ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Assessment of Vitamin A, Vitamin B 12 and Iron levels among tuberculosis patients and co-infected with HIV before and after TB treatment. Longitudinal cohort study

BY: MIHRET ALEMAYEHU [BSc]

A Research thesis to be submitted to the School of Graduate Studies of Addis Ababa University, Faculty of Medical Sciences, Department of Medical Laboratory Sciences for the Partial fulfillment for the Degree of Master (MSc) in Clinical Laboratory Sciences, Clinical Chemistry Specialty Track.

Oct, 2016

Addis Ababa, Ethiopia

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: Desta Kassa, PhD

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Lists of abbreviation

AIDS	Acquired Immune Deficiency Syndrome
AMac	Alveolar Macrophage
ART	Anti Retroviral Treatment
CR	Complement Receptor
FcR	Fc Receptor
HIV	Human Immuno Deficiency Virus
IL	Interleukin
MR	Mannose Receptor
MTB	Mycobacterium Tuberculosis
MTCT	Mother To Child Transmission
PMN	Polymorph Nuclear Neutrophil
PRR	Pattern Recognition Receptor
PTB	Pulmonary Tuberculosis
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
TB	Tuberculosis
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor

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Operational definition

Pulmonary Tuberculosis: tuberculosis of the lung
Case of tuberculosis: a patient in whom tuberculosis has been bacteriologic ally confirmed, or who has been diagnosed by a clinician.

Extra-pulmonary tuberculosis: Tuberculosis of organs other than the lungs: *eg*,pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges

Pulmonary tuberculosis, sputum smear positive(PTB+): (1) two or more initial sputum smear examinations positive for Acid-Fast Bacilli (AFB) or(2) one sputum smear examination positive for AFB plus radiological abnormalities consistent with active pulmonary tuberculosis as determined by a clinician, or (3) one sputum smear positive for AFB plus sputum culture positive for M. tuberculosis.

Pulmonary tuberculosis, sputum smear negative (PTB-): a case of pulmonary tuberculosis which does not meet the above definitions for smear positive TB

Abstract

Background: Tuberculosis (Tb) is major cause of morbidity and mortality, for millions of peoples worldwide. Of the different risk factors associated with Tb development and progress, deficiency of micronutrients (vitamins and minerals), mentioned one. In this regards association of micronutrients during development of Tb and then after Tb treatments scarcely found in Ethiopia. **Objective:** To assess the level of micronutrients (Vitamin A, Vitamin B12 and Iron) among M. tuberculosis patients with and without HIV confection at baseline and after anti TB treatment. **Material and method**: This study was conduct on serum samples, collected from M.TB patients with and without HIV infection, attending at different health centers in Addis Ababa. The collected samples were stored at EPHI at - 80^0 C, for about 10 years. Convenient sampling method used to select TB only (87), TB-HIV confection (57), latent TB (25) and healthy controls (21). Socio-demographic data was collected from the previously collected log book. The concentration of Iron and Vitamin B12 was measured by using chemiluminesense method; whereas concentration of retinol measured by using HPLC method. The collected data enter and analyzed by using SPSS statistical software version 20. P-values less than 0.05 considered as statistical significant. **Result:** of the total 190 participants, about 57 had both TB-HIV (HIV+TB+), 87 had TB only (HIV-TB+), 25 had latent TB (HIV-TST+) and 21 healthy controls (TB-HIV-) were are included in the study. The median age of study participants in each study groups with inter quartile range, 30(28-38) years in TB-HIV, 27 (21-32) years in TB only, 23(20-31) years in latent and 24(20-27) years were in healthy controls. Female study participants had major proportions in all cases, except in TB-HIV cases where males were dominant. The concentration of vitamin A, Vitamin B 12 and Iron had significant difference before and after Tb treatments both in Tb patients with and without HIV-co-infections. But these differences was not seen when Tb only patients (at 0 and after 6 month therapy) micronutrient concentrations compared with Tb with HIV co infected patients (at 0 and 6 months after treatments. On the other hand there was statistical significant different between M0 Tb patients with and without HIV co-infections compared with latent Tb and healthy controls on Iron, Vitamin A and Vitamin B12 concentration, while theses difference becomes non-significant after 6 months of Tb therapy.

Conclusion and recommendation: The present study demonstrated that micronutrient deficiencies may related with development of Tb diseases. Measurement of Micronutrients for those latent Tb patients might indicate the progress of overt Tb developments. Thus, micronutrient supplements during Tb treatment might help to enhance Tb cure rates for those Tb patients. But still large scale active longitudinal studies need to undertake to consolidates the present findings.

Key word: Tuberculosis, Vitamin B 12, Retinol, Iron, Micronutrients.

1. Introduction

1.1 Background Information

Tuberculosis (TB)is bacterial infectious disease caused by *Mycobacterium tuberculosis* (Mtb) species. TB is a curable infectious disease, if diagnosed early and treated properly .It is an airborne infectious disease primarily affects the lungs (pulmonary), and may also affect other extra pulmonary parts of the body such as kidneys, lymph nodes, spinal cord and the abdomen (1, 2).

Mtb is non-motile, nonsporulating, weakly gram-positive, acid-fast bacilli that appear microscopically as straight or slightly curved rods, 1 to 4 mm in length and 0.3to 0.6 mm wide. The bacteria express unique mycolic acids in the cell envelope that play a critical role in the structure and function of the cell wall. The waxycell wall confers many of the unique characteristics, acid-fastness, extreme hydrophobicity, resistance to drying, acidity/alkalinity, distinctive immune-stimulatory properties (3).

Most human infections with MTB occur through inhaled carrier droplets into the lower airways. There the microbe encounters the alveolar macrophage (AMac) and submucosal dendritic cell (DC). The outcome of the event will determine whether the infection will remain locally limited within the engulfing cells of the innate immune system, or will continue to spread, causing the individual to become a clinically active TB patient (4).

Mtb interacts with macrophages through multiple phagocytic receptors. Ligation of the Fc receptor (FcR) led to production of reactive oxygen intermediates (ROIs) and phagolysosomal fusion, whereas ligation of complement receptor 3(CR3) or mannose receptor (MR) inhibited the respiratory burst and prevented phagosomal maturation Surface-expressed pattern recognition receptors (PRR),such as Toll-like receptor 2 (TLR2), also bind Mtb and initiate proinflammatory cytokine such as TNF-a, IL-1b, IL-6, and IL-12.TNF-a is a critical cytokine in the response to mycobacterial components in that it can stimulate alveolar neutrophils and macrophages in an

autocrine and paracrine fashion to stimulate apoptosis and ROI/RNI production, leading to the destruction of some phagocytosed bacilli(3).

Occurrence of HIV among Mtb patients may result spread of the tubercle bacilli dissemination from localized granulomas into different part of the body. Rapid progression from initial infection to TB disease may also occur in markedly immunosuppressed patients. On the other hand TB disease aggravates HIV disease progression by increasing viral load level via immune activation. It can result in the reduction of the CD4 cells and increase in viral load .Therefore accelerating the progression of HIV infection to AIDS. Patients with active TB who are HIV-positive have a higher risk of dying from TB than those without HIV (5).

Nutritional status is one of the most important determinants of resistance to infection. It is well established that nutritional deficiency is associated with impaired immune functions. While malnutrition limits cell mediated immunity and increases susceptibility to infection, infection can lead to nutritional stress and weight loss, thereby weakening immune function. Micronutrient such as Vitamins A, vitamin B12 and iron all have key roles in metabolic pathways, cellular function, and immune competence (6,7).

There is an association between TB and malnutrition. Malnutrition enhances the development of active TB. It has been suggested that generalized malnutrition by reducing the expression of gamma interferon, tumor necrosis factor alpha, and other mycobactericidal substances may selectively compromise portions of the cell-mediated response that are important for containing and restricting TB (6,7).

Despite effective tuberculosis (TB) chemotherapy, a treatment period of 6–8 months is required for cure treatment outcome depends on a well-functioning cell-mediated immune system. A number of specific micronutrient deficiencies in TB patients which may impair the immune system and hence affect response to chemotherapy (8).

Vitamin A metabolites are essential for normal growth and development. This increase both innate immunity as well as the adaptive immunity . Retinoic acid, a vitamin-A metabolite has

been shown to inhibit expression of toll like receptor-II (TLR-II) on the cellular surface and thus affect the TLR-II signalling pathway and prevents *Mycobacterium tuberculosis* and other gram positive bacteria cause human infections. It also increases the phagocytic activity of human macrophages.Vitamin A helps in the normal function of immune cells and also enhances the synthesis of iNOS and other essential cytokines with antitubercular activity .So deficiency in dietary vitamin-A can hamper the activity of both innate and adaptive (9).

Vitamin A, which is usually assessed using serum retinol, plays important roles in lymphocyte proliferation, generation of antibody responses, and maintenance of mucosal surfaces and epithelial function(6) .

Vitamin A has immunocompetent role in human tuberculosis. Vitamin A has a vital role in lymphocyte proliferation and in maintaining the function of epithelial tissues. Vitamin A is essential for normal functioning of T and B lymphocytes, macrophage activity, and generation of antibody response (10).

Iron is a critical element for all organisms as it functions as an essential cofactor for metabolic pathways and enzyme function. Cells invest in complex systems to control iron reactivity, availability, and flux to prevent free radical damage to proteins, ribonucleic acids, and cell membranes. Iron is essential and iron deficiency significantly impairs cell proliferation and immune function (11).

Iron status can have a significant impact on susceptibility to infectious disease, and conversely, that infection and inflammation can alter iron homeostasis .Macrophages can produce hepcidin in response to bacterial infection. Hepcidin an antimicrobial peptide, is responsible for the regulation of iron homeostasis. Under inflammatory conditions, hepcidin is secreted by the liver in response to the cytokines IL-1 and IL-6 and mediates the internalisation and destruction of ferroportin 1 present on the surface of macrophages and on the membranes of intracellular vesicles including pathogen-containing phagosomes (12,13,14).

Vitamin B12 is a water-soluble vitamin that is naturally present in some foods, added to others, and available as a dietary supplement and a prescription medication. Vitamin B12 exists in several forms and contains the mineral cobalt , so compounds with vitamin B12 activity are

collectively called "cobalamins". Methylcobalamin and 5-deoxyadenosyl cobalamin are the forms of vitamin B12 that are active in human metabolism (15).

Vitamin B12 is required for proper red blood cell formation, neurological function, and DNA synthesis , essential biochemical reaction in fat and protein metabolism and also required for hemoglobin synthesis.Pernicious anemia is an autoimmune disease that affects the gastric mucosa and results in gastric atrophy. This leads to the destruction of parietal cells and failure to produce intrinsic factor, resulting in vitamin B12 malabsorption . If pernicious anemia is left untreated, it causes vitamin B12 deficiency, leading to megaloblastic anemia and neurological disorders, even in the presence of adequate dietary intake of vitamin B12 (15).

Although malnutrition is known to be associated with development of diseases, specifically the contribution of micronutrients deficiencies on the occurrence of Tb (longitudinal studies) scarcely found in our country. This paper tried to address this gaps.

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1.2 Statement of the problem

Tuberculosis (TB) remains a major source of morbidity and mortality throughout the world; one-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* (MTB),whereby approximately nine million people develop the disease each year, and almost two million die annually as a result (16,17).

Tuberculosis is one of the deadliest bacterial killers affecting almost all corners of the globe. In-spite of the discovery of antitubercular antibiotics and an available vaccine (BCG vaccine) Moreover the increasing prevalence of HIV-AIDS is being proved to be providing predisposition to .The prevalence of TB has been rising in recent years globally. It remains a major source of morbidity and mortality throughout the world; one-third of the world's population is estimated to be infected withMtb. (5,18)

WHO estimated that, in the year 2012, 8.6 million people have developed tuberculosis and 1.3 million have died of the disease, including 320000 deaths of HIV-TB co-infected people . Long term multiple antibiotic therapy, which is associated with many adverse drug related events have diminished patient compliance with the anti-tubercular chemotherapy (9).

Latently Mtb infected immuno- competent individuals have a 10% lifetime chance of reactivation to active disease but higher chance (up to 90%) of reactivation to active TB in immuno-compromised individuals. About 60% of all TB cases occur in Africa and South East Asia , and this has been associated with extreme poverty, malnutrition, poor hygiene and HIV infection which predisposes to immunosuppression (19,20).

1.3 Significance of the study

Nutritional status is one of the most important determinants of resistance to infection. It is well established that nutritional deficiency is associated with impaired immune functions. While malnutrition limits cell mediated immunity and increases susceptibility to infection, infection can lead to nutritional stress and weight loss, as a result weakening immune function. Micronutrients are important portion of nutrition that help for the development of the immune system of the body. Micronutrients increase ability to produce immunoglobulin and activating lymphocyte transformation for immune protection. The populations of T cells, B cells, and antibodies are increase and their functions also activated. Micronutrients such as Vitamins A have key roles in metabolic pathways, cellular function, and immune competence

Therefore this study will give update information on the micronutrients such as vitamin A , vitamin B_{12} and Iron and in pulmonary tuberculosis with and without HIV before and after TB treatment in Ethiopia.

2 Literature review

Micronutrients and vitamins are important for development of immune cells. TB and HIV often coincide with malnutrition. TB patients frequently suffer from deficiencies of nutrients ,which are fundamental to the integrity of the immune response, especially the host immuneresponse toward Mycobacterium (21).

The study was designed as a case-control study done in Netherlands in2000. The sample size was based on the ability to determine a difference with a 5 0.05 and 1-b 5 0.95 using a one-tailed test for concentrations of serum retinol and zinc and of blood hemoglobin. Because serum zinc concentration was the variable requiring the largest sample size, we calculated that with a sample size of 35 in each group, a between-group difference of 0.46 mmol/L in Zn could be detected. We recruited 45 subjects for each group because we assumed that 25% of patients might not meet the inclusion criteria. 41 (25 men and 16 women) active pulmonary TB patients (cases) and 41 healthy control subjects (25 men and 16 women) aged 28 6 9 y (mean 6 SD) were included in the study. Thirty-four patients (83%) compared with 36 controls (88%) had a BCG-scar on clinical examination. Symptoms and signs of patients were presented as follows: fever ($38°C) (54%), cough . 1 mo (93%), night sweats (61%), hemoptysis (51%), dyspnea (68%), chest pain (63%) and loss of appetite (76%). Of the cases, 26 (63%) had three positive smears and a remaining 15 (37%) had two positive smears for acid-fast bacilli, whereas 24 (59%) of cases had a positive sputum (22).

A longitudinal study of 1,100 men was conducted in a low-income area of Philadelphia, USA vitamin A, vitamin C, hemoglobin, albumin, calcium, and phosphorus were measured, and all subjects had chest x-rays to exclude those with evidence of tuberculosis. The subjects were followed for 7 years, and 28men subsequently developed tuberculosis. Participants with low

initial plasma levels of both vitamins A and Cat baseline had a significantly increased risk of developing active tuberculosis during the follow-up period (23).

A case-control study was conducted in India in 2011 on deficiency of micronutrients on pulmonary tuberculosis patients. The sample size was based on the ability to determine a difference with $\alpha = 0.05$ and $1-\beta = 0.95$.Using a one-tailed test for concentrations of serum retinol and zinc and of blood haemoglobin. Total 43 pulmonary tuberculosis (27 men & 16 women) age 18 to 55 enrolled for study. All the patients have fever, cough, haemoptysis, chest pain and loss of appetite. Of these cases 59% patients had three sputum smear positive and 41% patients had two sputum smear positive. Concentration of haemoglobin, serum albumin, serum retinol and serum zinc was significantly lower in pulmonary tuberculosis patients rather than in control. Erythrocyte sedimentation rate and WBC count was higher in pulmonary tuberculosis patients rather in control (24).

The design of the study was a double-blind, randomized community trial in Indonesia in 2010 on Zinc and vitamin A supplementation fails to reduce sputum conversion time in severely malnourished pulmonary tuberculosis patients. On a total of 300 patients were enrolled into the study and randomly grouped into 4 categories of intervention, i.e. supplementation of zinc (n = 76), vitamin A (n = 72),combined zinc + vitamin A (n = 66) and placebo (n = 86). After two months 274 patients were still in the study and analyzed, while 255 patients completed the study after 6 months and were analyzed. (25).

In a cross-sectional study done in Malawi in 2004 involving 579 HIV-positive and 222 HIV-negative adults with pulmonary tuberculosis in Zomba, Malawi, anthropometry, plasma HIV load and plasma micronutrient concentrations (retinol, α-tocopherol, carotenoids, zinc, and selenium) were measured. The risk of micronutrient deficiencies was examined at different severity levels of wasting. Body mass index (BMI), plasma retinol, carotenoid and selenium concentrations significantly decreased by increasing tertile of plasma HIV load. There were no significant differences in plasma micronutrient concentrations between HIV-negative individuals and HIV positiveindividuals who were in the lowest tertile of plasma HIV load. Plasma vitamin A concentrations <0.70 μmol/L occurred in 61%, and zinc and selenium deficiency occurred in

85%and 87% respectively. Wasting, defined as BMI<18.5 was present in 59% of study participants and was independently associated with a higher risk of low carotenoids, and vitamin A and selenium deficiency. Severe wasting, defined as BMI<16.0 showed the strongest associations with deficiencies in vitamin A, selenium and plasma carotenoids (26).

Intervention study done in Ghana in 2008 on Six hundred and ten (610) new adult pulmonary TBpatients were enrolled during the study period. Of these, 40 (6.6%) were excluded from the analysis because they were not available for their weights to be measured at the end of the intensive phase of treatment for various reasons: 18 defaulted from treatment, 12 died and 10 were transferred out to other treatment centres. Of the 570 patients included in the analysis, 369 (65%) were males and 201 were females. The mean age for all the patients was 39 years; 37 and 41 years for female and male patients respectively (p=0.12). Five hundred and seven (89%) were registered as sputum smear positive and 63 (11%) as smear negative pulmonary TB patients. The mean BMI at registration was 18.7 kg/m2; 51% were malnourished; 24%, 12% and 15% respectively had mild, moderate and severe malnutrition. Two months after starting treatment, the mean BMI was 19.5 kg/m2; 40% were malnourished; 21%, 11% and 8% respectively had mild, moderate and severe malnutrition. Using univariate regression analysis, nutritional status was significantly associated with marital status, income per month, educational level, believe in avoiding certain food types and immediate family size at the time starting TB treatment. Two months after starting treatment, change in BMI was significantly associatedwith age group, marital status, employment status, educational level and belief in avoiding certain food types (27).

This was across sectional study of patients from selected government TB diagnostic and treatment units in 2012-1013 on 365 adult TB patients in Uganda and used descriptive statistics to summarize their socio-demographic, clinical, radiological, sputum mycobacteriology and TB risk factors (HIV, diabetes, TB contact, alcohol use, tobacco smoking, poverty and overcrowding) data. A total of 158 (43.3%) patients were male and the median age was 29 (IQR 28–30). Majority of the patients (89.2%) had pulmonary TB, 86.9% were new and 13.2% were retreatment. Wasting (i.e. body mass index of <18.5 kg/m2) was found in 38.5% of the patients and 63% presented with cough. Constitutional symptoms (fever, anorexia, night sweats and

weight loss) were reported by 32.1%. Most patients (78.6%) presented with non-cavity lung parenchyma disease but 35.2% had cavity disease. Pleural disease was detected in 19.3% of patients. Positive smear microscopy and culture (irrespective of month of treatment) was found in 52.7% and 36.5% of patients respectively. Any drug resistance was detected in 21.1% of patients while multidrug resistance (MDR) TB defined as resistance to rifampicin and isoniazid was detected in 6.3% of patients. All MDR patients were new patients. The prevalence of TB risk factors were as follows: HIV 41.4%, diabetes 5.4%, close contact 11.5%, family history 17.5%, smoking 26.37%, poverty 39.5%, overcrowding 57.3% and alcohol use 50.7%. Overcrowding increased smear positive rate, prevalence ratio 1.22, $p = 0.09$ but all the other studied risk factors did not affect clinical, radiological and mycobacteriological study patient characteristics (28).

A systematic review on quadruple burden trouble of HIV, TB, intestinal parasitic infections, and multiple micronutrient deficiencies to describe immune-modulating effects related to disorders done in Ethiopia in 2014. A review focus on Human immunodeficiency virus (HIV), tuberculosis (TB), and helminthes infections are among the commonest public health problems in the sub-Saharan African countries like Ethiopia. Multiple micronutrient deficiencies are common in people living in these countries either playing a role in their pathogenesis or as consequences. This results in a vicious cycle of multiple micronutrient deficiencies and infection/disease progression. As infection is profoundly associated with nutritional status resulting from decreased nutrient intake, decreased nutrient absorption, and nutrient losses, micronutrient deficiencies affect immune system and impact infection and diseases progression. As a result, micronutrients, immunity, and infection are interrelated (29).

A study done in India on a total 30 pulmonary tuberculosis (19 men and 11 women) age 18 to 55 enrolled for study.All the patients have fever, cough, hemoptysis, chest pain and loss of appetite. Of these cases, 59% of the patients had three sputum smear positive and 41% of the patients had two sputum smear positive . Concentration of serum retinol was significantly ($p<0.0001$) lower in pulmonary tuberculosis patients (0.58 ± 0.25) as compared to controls (1.75 ± 0.80). The level of serum zinc was also significantly ($p<0.0001$) lower in pulmonary tuberculosis patients (6.8 ± 3.15) when compared with controls (13.1 ± 3.4). Similarly, the level of serum albumin was

significantly ($p<0.0001$) lower in pulmonary tuberculosis patients (2.5±1.2) as compared to controls (4.0±0.5) (30).

A study done in Ethiopia on A A total of 155 TB patients (81 HIV seronegative and 74 HIV seropositive) and 31 healthy controls were included in the study. Compared with the control group, the concentrations of iron, zinc and selenium were significantly lower ($P<0.05$) while that of copper and copper/zinc ratio was significantly higher ($P<0.05$) in the serum of TB patients. TB patients with HIV coinfection had significantly lower serum zinc and selenium concentrations and significantly higher copper/zinc ratio compared to that in TB patients without HIV coinfection ($P<0.05$). The serum concentration of zinc had significantly increased at the end of intensive phase of anti-TB chemotherapy in patients without HIV coinfection ($P<0.05$). An increase in serum selenium level was observed in TB patients with or without HIV coinfection after therapy. On the contrary, serum copper concentration and copper/zinc ratio declined significantly after anti-TB chemotherapy irrespective of HIV serostatus ($P<0.05$)(31).

A study done in Indonesia on forty of 54 patients (74%) in the micronutrient group and 40 of 56 (71%) in the placebo group completed the study At baseline, 64% of patients had a body mass index (in kg/m2) < 18.5, 32% had plasma retinol concentrations < 0.70 _mol/L, and 30% had plasma zinc concentrations < 10.7 _mol/L. After antituberculosis treatment, plasma zinc concentrations were not significantly different between groups. Plasma retinol concentrations were significantly higher in the micronutrient group than in the placebo group after 6 mo ($P <$ 0.05). Sputum conversion ($P < 0.05$) and resolution of X-ray lesion area ($P < 0.01$) occurred earlier in the micronutrient group (32).

A study done in indonesiaon in 2010 Initially, 300 patients were enrolled, and 255 finished the treatment. Most patients were severely malnourished (mean BMI 16.5 ± 2.2 Kg/m2). Patients in the zinc + vitamin A group showed earlier sputum conversion time (mean 1.9 weeks) compared with that in the other groups; however the difference was not significant. Also, no benefit could be demonstrated of any of the used supplementations on clinical, nutritional, chest x-ray, or laboratory findings (33).

3. Objective of the study

3.1. General Objective

- To assess the level of micronutrients (Vitamin A, Vitamin B12 and Iron) among M. tuberculosis patients and with HIV confection at baseline and after anti TB treatment

3.2. Specific Objectives

- To assess the level of micronutrient on TB patients
- To assess the effects of HIV on micronutrients in TB patients
- To assess the effects of TB treatment on micronutrient level among TB patients

4. Methods and Materials

4.1. Study area and study periods

This study was conducted from March to October 2016 at EPHI laboratories, Addis Ababa.

4.2. Study design

Retrospective Longitudinal cohort study was conducted.

4.3. Study samples

This study was conduct on serum samples, collected from M.TB patients who were attending at different health centers in Addis Ababa and stored at EPHI at - 80^0 C, for about 10 years.

4.4. Study Population

4.4.1 Source population

All patients with pulmonary tuberculosis patients in Addis Ababa, and had access to come to the sample collection sites (health institutions).

4.4.2 Study population and

Tb patients who give sample in the sample collection sites during the sample collection periods.

4.4.3 Sample size

Of these collected samples the current was carried out in 190 patients in different group like the patients with TB only (87) , TB-HIV confection (57), latent TB(25) and healthy controls (21).The sample size of the study depends on the sample size of the G7 cohort study performed previously in EPHI .

4.5. Sampling techniques

Convenient sampling techniques was employed to select study samples from the stored samples

4.6. Study variables

4.6.1 Dependent variables

- ✓ Vitamin A
- ✓ Vitamin B12
- ✓ Iron

4.6.2 Independent variables

- ✓ Age
- ✓ sex
- ✓ Tb diagnosis

4.7. Data collection techniques

4.7.1 Socio demographic data

Socio demographic data was extracted from data base log book at EPHI.

4.7.2 Laboratory data

Micronutrient measurement: Retinol concentration was measure by high performance liquid chromatography (HPLC),method..Materials and reagents used for retinol measurement were HPLC Grade n-hexne , HPLC Grade Ethanol , HPLC Grade methnol, All trans Retinol standard , HPLC Grade water, Retinol acetate and saline solution(0.9%) ;supplies were test tubes , tips ,tissue paper ,nitrogen gas, glass pipette, and filter paper; and equipment's HPLC, UV-Vis detector , analytical balance , centrifuge , micropipette, quartz cuvette ,sample rack ,vortex mixer , supelcoLC- 18 HPLC column applied during test procedure.

Principle of retinol test

Serum or plasma is diluted 1 to 2 with retnyl acetate solution in ethanol .the retnyl acetate act as internal standard and the ethanol precipitate the protein which release the retinol then extract with hexane .the extract evaporated under the stream of nitrogen and resuspended in methanol .retinol is separated by HPLC using reversed phase C18 column in methanol as mobile phase .retinol is detected with ultra violate detector at 325nm.Its concentration is determined from the ratio of its peak area to that of retnyl acetate

Reagent stability and storage

- The retinol standard and retinol acetate store in deep freezer at -80^0 C

- The others in cool and dark place

❖ Retinol values less than 0.70 μmol/L regarded as indicating deficiency, and values between 0.70 μmol/L and 1.4 μmol/L indicates marginal deficiency.

❖ Retinol is stable At storage temperature as warm as-20 ° Celsius (34).

Vitamin B12 test principle

Competition principle.

- 1st incubation: By incubating the sample (15 μL) with the vitamin B12 pretreatment 1 and pretreatment 2, bound vitamin B12 is released.
- 2nd incubation: By incubating the pretreated sample with the ruthenium labeled intrinsic factor, a vitamin B12-binding protein complex is formed, the amount of which is dependent upon the analyte concentration in the sample.
- 3rd incubation: After addition of streptavidin-coated microparticles and vitamin B12 labeled with biotin, the still-vacant sites of the ruthenium labeled intrinsic factor become occupied, with formation of a ruthenium labeled intrinsic factor vitamin B12 biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents stability and storage:

- Unopened at 2-8ºC up to the stated expiry date
- After opening at 2-8ºC -12 weeks
- On board -5 weeks

Stability of vitamin B12 (cyanocoblamin): Jansen and colleagues compared stability at -196° C, -80° C , and -20°C over a year and reported it to be “stable” at all temperatures. Stability

evidence with measurements of vitamin B12 increasing 0.35% annually at -25° C and increasing 1.9% annually at -20° C. Finally -0.09% annual change at -20° C (35) .

IronTest principle

FerroZine method

Under acidic conditions, iron is liberated from transferrin. Lipemic samples are clarified by the detergent. Ascorbate reduces the released Fe3+ ions to Fe2+ ions which then react with FerroZine to form a colored complex.

Transferrin-Fe complex ----------> apotransferrin + Fe3+ at PH < 2

Fe3+----------> Fe2+ in the presence of Ascorbate

FerroZine + Fe2+ ---------->colored complex

The color intensity is directly proportional to the iron concentration. It is determined by monitoring the increase in absorbance at 552 nm.

Iron is stable in serum sample for many years(35).

4.8. Data processing and analysis

A data were checked weather it is normally distributed or not by frequency distribution but it was not normally distributed. Mean and standard deviation (SD) are used for reporting normally distributed data, and median and 25th–75th percentiles are used for reporting non-normally distributed data. So use median and IQR non-normally distributed parameters were tested using the Mann-Whitney test and wilcoxon signed rank test. The SPSS software was used for all statistical analyses and a P-value 0.05 was considered significant.

4.9. Ethical consideration

The research was ethically cleared by EPHI ethics committee. Moreover, it will have also ethical clearance from departmental research and ethics committee of the department of medical laboratory science, college of allied health sciences, and department of medical laboratory science. Confidential identifiers will be used to code participant identities .result and any information regarding patient will be kept confidential during and after the completion of the research project by password, protected electronics and locking hard copy files.

4.10 Data management and Quality control

Data quality was ensured through use of standardized data collection materials, pretesting of the questionnaires, proper training before the start of data collection and intensive supervision during data collection by the principal investigator. For laboratory analysis Pre analytical, analytical and post analytical stages of quality assurance that is incorporated in Standard operating procedures (SOPs) of the EPHI clinical chemistry laboratory and nutrition department chemistry laboratory was strictly followed. In addition, well trained and experienced laboratory professionals were participate in the laboratory analysis procedure

5. Result of the study

of the total 190 participants , about 57 had both TB- HIV(HIV+TB+) ,87 had TB only (HIV-TB+), 25 had latent TB (HIV-TST+) and 21 healthy controls(TB-HIV-) were are included in the study . Table 1 show the median age of study participants in each study groups with inter quartile range ,30(28-38)yrs in TB-HIV , 27 (21-32)yrs in TB only , 23(20-31)yrs in latent and 24(20-27)yrs were in healthy controls . Female study participants had major proportions in all cases: TB only 48(55%), H only 46(65%), latent TB 17(68%) and healthy controls 12(57%), except in TB-HIV cases where males were dominant 32(56%).

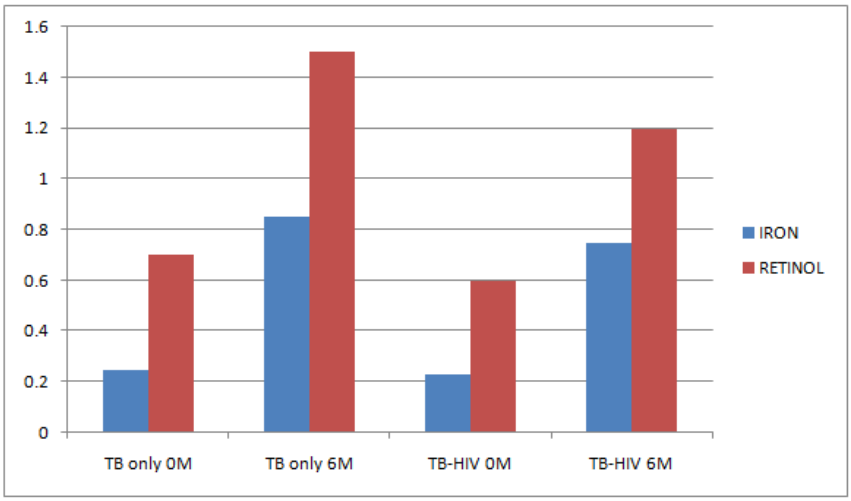
Table 1 Baseline demographic and anthropometric characteristics of study participants at EPHI, in 2016

Demographic and anthropometric data	TB only (87)	TB-HIV(57)	Latent(25)	Healthy control (21)
Median age ,years	27(21-32)	30(28-38)	23(20-31)	24(20-27)

Male ,%	39(45%)	32(56%)	8(32%)	9(43%)
Female ,%	48(55%)	25(44%)	17(68%)	12(57%)
Median BMI at 0M,kg/m2	28.5(17-20)	18.5(16.5-21.4)	22(19.4-24)	21.5(19.5-23)
BMI <18.5kg/m2,%	40	45	12	5

The median BMI of study participant in each group with inter quartile range was , 18.5 (17-20) kg/m2 in TB only patients , 18.5(16.5-21.4)kg/m2 of TB-HIV patients, 22(19.4-24)kg/m2 of latent and 21.5(19.5-23)kg/m2 of healthy controls. Malnutrition (BMI<18.5 kg/m2) was detected in 45% of TB-HIV patients, 40% in TB only patients,11% in HIV only patients , 12% in latent and 5% in health controls .

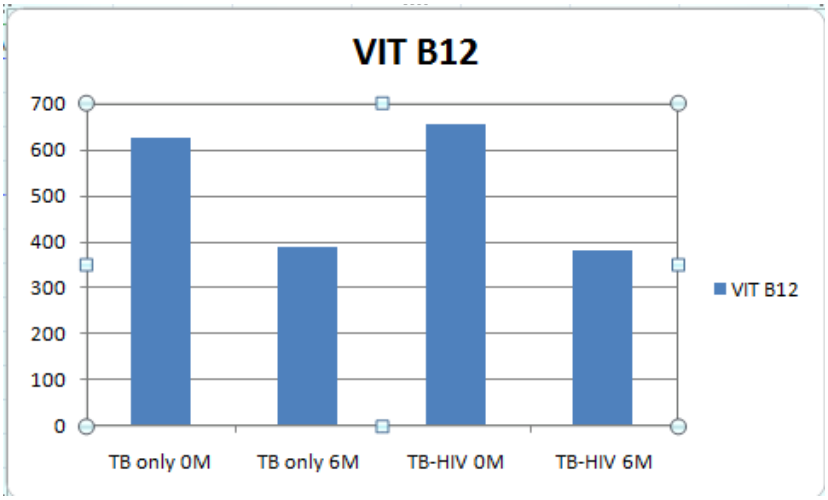
Figure 1: Iron and retinol level of TB only and TB-HIV patients at baseline and at 6th month of anti-TB treatment at EPHI, in 2016



As summarized in figure 1, the effect of anti-TB chemotherapy on the profile of micronutrient concentration was studied by comparing their level at baseline and at the end of intensive phase

of anti-TB chemotherapy. The concentration of iron and retinol in TB only and TB-HIV patients increased significantly after anti-TB chemotherapy (P<0.05).

Figure 2: Vitamin B12 level of TB only and TB-HIV patients at baseline and at 6th month of anti-TB treatment at EPHI, in 2016



As summarized in figure 2, effect of anti-TB chemotherapy on the profile of vitamin B12 concentration was studied by comparing their level at baseline and at the end of intensive phase of anti-TB chemotherapy. The concentration of vitamin B 12 in serum had significantly declined after anti-TB treatment compared to its pretreatment serum concentration both in TB only and TB-HIV patients, P<0.05.

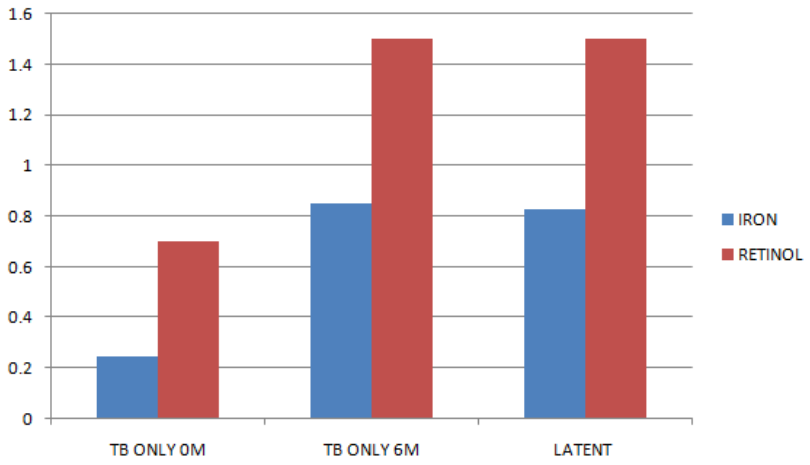
Table 2: Difference in Micronutrient level among TB only and TB-HIV at baseline and TB and TH-HIV at 6th month at EPHI in 2016

Types of micronutrient	TB only M0	TB-HIV M0	TB only M6	TB-HIV M6	TB only M0 Vs TB-HIV M0	TB only M6 Vs TB-HIV M6
Iron(µg/dl)	0.25(0.20-0.37)	0.23(0.19-0.37	0.85(0.51-1.22	0.75(0.54-1.45)	0.542	0.873
Vitamin B12	626.7(479.4	654.8(487.7	388.6(279.2	382.6(269.1	0.635	0.748

(pg/ml)	-829.4)	-873.2)	-565)	-534.6)		
Retinol	0.7(0.5-1.3)	0.6(0.3-0.9)	1.5(1.1-1.9)	1.2(0.7-1.5)	0.109	0.038

As shown in table 2 micronutrient level among TB only and TB-HIV at baseline and TB and TB-HIV after anti-TB treatment, The level of iron, vitamin B12 and retinol among TB only and TB-HIV at baseline had p value of 0.542, 0.635 and 0.109 respectively, . It implies that the three micronutrient level had no significance difference among TB only and TB-HIV at baseline. Measurement of iron and vitamin B 12 among TB only and TB-HIV patients after anti-TB treatment had value of 0.873 and 0.748, and it shows that there was no significance difference of iron and vitamin B12 among TB only and TB-HIV after anti-TB-treatment but there was a significance difference of retinol between the two study group p value 0.038 .

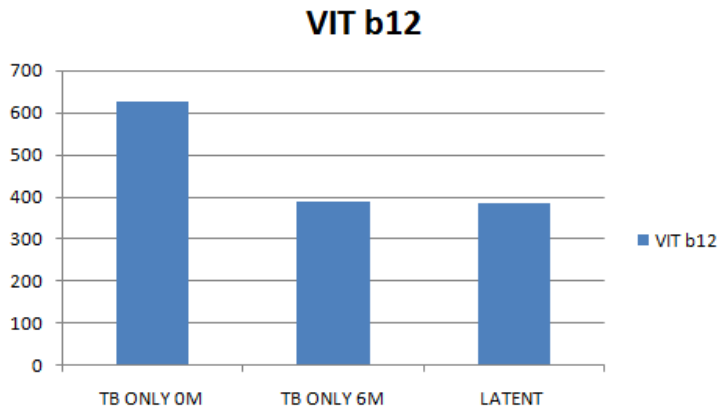
Figure 3.Iron and retinol level among TB only at baseline , after 6th month and latent TB patients at EPHI in 2016



In figure 3 the level of iron and retinol among TB only at baseline and latent Tb cases p value were 0.001, this implies that there was a significance difference among TB only at 0M and latent

one .The level of iron and retinol had significantly high in latent TB group than TB only. In the case of 6th month of anti TB treatment level of iron and retinol among TB only and Latent had p value 0.831 and 0.746 respectively and it implies that there was no significance difference between them.

Figure 4: Vitamin B12 level among TB only at baseline, after 6th month and latent TB patients at EPHI in 2016



In figure 4 the level of Vitamin B 12 had significantly high in TB only patients at baseline than latent TB and after intensive case of anti-TB treatment concentration of vitamin B12 had no significance difference with compare to latent one.

Table 3: Micronutrient level among TB only patients at baseline and 6th month and healthy controls at EPHI in 2016

Types of micronutrient	TB only M0	TB only M6	control	TB only M0 Vs controls	TB only M6 Vs controls
Iron(µg/dl)	0.25(0.20-0.37)	0.85(0.51-1.22	0.89((0.57-1.02	0.001	0.749
Vitamin B12 (pg/ml)	626.7(479.4-829.4)	388.6(279.2-565)	360.4(256.7-622.9)	0.001	0.649
Retinol (µmol/L)	0.7(0.5-1.3)	1.5(1.1-1.9)	1.5(1.1-1.7)	0.001	0.407

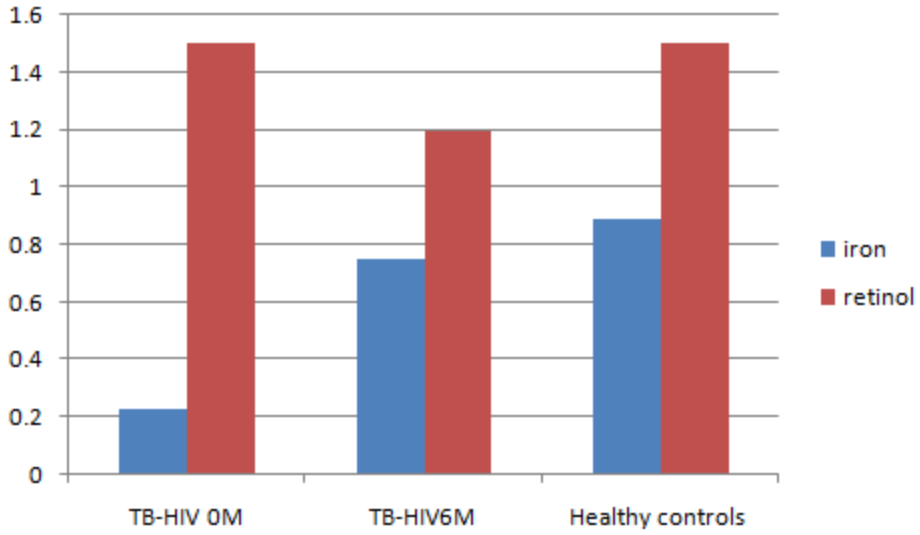
In table 3 when comparing the level of iron ,vitamin B12 and retinol among TB only at baseline and healthy controls, the p value was 0.001It implies that there was a significance difference among TB only and healthy controls at baseline .The level of iron and retinol had significantly high in control group than TB only but level of Vitamin B 12 had significantly high in TB only patients than control .In the case of 6th month of anti TB treatment level of iron ,vitamin B12 and retinol among TB only and healthy control had p value 0.749, 0.649 ,0.407 respectively ,and the three micronutrient level had no significance difference

Table 4: Micronutrient level among TB-HIV at baseline and 6th month and latent TB patients at EPHI in 2016

Types of micronutrient	TB-HIV M0	TB-HIV M6	Latent TB	TB-HIV M0 Vs latent	TB-HIV M6 Vs latent
Iron(µg/dl)	0.23(0.19-0.37	0.75(0.54-1.45)	0.83(0.58-1.07)	0.001	0.878
Vitamin B12 (pg/ml)	654.8(487.7-873.2)	382.6(269.1-534.6)	385.9(273.8-499.8)	0.001	0.838
Retinol (µmol/L)	1.5(1.1-1.9)	1.2(0.7-1.5)	1.5(1.3-1.8)	0.001	0.011

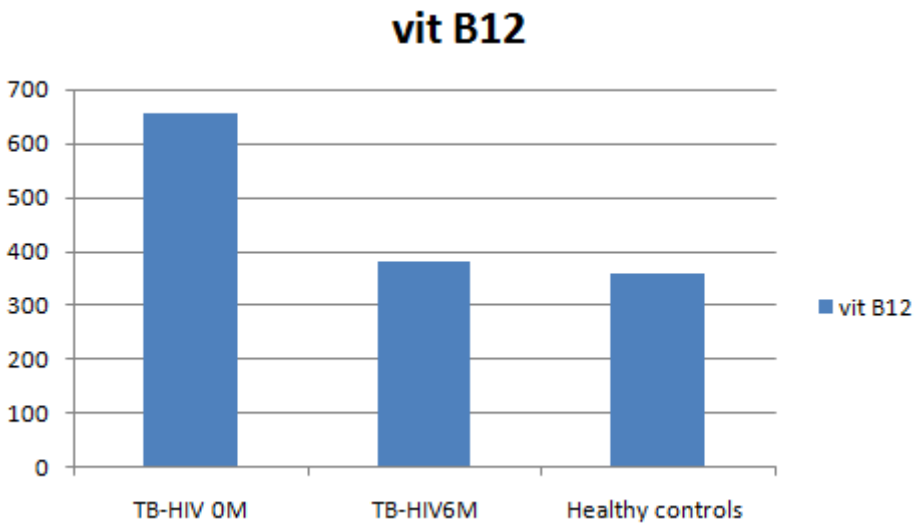
In table 4, when comparing the level of iron, vitamin B12 and retinol among TB-HIV at baseline and latent TB cases, p value was 0.001. It implies that there was a significance difference among TB-HIV and latent one .the level of iron and retinol had significantly high in latent TB group than TB-HIV but level of Vitamin B 12 was significantly high in TB-HIV patients than latent TB .In the case of 6th month of anti TB treatment level of iron and vitamin B12 among TB -HIV and Latent had p value 0.878 and 0.838 respectively. The two micronutrient level had no significance difference but the level of retinol had significance difference between the TB-HIV and latent one ,p value 0.011 .

Figure 5: Iron and retinol level among TB-HIV patients at baseline and 6th month and healthy controls at EPHI in 2016.



In figure 5 the level of iron and retinol among TB –HIV patients at baseline and healthy controls, p value was 0.001in two cases ,it implies that there was a significance difference among TB-HIV and healthy controls .The level of iron and retinol have significantly high in control group than TB-HIV . In the case of 6th month of anti TB treatment level of iron and retinol among TB-HIV and healthy control had p value of 0.703 and 0.083 respectively, and the two micronutrient level had no significance difference.

Figure 6: vitamin B12 level among TB-HIV patients at baseline and 6th month and healthy controls at EPHI in 2016



In figure 6 at baseline the level of Vitamin B 12 have significantly high in TB-HIV patients than control .In the case of 6th month of anti TB treatment level vitamin B12 among TB-HIV and healthy control had p value of 0.887 and had no significance difference.

6. Discussion

Nutritional status is an important determinant of resistance to infection, and malnutrition remains a major cause of immune suppression.

The present study demonstrated that Mycobacterium tuberculosis infection induces a reduction in serum retinol. This phenomenon was clearly shown by significant reduction in the concentration of retinol in pulmonary tuberculosis patients compared to healthy controls. A study done in India in 2011 was agreed with this result, and the concentration of serum retinol was significantly lower in pulmonary tuberculosis patients (0.58±0.25) as compared to healthy controls (1.75±0.80). The possible causes for the low level of serum retinol, in pulmonary tuberculosis patients were considered to be nutritional factors. (4). another study done in India in 2011, serum retinol was significantly lower in pulmonary tuberculosis patients rather than in control. Low concentrations of serum retinol can be due to a number of factors, including reduced intake or reduced absorption of fat. In addition, the infection itself can compromise vitamin A status in a number of ways. It can increase urinary excretion of vitamin A, and it had been shown in patients with fever and infection and increased utilization of retinol by tissues (9)

Mycobacterium tuberculosis infection induces a reduction in serum iron. The present study also demonstrated that serum iron was significantly reduced in pulmonary tuberculosis patients compared to healthy controls. A study done in northwest Ethiopia in 2005 showed low concentration of iron in TB patients than in healthy controls. The possible causes for the low level of serum iron, in pulmonary tuberculosis patients was considered to be nutritional factors (33).In addition the present study indicated that serum iron was significantly reduced in pulmonary tuberculosis co-infected with HIV patients compared to healthy controls .A similar study done in Ethiopia in 2005 also showed that Serum iron concentration was significantly higher in healthy controls than in TB patients with HIV co-infection ($P < 0.01$)(36).

Between TB only and TB-HIV patients measurement of serum iron level at baseline and after treatment had no significance difference at baseline in both groups of TB only and TB co-infected with HIV, before and after treatment .A study done in Ethiopia in 2005 demonstrated that the serum iron concentration in TB only patients and HIV coinfection was not also statistically significant ($P=0.07$).Serum iron level among TB only and TB-HIV patient had no difference due to HIV had no significance implication on iron metabolism (36).

Malnutrition is frequently observed in patients with pulmonary tuberculosis. Several studies reported that patients with active pulmonary TB were malnourished as indicated by reduction of several protein, anthropometric indexes and micronutrient status. In this study we had seen serum retinol concentration at baseline in TB only patients was low 0.7(0.5-1.3) μmol/L than after anti-TB treatment 1.5(1.1-1.9). This result supported by a study done in Indonesia in 2002 .the plasma retinol concentration < 0.7 μmol/L before anti TB treatment after 6thmonth the plasma retinol concentration increase. The possible mechanism of decrease in plasma retinol concentration during infection can be explained by impairment of hepatic release of vitamin A as a result of reduced synthesis of retinol binding protein and urinary loss of retinol may also have a role. The plasma retinol concentration after anti TB treatment become high and equivalent to healthy controls because of active TB decrease plasma retinol concentration (37).

Vitamin B12 absorption is a complex process, involving a series of steps that can be affected adversely by intestinal disease, infections, and medications. The most common explanations for poor vitamin B12 status are a low dietary intake of the vitamin (animal-source foods) and malabsorption. Malabsorption of the vitamin is most commonly observed as food-bound cobalamin malabsorption due to gastric infection. There is growing evidence that gene polymorphisms in transcobalamins affect plasma vitamin B12 concentrations (37).

Serum concentration of vitamin B12 among active TB patients and TB-HIV coinfection at baseline have significantly high value than after anti TB treatment, latent TB and healthy controls. This medications may interfere with the absorption or metabolism of vitamin B 12 in the case of TB only and TB-HIV coinfection (35).but in the case of latent TB and healthy controls may affected by Pernicious anemia is an autoimmune disease that affects the gastric mucosa and results in gastric atrophy. This leads to the destruction of parietal cells and failure to produce intrinsic factor, resulting in vitamin B12 malabsorption (15).

7. Strengths and weaknesses of the study

7.1 Strength

- The study was conducted from old samples, which was rarely done in other studies

7.1 Weakness

- Frequent thawing
- Do not know ART status of TB-HIV patients
- Small sample volumes to use all collected samples

8. Conclusion and Recommendation

8.1 Conclusion

- This study shows that the micronutritional status of patients with active pulmonary TB with and without HIV co-infections was poor compared with healthy controls. The

prevalence of low concentrations of serum retinol and iron was significantly higher in patients than in controls.

8.2 Recommendation

- Micronutrients, as played significant role in boosting immune responses, supplement of vitamins and minerals for Tb patients (with or without HIV co infection) should be considered in all Tb treatment regimes. Moreover, to consolidate the findings of the present study, active longitudinal studies should undertake for the future

9. References

1 Getahun H, Gunneberg C, Granich R, et al. HIV infection associated tuberculosis the epidemiology and the response. Clin Infect Dis. 2010;50(s3):S201–7

2 Treatment outcome of tuberculosis patients at National Hospital Abuja Nigeria:a five year retrospective study. *South African Family Practice* .2015; 57(1):50-56
3 Sakamoto.K . The Pathology of Mycobacterium tuberculosis Infection.Veterinary Pathology.2012;00(0) 1-17
4 Handzel ZT. The immune response to Mycobacterium tuberculosis infections in humans .accessed from http://dx.doi.org/10.5772/ accessed date 22 oct 2016
5 Garcia –Elorriaga and Del Rey-Pineda G .Type 2 Diabetes mellitus as a risk factor for tuberculosis .J Mycobac Dis .2014;4:2
6 tuberculosis (tb) and nutrition .Accessed from http://www.sun.ac.za/nicus/ and accessed date July 05, 2015
7 Papathakis P and Piwaz E .Nutretion and tuberculosis. A revinew the litreture consideration for tb control program.*USAID*.2010;19-25
8 Range N, Anderson AB, MagnussenP,MugeomelaA,FriisH.The effect of micronutrients supplementation on treatment outcome in patients with pulmonary tuberculosis in rondemised control trial in Tanzzania.TroMediInfe Health.2005;9:826-832
9 Chakraborty S ,Syal.K, Bhattacharyya R, Banerjee D. Vitamin deficiency and tuberculosis .need urgent clinical trial for management of tuberculosis .J nutrition health food sci.2014;2(2):1-6
10 Gupta KB, Gupta R, Atreja A, Verma M, and Vishvkarma S. Tuberculosis and nutrition.Lung India. 2009 ; 26(1): 9–16
11 Doherty C P. Host pathogen interaction: the role of iron[1-3].J Nurt.2007;137: 1341-1344
12 Collins HL. Withholding iron as cellular defense mechanism friend or foe .Eur .J. Immunology.2008 ;38:1803-1806
13 HIV/AIDS .TB and NUTRITION Accessed from http://www.assaf.org.za and accessed date 26/7/2015
14 Micronutrients and HIV infection: a review of current evidence WHO .2005
15 Dietary supplement fact sheeth accessed from ods.od.nid.gov/contact. accessed date 22 oct 2016 (15)
16 García-Elorriaga and Del Rey-Pineda .Type 2 Diabetes Mellitus as a Risk Factor for Tuberculosis.JMycobac Dis. 2014; 4:2
17 World Heath Organization: Global tuberculosis control 2009: epidemiology, strategy, financing. Geneva, Switzerland; 2010 WHO/HTM/TB/2009.411
18 Baghaei P, Marjani M, Javanmard P, Tabarsi P and Masjedi MR. Diabetes mellitus and tuberculosis facts and controversies .*J of Diabetes & Metabolic Disorders*. 2013; 12:58
19 Edem VF, Ige O, ArinolaOG.Plasma vitamins and essential trace elements in newly diagnosed pulmonary tuberculosis patients at sifferent duration of anti tuberculosis chemotherapy .*Egypt.J.Chest .Dis* .Tuberc.2015

20 Assessing the Outcome of Tuberculosis Treatment in the Cameroon Baptist Convention Health Board Tuberculosis Treatment Centers. accessed from www.phmed.umu.se/english/divisions/epidemiology accessed date 4/7/2015

21 Wang Q, Ma A, BygbjergIb C, Han X, Liu Y, Zhao S etal. Rationale and design of a randomized controlled trial of the effect of retinol and vitamin D supplementation on treatment in active pulmonary tuberculosis patients with diabetes.BMC Infectious Diseases.2013; 13:104

22 Karyadi E , Schultink W , Nelwan R H Hetal .Poor Micronutrient Status of Active Pulmonary Tuberculosis Patients in Indonesia. J. Nutr. 2000; 130: 2953–2958

23 Samba RD, Darton-Hill I and Pee SD. Addressing tuberculosis in the context malnutrition and HIV co-infection .Food and Nutrition Bulletin .2010;31(4):345-350.

24 Ahmad I, Srivastava VK, Prasad R ,YesufM,Sofiya, Saleem M. Dificiency of micronutrient status in pulmonary tuberculosis patients in north India. Biomedical research .2011;22(4):449-449

25 Pasaki TA, Karyadi E, SuratihNMS ,etal. Zinc and vitamin A supplementation fail to reduce sputum conversion time in severly malnourished pulmonary tuberculosis patients in Indonesia .nutrition journal .2010;9:4

26 Dijistra EE, Clark TD,Taha ET, Semba RD ,etal. Micronutrents malnutrition and wasting in adults with pulmonary tuberculosis with and without HIV co infection in Malawi .BMC infectious disease .2004;4:61

27 Dodor EA. Evaluation of nutritional status of new tuberculosis patients of the Effia-Nkwanta hospital .Ghana medical journal.2008;42(1):22-28.

28 Amare H, Gelaw A, Anagaw B ,Gelaw B. Smear positive pulmonary tuberculosis among diabetes patients at dessie referral hospital ,North east Ethiopia .*Infe.Dise.Pove*.2013;2:6

29 Kibirige D and MwebazeR.Vitamin B12 deficiency among patient with diabetes mellitus .is routine screening supplementation justified .Joumetabo diso.2013;12(17):

30 Ahmed I ,Saleem M ,Safiya, SirajP,Ali W, ,Srivastava VK.A comparative study of serum retinol ,serum zinc and albumin level in active pulmonary tuberculosis patients .Indian J of sci res.2011;2(3):119-121

31 Kassu A , Yabutani T, Mohammed ZH, Mohamed A ,Nguyan N, Huong BTM. etal. Alternation in serum level of trace elements in tuberculosis and HIV infections .European Jou .Nut.2006;60: 580-586.

32 Karyadi ,West CE, Schaltink W, Nelwan RH ,Gross R, Amin Z etal.A double blind placebo controlled study of vitamin A and Zinc supplementation in persons with

tuberculosis in Indonesia . effects on clinical response and nutritional status .Am J clini Nutrit.2002;75:720-727.

33 Pakasi T ,Karyadi E, SuratihNMD,SaleanM,Darmawidjaja N ,Bar H etal. Zinc and vitamin a supplementation fail to reduce sputum conversion time in severly malnourished pulmonary tuberculosis patients in indonesia .Nutrition Jour.2010 ;9:41

34 Comstock GW,Alberg AT and Helzlsouer KJ. Reported effect of long term freezer storage on concentration of retinol B-carotene and alpha tecopherol in serum or plasma summarized .Clini. chem..1993;39(6):1075-1078

35 Jansen, E., Beekhof, P.K., Cremers, J., Schenk, E., Long-term (in)stability of folate and vitamin B12 in human serum, Clin Chem Lab Med 50(10) (2012) 1761-1763

36 Allen LH.cause of vitamin b12 and folate deficiency. food and Nutrition Bulletin . 2008;29(2):520-534.

37 Leary FO, Samman S .Vitamin B12 in health and disease .Journal Nutrition.2010;2:299-316

10. Annexes

Annexe 1: Procedure for tests

I. Procedure for determination serum retinol by HPLC

Principle

Serum or plasma is diluted 1 to 2 with retnyl acetate solution in ethanol .the retnyl acetate act as internal standard and the ethanol precipitate the protein which release the retinol then extract with hexane .the extract evaporated under the stream of nitrogen and resuspended in methanol .retinol is separated by HPLC using reversed phase C18 column in methanol as mobile phase .retinol is detected with ultra violate detector at 325nm.Its concentration is determined from the ratio of its peak area to that of retnyl acetate

Reagent stability and storage

- The retinol standard and retinol acetate store in deep freezer at -80^0 C

- The others in cool and dark place

1 Rinse clean test tubes with HPLC grade /purified water followed by HPLC –grade methanol and bake over night or flush with nitrogen
2 Label tubes with arrange them in rack 5 standard (S1-S5) ,2 internal pooled control (C1-C2) and samples tubes 1 to n(the same number of tubes are needed for hexane extract)
3 Add 300 µL saline to standared and control tubes ,and 300 µL serum to the sample tubes respectively
4 Add 300 µL standard retinol (vitamin A) solution to each standard tubes using micro pipette shake tubes gently to mix
5 Add 300 µL ethanol to each sample tubes using micro pipette shack tubes gently to mix
6 300 µL standard retinyl acetate (vitamin A acetate) solution to all tubes using micropipette ;shake tubes gently to mix
7 Add 2.4 ml hexane (HPLC-grade) to all tubes using dispenser and cap the tube .vortex all the tube for 45 sec
8 Centrifuge all tubes for 15 min at 3000 rpm
9 Carefully remove the upper hexane layer with micropipette (800 µL twice) and transfer to a second capped tubes
10 Repeat steps 7 and 8
11 Carefully remove the upper layer with micropipette (800 µL three times) and transfer to the second capped tubes
12 Cap the tube (containing hexane layer) and store in freezer(-70)until required
13 Before analysis the hexane is evaporated with a stream of pure dry nitrogen and reconstitute immediately in 800 µL methanol
14 The tube should capped quickly to preserve the atmosphere of nitrogen within
15 Carefully transfer the standard and the sample into auto sampler vial which is then loaded into auto sampler tray .first standard (S1-S5) next the internal control (C1-C2) and finally the sample
16 Prepare the column by pumping methanol at 1ml/min for 10 minutes ,then switch the solvent volume to 1.5 ml/min. Check the whole HPLC system properly function
17 Set the auto sampler to Inject 40 µL from each vial and initiate the analytical run monitoring at 325 nm

II. Procedure for determination of serum vitamin B12

Competition principle.

- 1st incubation: By incubating the sample (15 µL) with the vitamin B12 pretreatment 1 and pretreatment 2, bound vitamin B12 is released.
- 2nd incubation: By incubating the pretreated sample with the ruthenium labeled intrinsic factor, a vitamin B12-binding protein complex is formed, the amount of which is dependent upon the analyte concentration in the sample.
- 3rd incubation: After addition of streptavidin-coated microparticles and vitamin B12 labeled with biotin, the still-vacant sites of the ruthenium labeled intrinsic factor become occupied, with formation of a ruthenium labeled intrinsic factor vitamin B12 biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell . Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents stability and storage:

- Unopened at 2-8ºC up to the stated expiry date
- After opening at 2-8ºC -12 weeks
- On board -5 weeks

III. Procedure determination for serum iron

Test principle

Ferro Zine method

Under acidic conditions, iron is liberated from transferrin. Lipemic samples are clarified by the detergent. Ascorbate reduces the released Fe3+ ions to Fe2+ ions which then react with FerroZine to form a colored complex.

Transferrin-Fe complex ----------> apotransferrin + Fe3+ at PH < 2

Fe3+----------> Fe2+ in the presence of Ascorbate

FerroZine + Fe2+ ---------->colored complex

The color intensity is directly proportional to the iron concentration. It is determined by monitoring the increase in absorbance at 552 nm