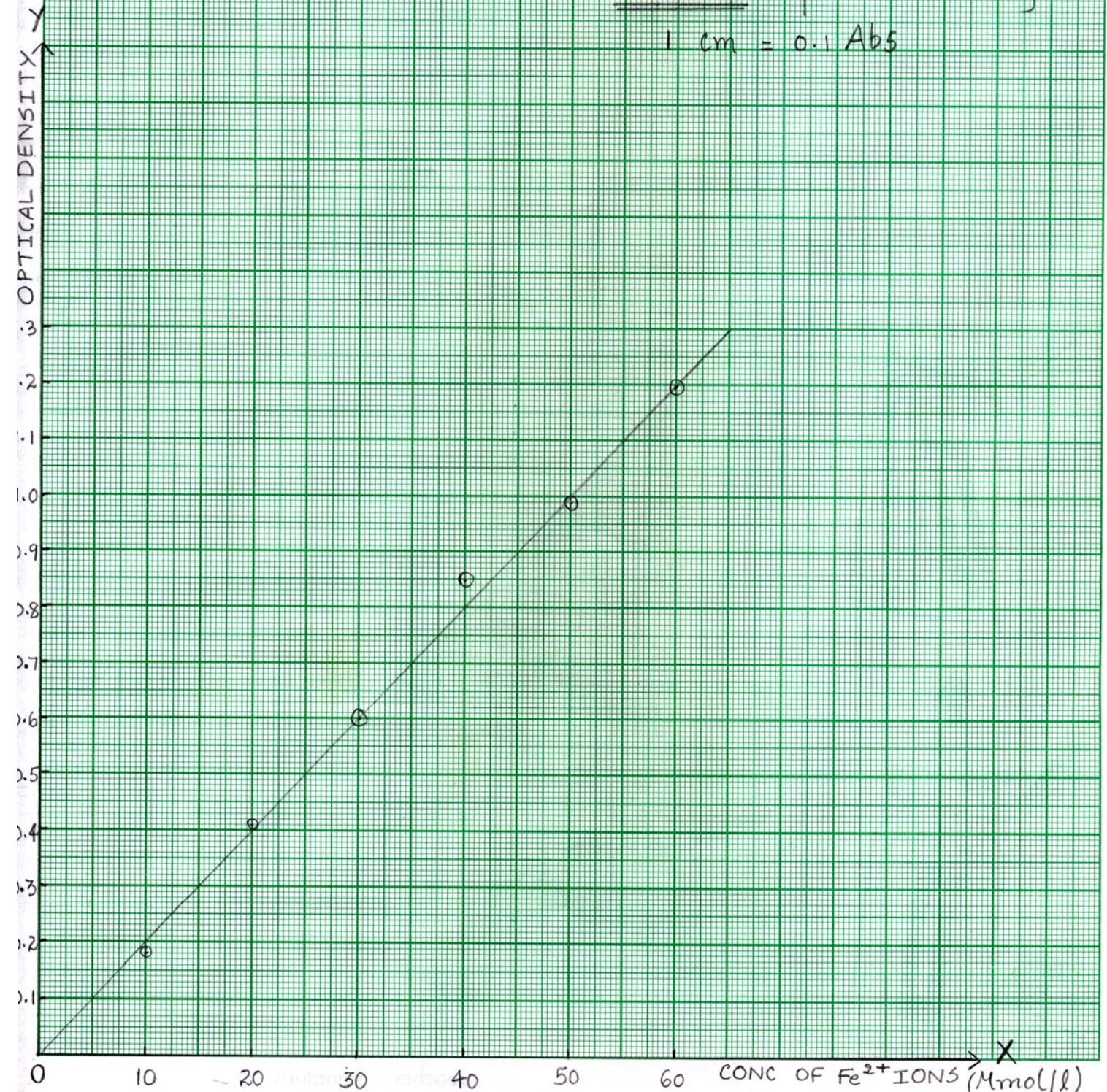
SCALE

X axis: Concentration of Fe^{2+} ions

2 cm = 10 Mmol/l

Y axis: Optical density

1 cm = 0.1 Abs



PROFORMA

**Stanley Medical College Hospital
Chennai.**

Date :

Name:

OP/IP No:

Age:

Sex:

Occupation:

Clinical History:

On Examination

Height:

Blood Pressure:

Heart rate:

Weight:

Respiratory rate:

Cardiovascular system:

Central nervous system:

Respiratory system:

Abdomen:

Investigations:

1. Serum Ferroxidase:

6. Serum Creatinine:

2. Serum Albumin:

7. Serum AST:

3. Serum Ferroxidase/Albumin ratio:

8. Serum ALT:

4. Serum Total Protein:

9. Serum Alkaline Phosphatase

5. Serum Total Bilirubin:

10. Blood Urea:

TABLE NO.1
MEAN VALUES OF VARIABLES IN GROUPS

S.No	Variables	Group	N	Mean±SD
1	Ferroxidase	Group I	40	1616.02±216
		Group II	40	980.22±189
		Controls	40	800.87±130
		Total	120	1132.37±395
2	Albumin	Group I	40	32.20±3
		Group II	40	38.87±4
		Controls	40	45.17±3.2
		Total	120	40.08±4.84
3	F/A Ratio	Group I	40	50.06±10
		Group II	40	26.07±7
		Controls	40	20.21±4
		Total	120	32.11±15.2
4	T.Protein	Group I	40	8.72±0.38
		Group II	40	7.70±0.35
		Controls	40	7.50±0.46
		Total	120	7.97±0.66
5	T.Bilirubin	Group I	40	0.83±0.2
		Group II	40	0.71±0.3
		Controls	40	0.79±0.2
		Total	120	0.78±0.2
6	SGOT	Group I	40	21.9±6.1
		Group II	40	23.6±6.0
		Controls	40	20.9±6.5
		Total	120	23.1±6.2
7	SGPT	Group I	40	26.3±7.5
		Group II	40	26.6±7.3
		Controls	40	27.1±7.8
		Total	120	26.7±7.5
8	Alk.phosp	Group I	40	92.2±22.9
		Group II	40	85.72±22.2
		Controls	40	89.7±21.3
		Total	120	92.9±22.0
9	Urea	Group I	40	19.2±5.5
		Group II	40	20.5±6.1
		Controls	40	19.9±7.0
		Total	120	24.6±6.2
10	Creatinine	Group I	40	1.1±1.1
		Group II	40	0.9±0.1
		Controls	40	0.8±0.1
		Total	120	1.0±0.6

TABLE NO.2

ANOVA

COMPARISON BETWEEN AND WITHIN GROUPS

S.No	Variables	Groups	Mean square	f	Significance
1	Ferroxidase	Between groups	7339183.9	220.1	0.001
		Within groups	33337.9		
		Total			
2	Albumin	Between groups	570.308	40.4	0.001
		Within groups	14.107		
		Total			
3	F/A Ratio	Between groups	10002.2	153	0.001
		Within groups	65.33		
		Total			
4	T.Protein	Between groups	17.2	105.9	0.001
		Within groups	0.16		
		Total			
5	T.Bilirubin	Between groups	0.146	2.277	0.107
		Within groups	0.064		
		Total			
6	SGOT	Between groups	45.033	1.154	0.319
		Within groups	39.01		
		Total			
7	SGPT	Between groups	6.35	0.111	0.895
		Within groups	57.53		
		Total			
8	Alk.phosp	Between groups	21.22	0.043	0.958
		Within groups	493.2		
		Total			
9	Urea	Between groups	12.4	0.314	0.731
		Within groups	39.4		
		Total			
10	Creatinine	Between groups	0.51	1.168	0.315
		Within groups	0.44		
		Total			

TABLE NO.3

SEXWISE COMPARISON BETWEEN GROUP I AND CONTROLS

(MALE)

S.No	Variable	Group I	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	1605.70±209.06	793.47±132.13	0.001
2	Albumin	32.65±4.29	41.28±3.22	0.001
3	F/A Ratio	50.260±11.81	19.97±4.71	0.001

P<0.05=significant

TABLE NO.4

SEXWISE COMPARISON BETWEEN GROUP II AND CONTROLS

(MALE)

S.No	Variable	Group II	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	992.05±197.18	793.47±132.13	0.001
2	Albumin	40.75±3.52	43.28±3.22	0.001
3	F/A Ratio	26.38±7.52	19.97±4.71	0.001

P<0.05=significant

TABLE NO.5

**SEXWISE COMPARISON BETWEEN GROUP I AND CONTROLS
(FEMALE)**

S.No	Variable	Group I	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	1626.35±228.73	809.05±131.87	0.001
2	Albumin	33.10±4.01	43.05±3.29	0.001
3	F/A Ratio	49.86±9.9	20.48±4.7	0.001

P<0.05=significant

TABLE NO.6

**SEXWISE COMPARISON BETWEEN GROUP II AND CONTROLS
(FEMALE)**

S.No	Variable	Group II	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	968.40±186.77	809.05±131.87	0.001
2	Albumin	38.65±4.25	43.05±3.29	0.001
3	F/A Ratio	25.77±7.8	20.48±4.7	0.001

P<0.05=significant

TABLE NO.7

**AGEWISE COMPARISON BETWEEN GROUP I AND CONTROLS
(upto 40)**

S.No	Variable	Group I	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	1548.87±225.32	797.22±147.45	0.001
2	Albumin	32.83±3.49	43.22±3.05	0.001
3	F/A Ratio	47.64±8.81	20.11±5.07	0.001

P<0.05=significant

TABLE NO.8

**AGEWISE COMPARISON BETWEEN GROUP II AND CONTROLS
(upto 40)**

S.No	Variable	Group II	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	986.04±210.44	797.22±147.45	0.001
2	Albumin	38.81±3.97	43.22±3.05	0.001
3	F/A Ratio	26.40±8.44	20.11±5.07	0.001

P<0.05=significant

TABLE NO.9

**AGEWISE COMPARISON BETWEEN GROUP I AND CONTROLS
(above 40)**

S.No	Variable	Group I	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	1716.75±161.34	803.86±118.43	0.001
2	Albumin	32.93±5.01	42.13±3.41	0.001
3	F/A Ratio	53.69±12.66	20.30±4.4	0.001

P<0.05=significant

TABLE NO.10

**AGEWISE COMPARISON BETWEEN GROUP II AND CONTROLS
(above 40)**

S.No	Variable	Group II	Control	P Value
		Mean±SD	Mean±SD	
1	Ferroxidase	973.11±167.24	803.86±118.43	0.001
2	Albumin	39.66±3.83	42.13±3.41	0.001
3	F/A Ratio	25.68±6.62	20.30±4.4	0.001

P<0.05=significant

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