

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



**LIPIDS AS INDIRECT BIOMARKERS OF PULMONARY TUBERCULOSIS
IN PATIENTS WITH AND WITHOUT HIV INFECTION IN ADDIS ABABA,
ETHIOPIA**

By

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List of Abbreviations

ANOVA	Analysis of Variance
ART	Anti-retroviral Treatment
ATT	Anti-tuberculosis Treatment
BMI	Body Mass Index
CD4+ cells	Cluster Differentiation
°C	Degree Centigrade
CHD	Chronic Heart Disease
EDTA	Ethylene Diamine Tetra-acetate
EHNRI	Ethiopian Health and Nutrition Research Institute
G	Gravity
HDL-C	High Density Lipoprotein Cholesterol
HIV	Human Immune deficiency Virus
HIV+/TB+	patients positive with both HIV and TB infections
HIV-/TB+	Patients positive with TB but negative for HIV infection
HIV+/TST+	Patients positive both for HIV and Tuberculin Skin Test
HIV-/TST+	Subjects positive for Tuberculin Skin Test but Negative for HIV infection
HIV-/TST-	subjects negative for both HIV infection and Tuberculin Skin Test
Kg/m ²	Kilogram per meter square
LDL-C	Low Density Lipoprotein Cholesterol
Mg/dl	Milligram per deciliter
NHTRL	National HIV and TB Referral Laboratory
NCEP-ATP	National Cholesterol Education Program, Adult Treatment Panel
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitors
PLWHIV	People Living with HIV infection
R	correlation
SD	Standard Deviation
STC	Serum Total Cholesterol
TB	Tuberculosis
TC	total Cholesterol
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
Vs	Versus

WHO World Health Organization

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Abstract

Background: - Even though some studies have shown that lipids could be indirect markers of pulmonary tuberculosis (TB), there is no sufficient data from Ethiopia which characterize the level of these markers during TB disease and infection. Nonetheless, the effect of HIV co-infection and its treatment on the level of these markers is not well investigated.

Objective: - to determine whether lipids are indirect biomarkers of pulmonary TB in pts with or without HIV infection.

Methods: - fasting plasma samples collected from 159 untreated adults visiting various health institutions in Addis Ababa (44 HIV+/TB+, 49 HIV-/TB+, 17 HIV+/TST+, 24 HIV-/TST+ and 25 HIV-/TST-) at baseline, from 88 (30 HIV+/TB+, 47 HIV-/TB+ and 11 HIV+/TST+) groups after 6 month of ATT and ART and from 31 (20 HIV+/TB+ and 11 HIV+/TST+) groups after 18 months of ART and stored for five years at -80 °C until analyzed for total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) using Cobas Integra 400 plus. We used stata version 11 for statistical analysis.

Results: -at baseline, mean level of TC, LDL-C and HDL-C were significantly lower in HIV-TB+, HIV+TB+ and HIV+TST+ as compared to HIV-TST+ and HIV-TST-. TG was also significantly lower in HIV+/TB+, HIV-TB+ and HIV+/TST+ than in HIV-TST-. Patients with HIV-/TB+ were also significantly different from HIV-/TST+ groups for TG levels. On the other hand HIV-TB+, HIV+TB+ and HIV+TST+ did not differ significantly from each other for most lipid profiles except for HDL-C that was markedly lower in HIV+/TB+ than HIV-/TB+ and HIV+/TST+ groups. After 6 month of ATT for HIV-/TB+ groups, significant increases were observed in TC, LDL-C and HDL-C. Proportion of patients with abnormal high TC, TG and LDL-C after ATT was very few ($\approx$ 4%). For HIV+/TB+ patients, After 4 months of ART and 6 month of ATT, significant increases were observed in TC, TG and HDL-C levels. At 18 months, the proportion of patients with TC levels $\geq$ 200 mg/dl had increased significantly, from 0 to 20 % and TG levels $>$ 150 mg/dl from 0 to 30 %. However, the proportion of patients with HDL-C levels $<$ 40 mg/dl decreased significantly, from 95 % to 50 %. For HIV+/TST+ patients, After 6 months of ART, no significant change in all lipid levels. However, after 18 months of treatment, TC levels significantly increased by a mean of 61 mg/dl and LDL-C increased by 37 mg/dl

Conclusion and recommendations: In addition to cardiovascular risk prediction, if confirmed in further studies with larger sample size and combined with other prognostic clinical and laboratory

markers, measurement of plasma lipids may allow clinicians and investigators to target patients with pulmonary TB in whom microscopy is most likely to yield a pathogen.

Key words: TB, TB/HIV co-infection, biomarker, lipids, ATT, ART

1. Introduction

1.1 Background

Lipids are synthesized in liver and intestine and are transported to various tissues for their metabolic functions. The lipid profile includes cholesterol, triglycerides, high density lipoproteins (HDL), very low density (VLDL), low density lipoproteins and various risk classification for coronary heart disease (CHD), cholesterol to HDL ratio, and LDL to HDL ratio [1]. Cholesterol is a solid alcohol of high molecular weight and possesses the tetra cyclic perhydrocyclopentanophenanthrene skeleton. It is synthesized from diet and endogenously by the liver and tissue particularly from acetate. Drugs such as atorvastatin, lovastatin, mevastatin etc. suppress the rate limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and thereby lower serum cholesterol levels without the accumulation of water insoluble intermediate of cholesterol synthesis such as desmosterol. It is a compound that is necessary in the body for hormone regulation and basic cellular metabolism. LDL and HDL are carriers of cholesterol. Plasma levels of LDL cholesterol are mainly determined by the production of apo B, the apolipoprotein of LDL cholesterol, by the conversion of VLDL to LDL, and by LDL-receptor mediated clearance. LDL molecules are often informally called bad cholesterol because they can transport their content of many fat molecules into artery walls, attract macrophages, and thus drive atherosclerosis, whereas HDL carries cholesterol from tissues back to liver [2]. Total cholesterol is used to measure lipid status and metabolic disorders [1].

One third of our cell membrane lipids' content is covered by cholesterol. It participates in the fluidity of this structure, in the activity of membrane bound enzymes and in membrane functions, such as phagocytosis and cell growth [3, 4]. It is also an important anabolic precursor for the biosynthesis of bile acid, vitamin D and steroid hormones [5, 6]. Drabowsky et al. demonstrated that cholesterol content in the cell membrane of human lymphocytes is important for their cytotoxic function [6]. Moreover, in a work published by Kaul D. and Anand P, found that a clear derangement of the ability of the macrophage to phagocytose mycobacteria was observed when they were depleted of cholesterol [7]. All of these findings are important in patients with pulmonary tuberculosis, in as much as activated lymphocyte subsets, such as CD4, CD8, and T cells, recruit macrophages and release molecules, such as interferon and tumor necrosis factor that render them more efficient in killing mycobacteria [8, 9]. In addition, cytotoxic lymphocytes (either CD4 or CD8) undergo phagocytosis of macrophages that have already internalized mycobacteria [10, 11].

Tuberculosis and HIV co-infections are associated with special diagnostic and therapeutic challenges and constitute an immense burden on healthcare systems of heavily infected countries like Ethiopia [12]. It was found that HIV infection is one of the most important risk factors associated with an increased risk of latent TB infection progressing to active TB disease, and recorded that Lower CD4+ lymphocyte count was found to be the only predicting factor for co-infection [13]. Pérez-Guzmán C, and Vargas MH also found that hypocholesterolemia is major risk factors for tuberculosis [14]. This may have implications in tuberculosis control programs, especially in countries with high prevalence [15]. Rao S. found that most patients with pulmonary tuberculosis had low total serum cholesterol levels, and that values of about 90 mg/dl were strongly associated with mortality in those patients with miliary disease. HIV infection had negative effect on lipid levels. Both ATT and ART had positive effect on lipid profiles. In most previous other countries studies, lipids in Tuberculosis infection only or patients with HIV infection and its treatment were evaluated [16, 17, 18, and 19]. Data on lipid profiles from TB patients and their treatments and from TB/HIV co-infected patients and their treatments was obtained only from Benign and India respectively which needs to be confirmed by another study. There were some cross sectional published and unpublished studies on lipid profiles of Ethiopian HIV patients taking ART [20]. However, to the best of our knowledge, no cohort study has been done on lipid levels of Ethiopian TB/HIV co-infected patients treated with ART. Moreover data on lipid profiles of Ethiopian TB patients taking ATT was scanty.

The aim of this study was therefore, to examine if lipids are indirect biomarkers of active TB patients with (HIV+TB+) and without (HIV-TB+) HIV infection, latent TB infected individuals with (HIV+TST) and without (HIV-TST+) HIV infection; and healthy controls (HIV-TST-) at enrollment and whether they are prognostic markers of treatment response at follow up.

1.2 Statement of the Problem

According to data from the Ministry of Health, TB is the leading cause of morbidity, the third cause of hospital admission and the second cause of death in Ethiopia [21]. Ethiopia is also one of the country's worst affected by the HIV epidemic, with a total of 1.2 million people living with HIV in 2007 [22]. TB makes malnutrition worse and malnutrition weakens immunity, thereby increasing the likelihood that latent TB will develop into active disease. HIV is one of the most important factors contributing to the increase in active TB cases in sub-Saharan Africa [23]. In Ethiopia, The annual risk of developing

TB in people living with HIV infection (PLWHIV) who are co-infected with *M. tuberculosis* ranges from 5 to 15% as compared to a 5 to 10% life time risk for HIV negative individuals [24].

Early detection and effective treatment with regular follow-up until complete recovery is the most important strategy to control the spread of tuberculosis in the community. Ethiopia needs special attention to control TB as the country harbours a large number of TB patients and many of whom are associated with HIV infection [23]. Co-infection with HIV and TB poses an additional metabolic, physical, and nutritional burden, resulting in further increase in energy expenditure, malabsorption, and micronutrient deficiency [23]. The Mycobacteria activate the invaded macrophages resulting to free radical burst. High serum levels of these free radicals and high concentration of lipid peroxidation products are characteristics of by patients with advanced tuberculosis [25]. The peroxidation could cause reduced concentration of serum lipids and tissue inflammation [26]. Sasaki et al. (1994) reported that lipid peroxidation could cause reduced concentration of serum lipids [25]. Yamanaka et al. (2001) also reported significantly lower concentration of cholesterol in TB patients [26]. This resulted Low cholesterol level which impedes macrophage function and accelerates the disease process. This observation has important therapeutic implication in tuberculosis control programmes [27].

Ten years ago, our country researchers found lower antioxidant potential and greater lipid per-oxidation in Ethiopian tuberculosis patients than in Ethiopian control subjects, with particularly high concentrations of lipid per-oxidation products in those who were co-infected with HIV [28]. Lipids in Ethiopian working adults were determined as one factor to study the prevalence of metabolic syndrome [29], they were also determined in diabetes [30]. Erythrocyte sedimentation rate may be requested as an indicator for screening of tuberculosis patients for underlying HIV infection it is also used as diagnostic value for TB especially in developing countries [31]. Lipids are negatively affected by pulmonary tuberculosis [32]. To the best of our knowledge, however, no study had systematically evaluated the prevalence of lipids among Ethiopian tuberculosis patients before and after treatment. Therefore the aim of this project was to study whether lipids are indirect biomarkers of pulmonary tuberculosis in patients with and without HIV infection in Addis Ababa, Ethiopia.

2. Literature Review

Hypolipidemia is a common disorder affecting about 2 - 3% of apparently healthy individuals and up to 6% of hospitalized patients. Hypocholesterolemia in healthy men is reported to be associated with significantly fewer circulating lymphocytes, total T cells, and CD8+ cells. The prevalence of hypocholesterolemia (<130 mg/dl) from 1479 selected men was found to be 1.8 % in whites and 3.6 % in blacks. In another survey, involving 772 for fighters 3.6% of blacks and 2.9 % of whites were hypocholesterolemic [33]. Both surveys demonstrated that racial difference in the prevalence of hypocholesterolemia as it is more likely to be seen in blacks. In another study conducted among hospitalized patients the prevalence of hypocholesterolemia ranges from 0.5 to 6.2% [7, 34, 35, and 36]. Pulmonary TB is an infectious disease causing the highest mortality rate produced by a single microorganism [36]. A total of 8.8 million incident TB cases have been registered in 96 countries in 2010 [37]. The World Health Organization (WHO) global reports on TB showed that Ethiopia is among the ten top high burden countries in terms of prevalence or incidence cases of TB [38]. Among 509 consecutive PTB suspects attending the outpatient department of university hospital in Addis Ababa, 33% could be culture verified as having PTB [39]. Macrophages are the main effectors cells responsible for the destruction of mycobacteria. Martens GW and his colleagues expertly demonstrated that murine macrophages depleted cholesterol notably decreased their ability to phagocytose mycobacteria. This effect is specific for mycobacteria while phagocytosis of other microorganisms, such as *Escherichia*, *Yersinia*, *Salmonella* and *Lactobacillus* remained unaffected. Thus inability of macrophages to uptake mycobacteria due to a low cholesterol content of their cell membrane might constitute a key defect in the host defense system against tuberculosis, since it is evident that even if activated by T-cells and armed to kill mycobacteria, macrophages will not do it if they are incapable of phagocytosing those [39, 40]. Some years ago it was found that most patients with pulmonary tuberculosis had low serum cholesterol levels (<90 mg/dl). For example, Agomuoh D and his colleagues found low cholesterol levels in Nigerian tuberculous patients [41].

Hypocholesterolemia could reflect hypolipoproteinemia with impairment of the host defense against bacterial products provided by circulating lipoproteins. Cytokines in sepsis may impair lipoprotein production or facilitate their degradation with loss of their protective effect [42]. A study conducted in America College of Chest, the levels of STC, HDL, and LDL were found significantly lower in smear positive group when compared with the smear negative group. The value being TC (141 ± 26.4 g/dl versus 177.5 ± 33 , $P < 0.05$) HDL (31.45 ± 3.5 versus 37.32 ± 4.27 , $P < 0.05$) and LDL (101.52 ± 15.06 versus 119.37 ± 12.69 , $P < 0.05$) [43], similarly the levels of these lipids were found to be significantly lower in advanced TB (stage III) group as compared with stages II and I, the values being cholesterol (133.16 ± 22.59 versus 159.3 ± 16.94 versus 37.29 ± 4.2 , $P < 0.05$ and LDL (97.6 ± 13.92 versus 119.36 ± 4.11 versus 122.64 ± 13.86 , $P < 0.05$) [43]. Deniz, O and his colleagues reported that cholesterol was correlated ($r^2 = 0.25$) with triglycerides at $p < 0.0001$; but in another study it was found that severe hypocholesterolemia became sometimes associated with hypertriglyceridemia. This was especially found in septic patients in whom changes in plasma triglycerides may largely diverge from those of cholesterol [41, 43]. This object is a controversy.

Cholesterol and triglycerides levels do not only reflect their concentration but also altered lipoprotein patterns with modified lipoprotein compositions; it indicates that they are markers of severity of disease. Deniz, O., S. Gumus, et al found significant correlations between degree radiological extent disease and serum HDL-C concentration ($r = -0.6$, $P = 0.0001$) and between degree of smear positivity and LDL-C concentration ($r = -0.28$, $P = 0.011$) this indicated lower in patients with pulmonary tuberculosis than in healthy controls [43]. The reason for decreased serum TC and HDLC-C concentrations may be mainly due to inflammation caused by pulmonary tuberculosis [, 42, 43, and 44]. In America, a study conducted in 15 controls and 60 cases of pulmonary tuberculosis for serum total cholesterol level determination, and all types of cases of pulmonary tuberculosis clients showed diminished value [14]. Low STC levels are therefore, the main predisposing factors for the development of active pulmonary tuberculosis [39]. However in another study No marked difference between serum levels of cholesterol, TG, HDL-C and HDL-C b/n the two groups (patients with pulmonary TB infection and healthy controls) were observed ($P > 0.05$) [44]. Yamanaka et al reported that serum cholesterol was significantly lower in tuberculosis patients and was worse in homeless patients who were prone to starvation.

A study conducted from rural Ethiopia, from 243 suspected patients, 52 had confirmed with pulmonary tuberculosis [45]. Another study conducted at Gondar University Hospital (2012), from 400

HIV positive participants, 7.5% of them were found to have pulmonary tuberculosis [13]. In another part of the country, in 155 patients with active tuberculosis (81 HIV negative and 74 TB/HIV co infected, 31 controls), body mass index (BMI) <18.5 was common (65.4 % of TB patients, 71.6% of TB/HIV co infected [46], but their lipid parameters were not analyzed. In a recent randomized clinical trial in pulmonary tuberculosis patients (new cases) hospitalized during the intensive phase of the four drug anti-tubercular treatment, Guzman et al., 2005 demonstrated that a cholesterol-rich diet notably accelerated the bacteriological sterilization of sputum [47]. With anti-TB drug treatment, nutritional status usually improves. This may be for a variety of reasons, including improved appetite and food intake, reduced energy/nutrient demands, and improved metabolic efficiency [25]. With the currently available drugs, about 90% of pulmonary tuberculosis cases can be cured. However, the success of the treatment depends on the use of appropriate anti-tuberculous drugs, the adherence of the patient to treatment, the sensitivity of mycobacteria to drugs, and the control of associated diseases and metabolic complications [48].

An additional factor that could negatively affect the efficacy of the anti-tubercular treatment is a deficiency in cellular immunity, which in turn can be influenced by nutritional status [49]. Specifically, cholesterol is required for the phagocytosis of mycobacteria into macrophages. The sputum culture result became negative in 73% of study participants after the first month of [47]. However, the lipid status in Ethiopian PTB patients with or without HIV infection was not diagnosed so far.

3. Significance of the Study

Several diagnostics and diagnostic strategies have been endorsed by WHO and are being introduced into clinical use and national tuberculosis control programmes. But still Tuberculosis case detection remains difficult, partly because of inaccurate diagnostic methods. [49]. Lipids are needed as indirect predictors of TB reactivation and cure. These can indicate normal or pathogenic processes, or pharmacological responses to therapeutic intervention. The need for these biomarkers in tuberculosis is most crucial in three areas: in patients with active disease, to predict durable (non-relapsing) treatment success; in patients with latent *M tuberculosis* infection, to indicate reactivation risk and predict treatment success; and in people other than those with active disease, to indicate protection from tuberculosis by new vaccines.

Lipids could be used as a marker for screening of TB where diagnostic facilities are limited. This would save resources, reduce workload, and would thereby strengthen the country's tuberculosis control program by identifying more relationship between active and latent TB. . As Lipids are routinely done along with other basic investigations for the diagnosis of tuberculosis, we suggest that all physicians of similar situation look into the lipid value while initiating the treatment of their TB patients. To the best of our knowledge, the present study is the most comprehensive evaluation to date of circulating concentrations of lipids and is markers for development o f active diseases in pulmonary tuberculosis patients in developing countries. This research also was helpful to see the effect of HIV on lipid profile among TB patients. The purpose of this project was also to search the knowledge gaps and to guide nutritionists and others working in PTB control programs for the improvement of nutritional management of the active TB disease and to recommend to the concerned bodies based on the results.

4. Objectives

General objective

To examine whether lipids are indirect biomarkers of pulmonary tuberculosis in patients with or without HIV infection

Specific objective:

- To compare lipids between TB pts and healthy controls
- To assess the effect of HIV on lipid levels of TB patients
- To find out the effect of ATT on lipid profiles of untreated TB patients
- To determine the effect of ART on lipid parameters of TB/HIV co-infected ART naïve individuals
- To assess the effect of ART on lipid levels of HIV patients with latent TB infection

5. Methods

5.1 Study site

this observational retrospective cohort study was conducted in 2 public health centers (Akaki and Kaliti) and 1 public hospital (Saint Peter specialized TB referral hospital), which are located in different parts of the city, and they are representative in terms of service provision, staffing and cultural diversity, to determine whether lipids are indirect biomarkers of pulmonary TB so as to give recommendations to the concerned bodies for the management and control of the disease. Selection of these sites was based on pilot study done by ENHRI for the study of “Biomarkers of Immune Protective against TB in the Context of HIV/AIDS in Ethiopia”

5.2 Testing site

Samples stored at national HIV and TB referral laboratory (NHTRL), Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia were analyzed.

5.3 Patient selection

From June to December 2013 we studied 93 stored plasma samples of consecutively identified pulmonary tuberculosis patients with (HIV+/TB+, n = 44) or without (HIV-/TB+, n = 49) HIV infection in Ethiopia diagnosed during April 2007 - February 2009 with age of ≥ 15 years of both sex who were anti-tuberculosis treatment (ATT) and anti-retroviral treatment (ART) naïve patients at baseline and scheduled to visit the above clinics at six and at eighteen months. 17 stored plasma samples for HIV patients with latent TB infection (HIV+/TST+) were also included in our study. Tuberculosis was considered proven when a patient had signs of clinical and radiologic pulmonary tuberculosis, including positive Ziehl Neelsen staining of sputum showing acid-fast bacilli [10]. In this retrospective study, we took selected samples of study subjects included in the cohort study for biomarker of immune protective against TB in the context of HIV in Ethiopia conveniently based on inclusion and exclusion criteria. After recruited the samples for study, they were conveniently assigned for analysis of lipid parameters. Sex, age, height, weight, body mass index (BMI), smoking condition, smear result, X-ray findings, World Health Organization (WHO) disease staging, CD4+ cells, hemoglobin levels, HIV status, treatment out comes and other related sign and symptoms were taken from data management of Biomarkers of protective immunity against TB in the context of HIV/AIDS in Ethiopia.

Lipid parameters were later compared with the whole study population with respect to these variables. Twenty five healthy subjects (HIV-/TST-) (9 men, 16 women; $\bar{x} \pm \text{SD}$ age: 25 ± 7 y) with no complaints and no known diseases from the same area who were recruited and stored their plasma samples were analyzed their lipid profile as controls. As an additional control group, we included stored plasma samples from 24 participants with latent TB infection (HIV-/TST+) (10 men, 14 women; $\bar{x} \pm \text{SD}$ age: 25 ± 7 years). Before collection and storage of plasma specimen, the control groups underwent structured clinical and some basic laboratory examination related to TB and HIV sero-status

None of the tuberculosis patients, TB/HIV co-infected subjects or control subjects was using any kind of treatment or prophylaxis for chronic disease, such as hypertension, diabetes mellitus, coronary artery disease, HIV infection, or other diseases that could affect the results of our analysis. In the patient group, lipids were analyzed from untreated stored plasma samples at baseline and at follow up after treatment. Since the study was a sub-study project entitled as “Biomarkers of protective immunity against TB in the context of HIV/AIDS in Ethiopia” which had been ethically cleared institutionally, by the Ethiopian Health and Nutrition Research Institute (EHNRI), and National, by the Ethiopian Science and Technology Agency. In this context, therefore, informed consent of patients” and other related ethical protocols were considered before the initiation of the mother study project. This study had used the stored samples of the mother project at baseline and follow up which was available at the National HIV Lab, EHNRI after permission was obtained from EHNRI. The study protocol was approved by Department of Medical Laboratory Sciences, Addis Ababa University and Science and Ethics Review Office (SERO) of EHNRI.

Active TB is defined as when the immune system is weakened and causing symptoms and contagious disease. Latent TB is defined as where a patient is infected with *Mycobacterium tuberculosis*, but does not have active tuberculosis disease

Protocol for blood sampling, collection and transport: - Peripheral venous blood was drawn into sterile vacuum tubes with EDTA additives between 8:00 and 10:00 after the subjects had fasted overnight. The tubes were kept for 30 minutes to avoid hemolization and then centrifuged ($400 \times g$, 10 min); plasma was transported on dry ice to Ethiopian Health and Nutrition Research Institution (Addis Ababa) for storage at -80°C until analyzed. All samples were protected from light with aluminum foil during transport and processing and were thawed only once.

Inclusion criteria:- clients greater than ≥ 15 years

- Fully recorded target data
- properly preserved plasma specimen
- Individuals who don't take anti-TB treatment at baseline
- Participants who don't start anti-HIV treatment at baseline
- Study subjects that had above one vial of stored plasma samples on the deep freeze. This was because of need of vials for other studies

Exclusion criteria

- Incomplete data
- improperly preserved specimen
- patients who died or missed for any reason during follow up
- participants that had < 50 viral load at baseline
- incident samples (i.e. study subjects with these samples who were latent at baseline but changed in to active infection at follow up for example latent TB changed in to active TB)
- samples with < 1 ml volume

Study design:- experimental retrospective cohort study

5.4 Sample Size and Sampling Procedure

Since the study is the sub study of biomarkers of immune protective against TB in the context of HIV in Ethiopia in which sample size was determined based on the pilot study, we took 200 samples (50 HIV+/TB+, 50 HIV-/TB+, 50 HIV+/TST+, 25 HIV-/TST+ and 25 HIV-/TST-) conveniently at baseline and our sampling procedure was convenient sampling technique.

Biochemical analysis: - Their stored plasma samples were analyzed using fully automated machine (cobas integra 400+, Roche-Switzerland) for lipid profiles (TC, TG, LDL-C and HDL-C). Abnormal high lipid levels were defined as $TC \geq 200$ mg/dl, $HDL-c < 40$ mg/dl, $LDL-c \geq 130$ mg/dl, $TG \geq 150$ mg/dl and $TC/HDL-c$ ratio ≥ 5 by the United States National Cholesterol Education Program, Adult Treatment Panel (NCEP-ATP) III guidelines [51]. A cutoff value for the abnormal low lipid level was not defined in the NCEP guidelines and was therefore chosen as < 130 mg/dl for TC, < 90 mg/dl for

TG, < 90 mg/dl for LDL-C and < 40 mg/dl for HDL-C, on the basis of other published studies, to define a group at hypolipidemic [16].

5.5 Outcome Variables

The outcome variables were total-C, LDL-C, HDL-C, and TGs, defined (1) as continuous variables and (2) as unfavorable levels, as defined by the National Cholesterol Education Program (NCEP) [51]

5.6 Exposure Variables

The primary independent variables were HIV+/TB+, HIV-/TB+, HIV+/TST+, HIV-/TST+, HIV-/TST-, ATT and ART. Confounding factors: BMI, age, sex and cigarette smoking condition

5.7 Statistical analysis

Statistical analyses were performed by using Stata for windows; version 11 (College Station, Texas, USA). Descriptive results of continuous variables were expressed as mean $\pm$ SD. Lipid values among the five study groups were compared using one way analysis of variance (ANOVA) followed by Bonferroni test. Correlations between variables were calculated by Pearson's correlation test. Lipid levels at 6 and 18 months were compared with baseline levels using partial T- test. We compared the proportion of patients with lipid abnormality at baseline versus at 18 months using the McNemar test for correlated proportions. All tests used $\alpha=.05$ as the cutoff point for statistical significance.

5.8 Data Quality Assurance

Pre-test was done to the long term stored samples before regular use. Laboratory personnel had checked for deterioration of stored samples and their stability. Tests for total cholesterol, triglycerides, HDL-C and LDL-C was performed following the specimen reach room temperature. Sample rejection criteria, calibration techniques and quality controls were followed based on each test kits and machines. Data was checked for competence before analysis. Analysis and interpretation of the result was checked by biostatistician advisor.

6. Results

Of the 200 samples enrolled in to whether lipids are indirect biomarkers of pulmonary tuberculosis in patients with or without HIV infection, 159 (HIV+/TB+, n= 44; HIV-/TB+, n = 49; HIV+/TST+, n = 17; HIV-/TST+, n = 24; HIV-/TST-, n = 25) samples (45 for males and 65 for females) were eligible for the current analysis (41 were not randomized). Follow-up data were available for 88 (HIV-/TB+, n = 47; HIV+/TB+, n = 30; HIV+/TST+, n = 11) samples at 6 month (7 patients died, 7 were lost to follow up, and 8 missed study visits and not collected and stored their plasma samples) and 30 (HIV+/TB+, n = 20; HIV+/TST+, n = 10) samples at 18 month (3 patients died, 5 were lost to follow up, and 3 missed study visits and not collected their plasma specimens). Data from patients who died or were lost to follow-up were not included in this analysis.

Patient characteristics:- At baseline, the 44 HIV infected patients with TB (female sex, 52 %) had a mean age of 33 years, mean body weight of 50.2 kg, mean body mass index of 18.7 kg/m², mean hemoglobin 14 mg/dl, mean CD₄ + cell count of 159.2 cells/mm³; Patients with only TB infection (female sex, 56%) had mean age of 27 years, mean body weight of 51 kg, mean body mass index of 19 kg/m², hemoglobin mean 16 mg/dl and CD₄ + cell count of 444 cells/mm³. The HIV infected patients with latent TB infection (female sex, 71 %) had a mean age of 35 years, mean body weight of 53.2 kg, mean body mass index of 20.5 kg/m², mean hemoglobin level of 15.2 mg/dl, mean CD₄ + cell count of 351.4 cells/mm³. The participants with latent TB infection (female sex, 58.3 %) had a mean age of 26 years, mean body weight of 54.8 kg, body mass index 21.4, hemoglobin 16.4 mg/dl, and mean CD₄+ cell count 749.2 cells/mm³. Healthy controls (female sex, 64 %), had a mean age of 25 years, mean body weight of 56.9 kg, mean body mass index of 21.4 kg/m², hemoglobin level 18 mg/dl, mean CD₄+ cell count of 754.8 cells/mm³.

Before treatment, HIV+/TB+ patients were significantly different from HIV-/TST+ and HIV-/TST- with respect to age, BMI, and hemoglobin and CD₄+ cells and from HIV-TST- with respect to weight. The HIV-/TB+ patients were notably different from HIV-/TST+ and HIV-/TST- with respect to CD₄+ cells and BMI and from HIV-/TST- with respect to age and from HIV-/TST+ with respect to height. The HIV+/TST+ group were significantly different from HIV-/TST+ and HIV-/TST- with respect to age and CD₄+ (data not shown)

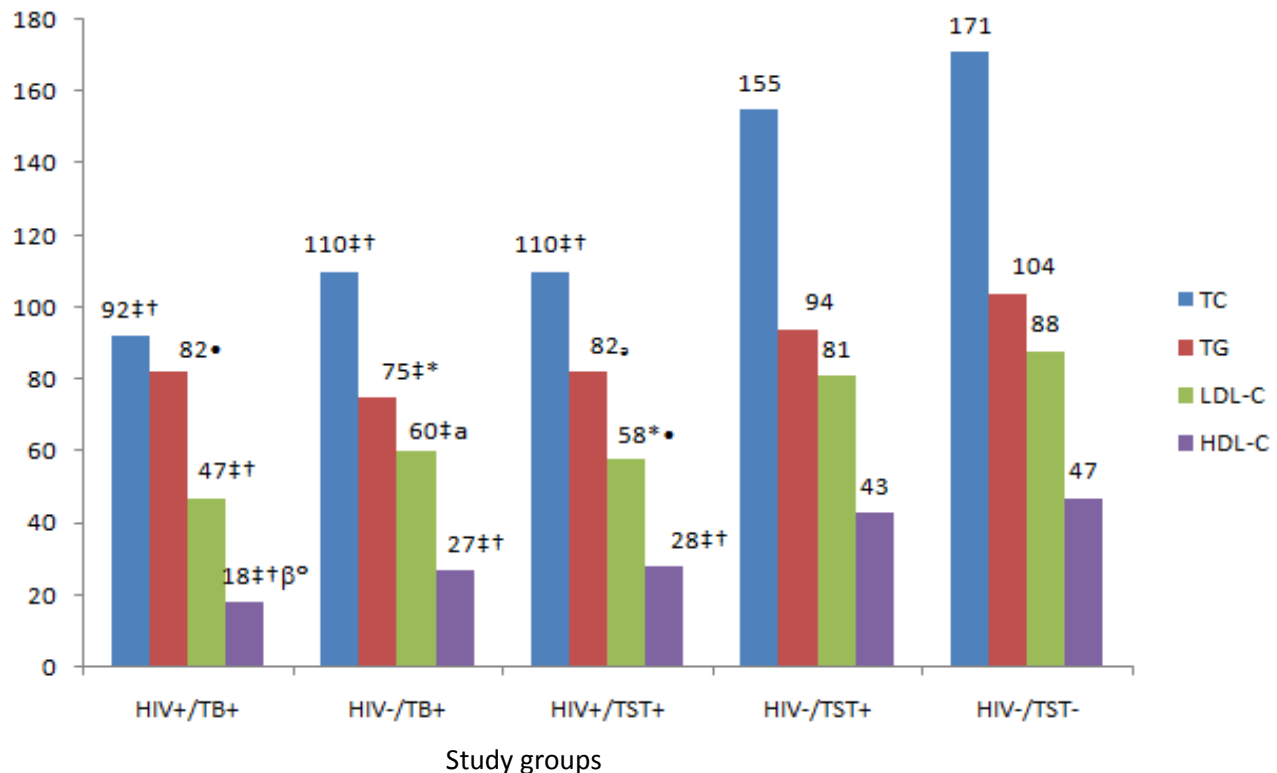


Fig.1. baseline Mean plasma levels of lipids in TB patients co-infected with HIV (HIV+/TB+, n = 44), pulmonary tuberculosis patients without HIV infection (HIV-/TB+, n = 49), HIV patients with Latent tuberculosis infection (HIV+/TST+, n = 17), HIV negative individuals with latent TB infection (HIV-/TST+, n = 24) and Healthy controls (HIV-/TST-, n = 25); ‡significantly different from HIV-/TST-, $P < .0001$; †significantly different from HIV-/TST+, $P < .0001$; •significantly different from HIV-/TST-, $P < .01$; a significantly different from HIV-/TST+, $P < .01$; °significantly different from HIV-/TST-, $P < .05$; *significantly different from HIV-/TST+, $P < .05$; β significantly different from HIV-/TB+, $P < .01$; °significantly different from HIV+/TST+, $P < .05$

Baseline lipids

The baseline lipid levels were comparable by co-infection, sex, and age and body mass index. mean level of TC and HDL-C were significantly lower in HIV-TB+, HIV+TB+ and HIV+TST+ as compared to HIV-TST+ and HIV-TST- ($P < 0.0001$ for all). TG was considerably lesser in HIV-TB+ than in HIV-TST+ ($P < 0.05$) and HIV-TST- ($P < 0.0001$). Additionally, TG was considerably lesser in HIV+/TB+ ($P < .01$) and in HIV+/TST+ ($P < .05$) than in HIV-/TST-. LDL-C in HIV+TB+ was lower than HIV-TST+ and HIV-TST- ($P < 0.0001$). Similarly LDL-C in HIV-TB+ was lower as compared to HIV-TST+ ($P < 0.01$) and HIV-TST- ($P < 0.0001$). Likewise, LDL for HIV+TST+ was lower than

HIV-TST+ at $P < 0.05$ and HIV-TST- at $P < 0.01$. On the other hand HIV-TB+, HIV+TB+ and HIV+TST+ did not differ significantly from each other for all lipid profiles except for HDL-C which was significantly lower in HIV+/TB+ than HIV-/TB+ and HIV+/TST+ groups. There was no also significant difference between HIV-TST+ and HIV-TST- ($P > 0.05$ for all).

TC levels were significantly higher with higher body mass index in HIV+/TB+ ($r = 0.36$, $P < 0.05$), in HIV-/TB+ ($r = .418$, $P < .01$), and in HIV+/TST+ ($r = 0.44$, $P < 0.05$). bmi had also positive correlation with TG in HIV+/TST+ ($r = 0.64$, $P < 0.01$), with LDL-C in HIV+/TB+ ($r = 0.38$, $P < 0.05$) and in HIV-/TB+ ($r = .372$, $P < .01$). In addition to these parameters, weight had direct relation with TC levels and LDL-C in HIV-/TB+ groups ($r = .32$, $P < .05$ for TC and $r = .306$, $P < .05$). However, weight of HIV+/TB+ patients decreased as their triglyceride level increased ($r = -0.421$, $P < .01$). CD4+ cells also had negative relation with triglyceride in HIV-/TB+ patients ($r = -0.409$, $P < .05$) (data not shown).

Longitudinal lipid values

For HIV-/TB+ patients, At 6 months of ATT, patient mean BMI was 20 kg/m^2 , and mean CD4+ cell count had increased from 496 to 703 cells/mm³ (data not shown). After 6 months of treatment, significant increases were observed in TC (110 mg/dl versus 143 mg/dl; $P < .0001$), LDL-C (60 Vs 73 mg/dl; $P < .05$) and HDL-C levels (27 mg/dl Vs 38 mg/dl; $P < .0001$) (figure2). At the end of treatment, which lasted six months, the levels of TC, TG and HDL-C ($P < .01$ for all) and LDL-C ($P < .05$) remained significantly lower in TB treated patients when compared to healthy controls. However when we compared the TB treated patients with that of subjects with latent TB infection, there was no significant difference for all lipid profiles between them (figure3). At 6 months, the proportion of patients with TC levels $< 130 \text{ mg/dl}$ had decreased significantly, from 72 to 40 % ($P < .01$). However, the proportion of patients with TG $< 90 \text{ mg/dl}$, LDL-C $< 100 \text{ mg/dl}$ and with HDL-C $< 40 \text{ mg/dl}$ did not change significantly (83 Vs 77 %, $P = .65$; 94 Vs 81%, $P = .15$; 43 Vs 43 %, $P = 1$ respectively), as shown in table2.

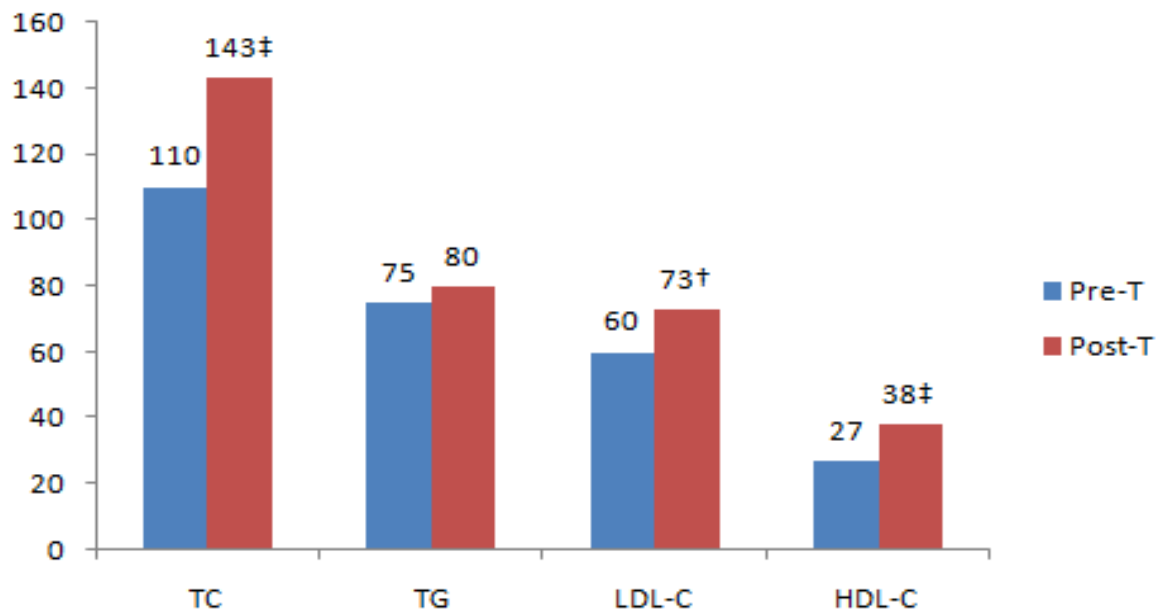


Fig.2. The mean levels of TC, TG, HDL-C and LDL-C of 47 tuberculosis patients (HIV-/TB+) prior to treatment (Pre-Treatment, Pre-T) and at the end of treatment (Post-Treatment, Post-T) were matched. ‡ Significantly different from pre-treatment, $P < 0.0001$; † significantly different from pre-treatment, $P < 0.05$.

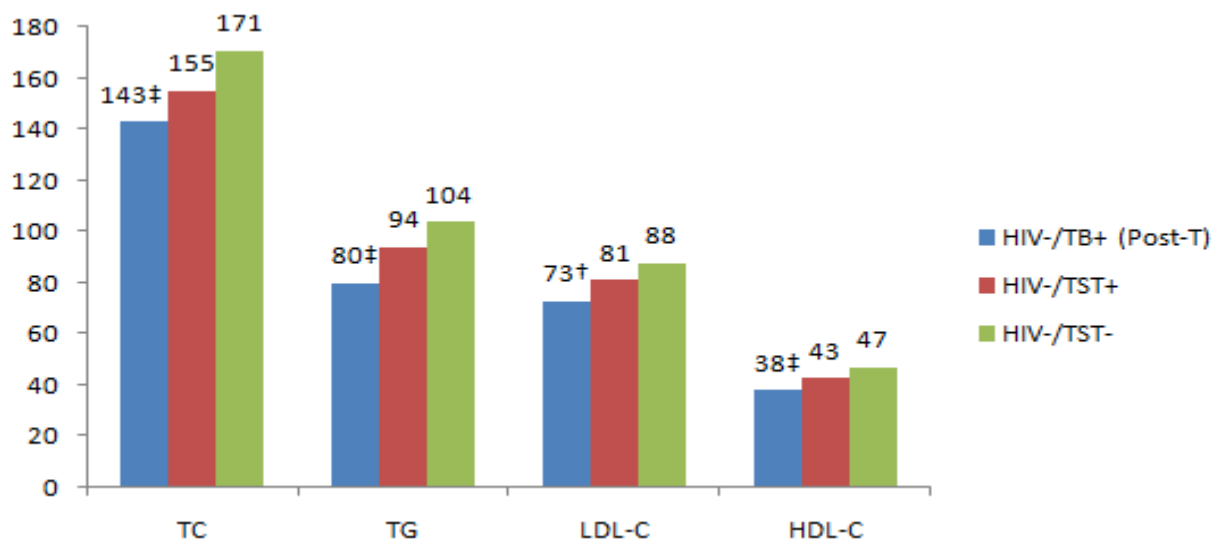


Fig3. The mean levels of TC, TG, HDL-C and LDL-C of 47 tuberculosis patients at the end of treatment (Post-Treatment, Post-T) were compared to that of 24 HIV-/TST+ and 24 HIV-/TST-

groups. [‡]Significantly different from HIV-/TST-, $p < .01$; [†]significantly different from HIV-/TST-, $P < .05$.

Table1 comparing proportion of patients with low lipid levels at baseline and after 6 months of ATT for HIV-/TB+ groups

Lipid parameter	No (%) of patients with low lipid levels				
	At	After 6 months of ATT (n = 47)			
	baseline (n = 47)	Total	Newly developed	Persisted from baseline	P ^a
Total cholesterol level < 130 mg/dl	34 (72)	19 (40)	4 (9)	15 (32)	< .01
Triglyceride level < 90 mg/dl	39 (83)	36 (77)	8 (17)	28 (60)	.65
LDL cholesterol level < 100 mg/dl	44 (94)	38 (81)	3 (6)	35 (75)	.15
HDL-cholesterol level < 40 mg/dl	43 (88)	43 (88)	0	43 (88)	1

NOTE: ATT, Anti-Tuberculosis Treatment

^a comparing the proportion of patients with low lipid levels at baseline and at 6 months with use of McNemar's test

for HIV+/TB+ patients, After 4 months of ART and 6 month of ATT, significant increases were observed in TC (94 vs 130 mg/dl; $P < .001$), TG (83 vs 110 mg/dl; $P < .01$) and HDL-C levels (19 vs 27 mg/dl; $P < .05$) (table 2). At 18 months of ART, patient mean BMI was 21 kg/m², and mean CD4+ cell count had increased from 157 to 258 cells/mm³ (data not shown). As shown in table 3, all the lipid levels were significantly higher at 18 months than at baseline. After 18 months of treatment, TC levels increased by a mean of 78 mg/dl, TG increased by 53 mg/dl, LDL-c by 33 mg/dl and HDL-C levels increased by 22 mg/dl.

At 18 months, the proportion of patients with TC levels ≥ 200 mg/dl had increased significantly, from 0 to 20 % ($P < .05$) and TG levels > 150 mg/dl from 0 to 30 % ($P < .01$). However, the proportion of patients with HDL-C levels < 40 mg/dl decreased significantly, from 95 % to 50 % ($P < .05$). The

proportion of patients with LDL-c levels > 130 mg/dl did not change significantly (0 vs 5 %, P = .08) (data not shown).

Table2. Pattern of change in plasma lipid levels for HIV+/TB+ groups receiving ATT + HAART

Lipid parameter (mg/dl)	Baseline (n = 30)	At 6 months (n = 30)	Change at 6 months	P ^a (6 months Vs baseline)
Total cholesterol	94 ± 28	130 ± 34	35 ± 41	< .001
Triglyceride	83 ± 17	110 ± 47	27 ± 48	< .01
LDL-C	50 ± 22	61 ± 27	11 ± 33	0.07
HDL-C	19 ± 13	27 ± 14	9 ± 18	< .05

^a comparing baseline and after 6 months data using paired partial t-test

Table3. Pattern of change in plasma lipid levels for HIV+/TB+ group receiving HAART

Lipid parameter (mg/dl)	Baseline (n = 20)	at 18 months (n = 20)	Change at 18 months (18 months -baseline)	P ^a (18 months Vs baseline)
Total cholesterol	99 ± 27	176 ± 45	78 ± 44	< .0001
Triglyceride	80 ± 18	133 ± 97	53 ± 99	< .05
LDL-C	53 ± 22	86 ± 27	33 ± 31	< .001
HDL-C	21 ± 15	43 ± 19	22 ± 27	< .01

^a comparing baseline and after 18 months data using paired partial t-test

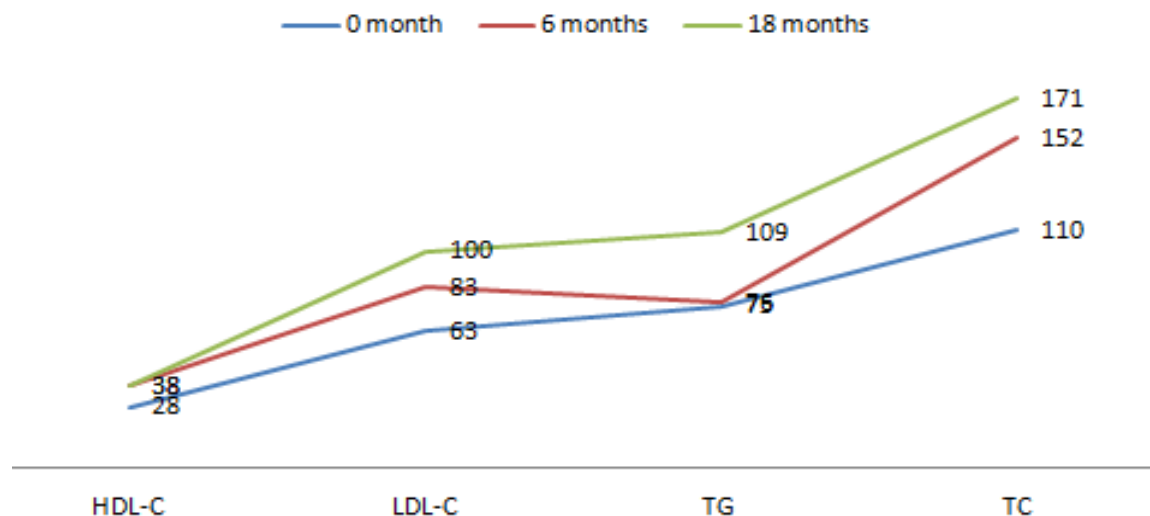


Fig.4. The mean levels of TC, TG, HDL-C and LDL-C of 9 HIV patients (HIV+/TST+) prior to treatment (0 month), 6 months and at the end of treatment (18 months) were matched.

for HIV+/TST+ patients, After 6 months of ART, no significant change in all lipid levels ($P > .05$ for all) (fig4). At 18 months of ART, patient mean BMI was 21 kg/m^2 , however, mean CD_4^+ cell count had decreased from 364 cells/mm^3 to 206 cells/mm^3 (data not shown). As shown in fig4, only TC and LDL-C levels were significantly higher at 18 months than at baseline. After 18 months of treatment, TC levels increased by a mean of 61 mg/dl and LDL-C increased by 37 mg/dl . At 18 months, number of patients who had TC levels $\geq 200 \text{ mg/dl}$, TG $> 150 \text{ mg/dl}$ and LDL-C levels $> 130 \text{ mg/dl}$ were 2 for all. But patients with HDL-C $< 40 \text{ mg/dl}$ were decreased from 89 % to 67 %. (data not shown).

7. Discussion

This study provided a unique opportunity to determine whether lipids are indirect biomarkers of pulmonary tuberculosis in patients with or without HIV infection. TB, TB/HIV co-infection and HIV patients with latent TB infection themselves, in the absences of treatments, were linked with lower TC, LDL-C and HDL-C levels when compared to healthy controls and individuals with latent TB infection. TG level was also lesser in these groups than did in healthy subjects. But comparing to HIV-/TST+ individuals, patients with only TB infection had significantly lower TG levels. HIV/TB co-infected patients had lower HDL-C levels than did subjects with TB infection and HIV patients with latent TB infection. However, lipid parameters in latent TB groups were not significantly different from healthy groups.

Previous studies showed that patients with pulmonary tuberculosis infection had decreased levels of TC, LDL-C and HDL-C, with the extent of low lipid levels correlating with disease severity [52]. Another study found that more advanced HIV disease was associated with less favorable lipid profiles which was similar with our findings [53]. The baseline lipid profiles may somewhat reflect the severity of nutritional and immunological compromise seen with these single and co-morbidity.

After 6 months of ATT for HIV-/TB+ groups, their lipid levels were significantly increased as compared to their baseline data. Results from Benin reported that tuberculosis treatment increases TC levels and normalizes HDL while reducing atherogenic indices to below levels of controls which supports our result. The only difference was that in their result, treated subjects had higher lipid parameters when compared to healthy controls [18], in contrast to our findings, in which individuals treated with ATT for six months had still lower lipid levels than did healthy participants. This difference may be due to genetic makeup, nutritional status, the method we used in which we analyzed the lipid profiles from stored samples. The other interesting finding to ours which was not included in the Benin's study was that, after 6 months of ATT, HIV-/TB+ groups had almost similar amount of lipid levels with the HIV-/TST+ groups. There was a decrease in the number of patients with low levels of TC (from 72 to 32 %) at the end of the study. Averagely, about 21 % of our study patients had normalized their lipid levels after taking anti-tuberculosis treatment for six months (data not shown). A study from Mexico City looked at a Cholesterol-Rich Diet for Bacteriologic Sterilization in Pulmonary Tuberculosis [44]. In that study, the sputum culture became negative in 73 % of their patients after 2 months of follow up. This increase in the proportion of patients who showed culture negative in the Mexico study may be attributable to differences in the method we used (they measured how many

patients in which their bacteriologic culture was sterilized in 8 weeks of follow up by giving cholesterol rich diet in addition to anti-tuberculosis treatment; but we studied how many patients had normal lipid value after 6 months of ATT treatment.), genetic makeup, socioeconomic status, and diet, all of which can affect lipid changes in patients receiving ATT. The baseline lipid profiles may somewhat reflect the severity of nutritional compromise seen with this morbidity. An adequate level of cholesterol is necessary for the proper functioning of the immune system against infection [54]. The changes that we observed in lipid profiles in this study (namely, increased TC, TG, HDL-C, and LDL-C levels) may therefore, at least in part; represent the return to normal lipid values when TB infection is treated, inflammation decreases, nutritional status and immune function improves.

After six months of ATT + ART for HIV+/TB+ groups, their lipid levels were significantly increased as compared to their baseline data. On the other hand, lipid profiles for HIV+/TST+ groups were not changed after 6 months of ART treatment. At 18 months of ART, significant change were observed for all lipid levels in HIV+/TB+ groups and for TC and LDL-C levels in HIV+/TST+ individuals. Previous researches reported that some antiretroviral drugs, such as stavudine (d4T) [55], and protease inhibitors (PIs) [56], increase the blood levels of TC, LDL-c, and TGs with variable effects on levels of HDL-c. Nevirapine (NVP) use is associated with increases in LDL-c [57].

There was an increase in the number of patients with abnormal TC and TG levels (20 & 30 % respectively) for HIV+/TB+ groups at the end of the study. A study from India, looked at the lipid profile of HIV/TB co-infected patients receiving non-nucleoside reverse transcriptase inhibitors (NNRTI) based ART for a mean of 12 months [19]. Their study population was roughly comparable by age to our study population but did not include before anti-TB treatments. In that study, 26 % of patients, compared with 20 % of patients with our study, developed TC-c levels ≥ 200 mg/dl. Whereas 32 % of their patients, similar with 30 % of our patients, developed TG levels > 150 mg/dl.

This increase in the proportion of patients who showed abnormal levels in the India study may be attributable to differences in genetic makeup, treatment regimens, and storage effect of plasma samples, socioeconomic status, and diet, all of which can affect lipid changes in patients receiving ART.

At 18 months for HIV+/TST+ study groups, number of patients who had TC levels ≥ 200 mg/dl, TG > 150 mg/dl and LDL-C levels > 130 mg/dl were 2 for all. But patients with HDL-C < 40 mg/dl were decreased from 89 % to 67 %. A study from Uganda in HIV patients received nevirapine or efavirenz

based and 24 months follow up, recorded a 10% increase in the number of patients with TC levels > 200 mg/dL, a 20% increase in patients with TG levels > 150 mg/dL, and 6% increase in the number of patients with LDL-c level > 130 mg/dL at the end of 24 months [58]. This increase in the proportion of patients who showed abnormal levels in the Uganda study may be due to differences in genetic makeup, treatment regimens, and storage effect of plasma samples, socioeconomic status, and diet, their long term follow up, sample size for the study, all of which can affect lipid changes in patients receiving ART. Earlier cross sectional study from Ethiopia reported that Total cholesterol $\geq$ 200 mg/dl occurred in 43.4% of ART and 15.9% pre-ART patients, whereas HDL-cholesterol below 40 mg/dl occurred in 43.4% and in 63.7% respectively. The LDL-cholesterol $\geq$ 130 mg/dl occurred in 33.6% of ART and 15% pre-ART patients, while triglycerides $\geq$ 150 mg/dl occurred in 55.8% and 31.0% respectively [20].

8. Limitation of our study

First, unfortunately, we do not know the preadmission plasma lipid values of our patients. Second, the number of patients enrolled in the study was low. A study with a larger group of patients is recommended. Third, we used a five year stored plasma samples which had little effect ($\approx 2\%$ decrement for TC, TG and HDL-C) of 7 year stored plasma samples studied in United States America (USA) [59]. Fourth, demographic, clinical and lipid related laboratory results were taken retrospectively which had some discrepancy. Fifth, our findings would be full enough to give complete recommendations if result of lipids had been associated with degree of smear positivity, degree of radiological extent report and culture results which were not found in the prospective study to say lipids are indirect biomarkers of PTB. Six, we were unable to compare lipid levels with other well known markers of inflammation (C-reactive protein (CRP), (albumin concentrations) and erythrocyte sedimentation rate (ESR) because no data from the prospective study to differentiate the most predictive factor for the infection. Seventh, no standardized cut off of value were found to decide hypo-lipidemic patients even though we classified based on the other published articles and comparing with healthy controls.

9. Conclusion and Recommendations

All of the patients in our study had low pretreatment lipid levels, likely resulting from TB infection, TB/HIV co-infection or, serious under nutrition, or poor dietary intake. This study also demonstrated that plasma lipid profiles decreased in active pulmonary TB patients than individuals with latent TB infection and healthy controls irrespective of HIV infection except for HDL-C which was lesser in active TB co-infected with HIV infection than did patients with active pulmonary TB only and HIV patients with latent TB infection. In addition to cardiovascular risk prediction, if confirmed in further studies with larger sample size and combined with other prognostic clinical and laboratory markers, measurement of plasma lipids may allow clinicians and investigators to target patients with pulmonary TB in whom microscopy is most likely to yield a pathogen. To our knowledge, the present study is the most comprehensive evaluation to date of circulating concentrations of lipids and is markers for development of active diseases in pulmonary tuberculosis patients in developing countries.

The finding of new approach, that is lipids as indirect markers of Pulmonary Tuberculosis, is a continuous need to prevent and management of the disease. Therefore, this protocol would be an interest study in which it could show the effect of pulmonary tuberculosis in patients with or without HIV infection on change of lipid profiles and level of lipid parameters after anti-TB and anti-HIV treatment which was not mentioned by other researchers so far in our country. These findings further support a link between hypo-lipidemia and TB and HIV or hyper-lipidemia and Anti-TB and anti-HIV treatment. Whether lipid rich nutrients supplementation will improve tuberculosis outcome or is of importance for its prevention, however, should be examined in future prospective studies. This research also would be helpful to see lipid values in Ethiopian healthy individuals to decide on nutritional status of the people.

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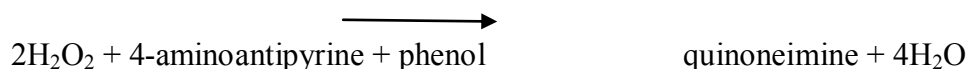
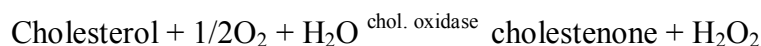
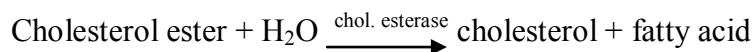
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Annex I: - Lipid Profile Analysis

1. Total Cholesterol

Principle: - free and esterified cholesterol in the sample originates, by means of the coupled reactions described below, a complex that can be measured by spectrophotometry



Reference value: - up to 200 mg/dl = 5.2 mmol/l

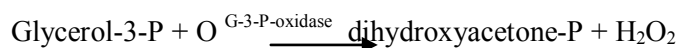
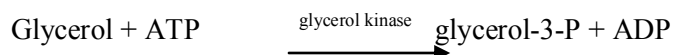
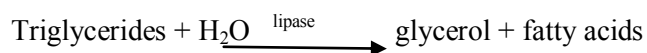
Sample rejection criteria: turbid, lipemic, hemolyzed or incomplete volume

Reagent rejection criteria: - turbidity, absorbance of the blank over 0.2 at 500 nm (1 cm cuvette)

Quality control: - biochemistry control serum level 1 will be used

2. Triglyceride Level Determination

Principle: - triglycerides in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by spectrophotometry



Materials required are the same to the cholesterol listed above

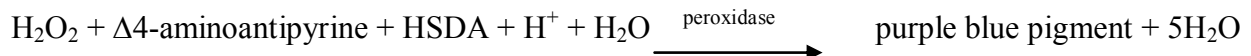
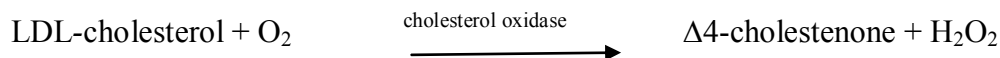
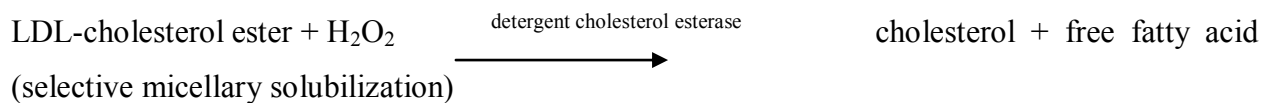
Reagents (5 mmol/l magnesium chloride, 6 mmol/l 4-chlorophenol, 100 U/ml Lipase, 1.5 U/ml glycerol kinase, 4 U/ml glycerol-3-P-oxidase, 0.8 U/ml peroxidase, 0.75 mmol/l 4-aminoantipyrine and 0.9 mmol/l ATP). Procedures and calibration method is the same with cholesterol level determination method.

Reference range: - up to 150 mg/dl

3. LDL-C analysis

Test principle:-homogeneous enzymatic-colorimetric assay.

This automated method for the direct determination of LDL-Cholesterol takes advantage of the selective micellary solubilization of LDL-Cholesterol by a nonionic detergent and the interaction of a sugar compound and lipoproteins (VLDL and chylomicrons). When a detergent is included in the enzymatic method for cholesterol determination (cholesterol esterase cholesterol oxidase coupling reaction), the relative reactivities of cholesterol in the lipoprotein fractions increase in this order: HDL< chylomicrons< VLDL< LDL. In the presence of Mg^{++} , a sugar compound markedly reduces the enzymatic reaction of the cholesterol measurement in VLDL and chylomicrons. The combination of a sugar compound with detergent enables the selective determination of LDL cholesterol in serum. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4 -cholestenone and hydrogen peroxide. This direct assay meets the 1995 NCEP goals of < 4% total CV, bias $\leq 4\%$ versus reference method, and $\leq 12\%$ total analytical error [42].



The color intensity of the blue quinoneimine dye formed is directly proportional to the LDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583 nm.

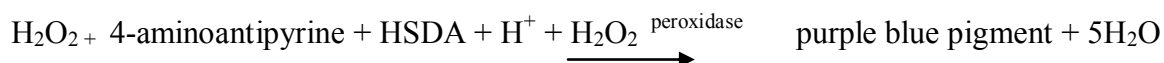
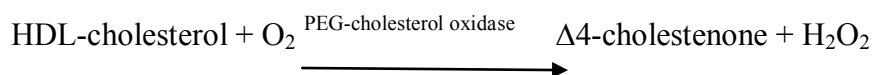
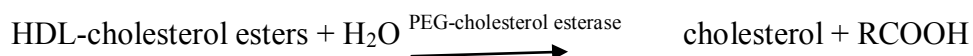
Specimen: - serum/plasma

Reagents and specific procedures are listed in the test kit of clinical chemistry analyzer.

4. HDL-cholesterol Analysis

Test principle: - Homogeneous enzymatic-colorimetric assay. In the presence of magnesium sulfate and dextran sulfate, water-soluble complexes with LDL, VLDL, and chylomicrons are formed which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-

cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40%). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4 -cholestenone and hydrogen peroxide. This direct assay meets the 1995 NCEP goals of 13% total analytical error.



The color intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583 nm.

Specimen: - serum/plasma

Reagents and procedure specified in the test kit of the specific of clinical chemistry analyzer.

Annex II Information Sheet for the Research and Informed Consent of Patients

Title of the project: lipids as indirect biomarkers of pulmonary tuberculosis patients with or without HIV infection visiting selected health institutions, Addis Ababa, Ethiopia

Since the study was conducted on stored serum samples and other related information was reviewed from data management of the study of biomarkers of immune protective against tuberculosis in the context of HIV infection in Ethiopia without contact with study participants, no need of preparing informed consent of patients and nothing will be translated to the local language.

Annex III Questionnaire

Study participants' enrollment form (case report form from data management)

Part one: - socio demographic status

Study site ----- date enrolled ----- address ----- city -----
Kifle ketema ----- kebele ----- house no -----

Sex: 1) male 2) female age at diagnosis (in years): ----- marital status: 1) single

2) Married 3) divorced 4) widowed 5) others

Educational status: 1) no education 2) primary 3) secondary 4) tertiary

Religion: 1) orthodox 2) muslim 3) catholic 4) protestant 5) others

Occupation: 1) ----- ethnicity: 1) Oromo 2) Amhara 3) Tigray 4) Gurage 5) others

Inclusion and exclusion criteria

1. Inclusion criteria: - tick all these requirements (exclude if any of the „No“ boxes is ticked)

Fully recorded target data: Yes ☐ No ☐

Enough volume and properly preserved serum specimen: Yes ☐ No ☐

Patients with ≥ 15 years or ≤ 64 years: Yes ☐ No ☐

2 Exclusion criteria: - tick all these requirements (exclude if any of the „Yes“ boxes is ticked)

Pregnant women Yes ☐ No ☐

Women taking contraceptive pills Yes ☐ No ☐

Yes ☐ No ☐

Anti HIV treatment during or after TB treatment Yes ☐ No ☐

Final decision on inclusion/exclusion: - included ☐ excluded ☐

Part two: - clinical data at baseline (at zero month)

Height (m) ----- weight at diagnosis (kg) -----

Body mass index -----

Patient's knowledge of tuberculosis: 1) symptom 2) transmission 3) treatment

4) All 5) none

Presence of cough more than three weeks: 1) yes 2) no fever: - 1) yes 2) no 3) night sweat :

1) yes 2) no

Coughing up blood: 1) yes 2) no chest pain: 1) yes 2) no

Previous anti-TB treatment: 1) yes 2) no

Classification of pulmonary tuberculosis based on chest-X ray findings as:

1) Minimal 2) mild 3) moderate 4) advanced

Date of seen by nurse:-

Part three: - laboratory data

Initial sputum smear for TB: 1) positive 2) negative

TB culture: 1) positive 2) negative

Classification of mycobacterium tuberculosis infection:

1) Pulmonary 2) extra pulmonary

Type of pulmonary tuberculosis: 1) Smear positive pulmonary tuberculosis 2)

smear negative pulmonary tuberculosis

Stage of mycobacterium tuberculosis

Active disease ☐ latent disease ☐

HIV rapid test: positive ☐ negative ☐

ELISA for HIV: positive ☐ negative ☐

HBS-Ag reaction: reactive ☐ non reactive ☐

HC-anti-antibody reaction: reactive ☐ non reactive ☐

Other opportunistic infections: positive ☐ negative ☐

Tick one of them if it is grouped under the following classes:

HIV+/TB+ ☐ HIV-/TB+ ☐ HIV+/TST+ ☐

HIV-/TST+ ☐ HIV-/TST- ☐

Baseline laboratory results (at zero months)

Hematological result: TWBC (cells/centimeter cube) ----- lymphocytes

(cells/centimeter cube) ----- hemoglobin (mg/dl) -----

CD4 cell count -----

Clinical chemistry tests (if any)

Albumin (g/dl) ----- total protein (g/dl) ----- glucose level (mg/dl) ----

Lipid Profile Results (from stored samples)

TC (mg/dl) ----- TG (mg/dl) ----- HDL (mg/dl) ----- LDL (mg/dl) -----

TC to HDL ratio ----- LDL to HDL ratio -----

Part four: - Treatment Data

Anti-TB treatment: rifampin and isoniazid ☐ ethambutol and pyrazinamide ☐

Received DOT for all doses ☐ Received partial dose ☐

Self administered therapy: Yes ☐ No ☐

Adherent to drug regimen: Yes ☐ No ☐

Received six month therapy: Yes ☐ No ☐

Follow up Laboratory Results (after six months)

Tick one of them if it is grouped under the following classes:

Patients taking anti TB: HIV negative individuals ☐ HIV positive individuals ☐

Hematological result (if any): TWBC (cells/centimeter cube) ----- lymphocytes

(cells/centimeter cube) ----- hemoglobin (mg/dl) -----

CD4 cell count/mm³ -----

Clinical chemistry tests (if any)

Albumin (g/dl) ----- total protein (g/dl) ----- glucose level (mg/dl) ----

Lipid Profile Results (from stored samples)

TC (mg/dl) ----- TG (mg/dl) ----- HDL (mg/dl) ----- LDL (mg/dl) -----

TC to HDL ratio ----- LDL to HDL ratio -----

Follow up Laboratory Results (after eighteen months)

Tick one of them if it is grouped under the following classes:

Patients taking anti TB: HIV negative individuals ☐ HIV positive individuals ☐

Hematological result (if any): TWBC (cells/centimeter cube) ----- lymphocytes

(cells/centimeter cube) ----- hemoglobin (mg/dl) -----

CD4 cell count/mm³ -----

Clinical chemistry tests (if any)

Albumin (g/dl) ----- total protein (g/dl) ----- glucose level (mg/dl) ----

Lipid Profile Results (from stored samples)

TC (mg/dl) ----- TG (mg/dl) ----- HDL (mg/dl) ----- LDL (mg/dl) -----

TC to HDL ratio ----- LDL to HDL ratio -----