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FREE FATTY ACID AND LIPID PROFILE IN ACTIVE TB, LATENT TB, ACTIVE TB AND HIV, ACTIVE TB AND MALARIA PARASITE IN SUBJECTS IN ANAMBRA

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ABSTRACT

Background: *M. tuberculosis* infection is a serious public health challenge especially in developing countries for instance Nigeria. This cross sectional research investigated the effect of tuberculosis (TB) infection on free fatty acid and lipid profile of subjects in Anambra State. **Methods:** Free Fatty acid (FFA) and Lipid profile levels were determined from 239 study subjects composed of 120 treatment naïve active TB patients [26 HIV co infected (TB+HIV+) and 82 HIV negative (TB+HIV−), 12malaria parasite co-infected (TB+MP+)], 26 latent TB infected (LTBI) and 105 healthy control(TB-HIV−TST−). Questionnaire was used to obtain personal data and relevant medical history. The weight and height of all the subjects were measured and used to determine their Body Mass index. TB infection was determined by Ziehl-Neelsen (ZN) sputum smeared microscopy and confirmed positive using gene Xpert. FFA and Lipid profile were estimated by colorimetric method employing biovision FFA and Randox kits UK respectively. **Results:** The levels of FFA, total cholesterol (TC), triglyceride(TG), low-density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C) and very low-density lipoprotein cholesterol (VLDL-C) in TB+, TB+HIV+ and TB+MP+ subjects were significantly lower compared to healthy control and LTBI subjects ($P<0.05$). Further, there was no significantly lower difference in the levels of FFA,TG,TC,HDL-C, LDL-C, and VLDL-C in LTBI subjects compared to healthy control subjects ($P>0.05$). Also, there was no significant difference in the levels of FFA,TG,TC, LDL-C,VLDL-C and HDL-C in TB subjects compared to TB+ HIV+ and TB+MP+ subjects ($P>0.05$) respectively. **Conclusion:** Low level of free fatty acid and hypolipidaemia in TB subjects could be attributed to tuberculosis especially in active TB infection.

KEYWORDS: Tuberculosis, lipid profile, malaria parasite.

INTRODUCTION

M. tuberculosis infection is a serious public health challenge especially in developing countries for instance Nigeria.[1,2] The main cause of TB is *Mycobacterium tuberculosis*, a small, aerobic, rod shaped, non motile bacillus that does not form spores.[3] Tuberculosis may infect any part of the body, but most commonly occurs in the lungs (known as pulmonary tuberculosis). Extrapulmonary TB occurs when tuberculosis develops outside of the lungs, although extrapulmonary TB may coexist with pulmonary TB.[3,4] The high lipid content of this pathogen accounts for many of its unique clinical characteristics. It divides every 16 to 20 hours, which is an extremely slow rate compared with other bacteria, which usually divide in less than an hour.[5] Cholesterol is found almost exclusively in animals and is a key membrane component of all cells.[6] It is a steroid alcohol with 27 carbon atoms that are arranged in a tetracyclical sterane ring system, with a C-H side chain. Tuberculosis and malnutrition are synergistically associated to each other. Any inflammatory condition following infection causes release of free radicals and reactive oxygen species (ROS) which adversely affects host lipid by causing enhanced lipid peroxidation. Association between tuberculosis (TB) and malnutrition is well recognized. TB can lead to malnutrition and malnutrition may predispose to TB. Lipids are more important constituents that determine nutritional status and at the same time participate in immune function.[7] Increasing evidence indicates link between low cholesterol and a number of human diseases including TB.[8,9,10] Despite this fact, it is not known to which extent MTB infection

affects lipid parameters. This study was conducted to determine the effect of tuberculosis infection on the concentration of free fatty acid and lipid profile in active TB, latent TB, active TB and HIV, active TB and malaria parasite in subjects in Anambra.

Informed Consent
The aim, benefits and purpose of the study were explained to the subjects. Participation was voluntary and informed consent was obtained from all the subjects. The subjects were allowed at any time they so desired to discontinue and that would not in any way affect their care. The information obtained from the subjects was kept highly confidential in observance of the privacy act.

Ethical Issues and Approval
Ethical approval for the study was obtained from Nnamdi Azikiwe University Teaching Hospital Ethics Committee (NAUTHEC) Nnewi, St. Charles Boromeo Hospital and Immaculate Heart Hospital Ethics Committee (IHHEC) Nkpor.

Recruitment Procedure
Subjects were recruited consecutively as they attend the TB DOT centre.

Inclusion Criteria
Newly diagnosed TB positive subjects with or without mp, and or HIV co infections were recruited.The subjects above were Category one, first line TB positive subjects of all ages, attending the TB DOTS Clinic, NAUTH, Nnewi, St. Charles Boromeo Hospital and IHHN.

Exclusion Criteria
TB positive subjects on antiretroviral therapy were excluded. Pregnant women, smokers and subjects who had other clinical problems such as diabetics, cancers, and cardiovascular diseases were excluded from the study.

Sample Size
Prevalence rate of tuberculosis in NAUTH TB DOTS Clinic for 2013 was calculated using the figures below.
Number of suspected TB patients of NAUTH TB DOTS Clinic for 2013= 2112
Number of TB positive patients of NAUTH TB DOTS Clinic for 2013 = 169
TB prevalence rate for suspected TB positive patients = 169/2112 X 100% = 8% (NAUTH, 2013).
Sample size was determined using the formula of Araoye, (2004).
$N=Z^2Pq\ (1-P)/D^2$

Where;
N=minimum sample size
P= expected prevalence rate (%) of NAUTH TB DOT Clinic for 2013=169/2112 X 100% = 8%
D= desired level of significance = 0.05 (5%)
Z= standard normal deviation set at 1.96 which corresponds to the 95% confidence level;
q= alternate proportion (1-p) (1 - 0.08 =0.92)
Substituting in the above formula
$N= (1.96)^2 \times 0.08\ X\ 0.92)/\ (0.05)^2$
= 3.842 X 0.08X 0.92/0.0025
=0.2827712/0.0025
=113. A total sample size of 113 was calculated. In order to take care of possible attrition, a total of 159 TB positive subjects were recruited for the study.

Sample Collection
Necessary precautions were taken on proper collection, separation and processing of samples.

Sputum was collected twice and blood samples were collected from TB positive subjects. Firstly, immediately the subject(s) was confirmed of being acid fast bacilli positive (AFB +), before the initiation of Anti tuberculosis treatment (ATT). Blood samples were collected from latent TB and normal control subjects once. Ten milliliters (10mls) of blood was collected from each subjects at each period of blood collection, thick and thin blood films were made for microscopic detection of *P. falciparium on* recruitment and malaria *plasmodium falciparum/*pan rapid test device (Startcare™ Accessio USA) which is a chromatographic immunoassay for the qualitative detection of circulating *P. falciparum* antigen in whole blood was also used. The thick blood film concentrated red blood cells (RBCs) on a small surface and was more sensitive than the thin film method in detecting low levels of parasitaemia. Giemsa stain, an alcohol based Romanowsky stain was used. It is a mixture of eosin which stains the parasite chromatin red or pink and methylene blue which stains the parasite cytoplasm blue. The red blood cells were lysed during the staining process leaving white blood cells, platelets and any parasites, five milliliters (5ml) of blood was dispensed in lithium heparin bottle and five milliliters (5ml) of blood was dispensed in plain tube to separate serum for various biochemical assays.[11] The blood in the plain tube was allowed to stand for 30 minutes to clot, and further centrifuged at 3500 rpm for 5 minutes using Wisperfuge model 1384 centrifuge (Samson, Holland). Serum was separated from clot with micropipette into sterile serum sample bottle for the measurement of biochemical parameters. Each subject's blood sample was stored in aliquot, in three vials to avoid repeated thawing and storing that would affect the result of the analysis.

Sputum collection
Sputum samples were collected using the Directly Observed treatment short Course (DOTs) strategy specification. This involved collecting two (02) samples:
(1) On the spot assessment of subjects.
(2) Another sample early morning next day.

Sputum Processing
Sputum samples were processed using the Ziehl Neelsen Staining Method and confirmed using the Gene Xpert by Cepheid, especially for HIV positive cases with cough.

The positive TB subjects were referred to the DOTs clinic where they commenced treatment.

Anthropometric measurements

The following anthropometric indices were measured for each subject using standardized procedures.[12,13] Weight was measured to the nearest 0.5 kilogram using a standard weighing scale with the subjects wearing light clothes and on bare foot. The scale was on a hard and flat surface, and calibrated frequently using known standard 10kg weight, while the pointer of the scale was adjusted to zero before each measurement.

a. Height was measured to the nearest 0.1 cm using a stadiometer in an erect position against the wall without foot wears, head scarf or caps.
b. Body Mass Index (BMI) was then calculated by dividing the weight (W) in kg by the square of the participant's height (H^2) in meters i.e. BMI = W/H^2 in (kg/m^2) and all values were taken to the nearest one decimal place.

MATERIALS AND METHODS

This cross sectional research was conducted at Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi and Immaculate Heart Hospital Nkpor, both in Anambra State. 239 study subjects composed of 120 treatment naïve active TB patients [26 HIV co infected (TB+HIV+) and 82 HIV negative (TB+HIV−), 12malaria parasite co-infected (TB+MP+)], 26 latent TB infected (LTBI) and 105 healthy control(TB-HIV−TST−) were recruited. At recruitment, all study subjects were interviewed using a standard questionnaire and demographic data were collected. The weight and height of all the subjects were measured and used to determine their Body Mass index. TB infection was determined by Ziehl-Neelsen (ZN) sputum smeared microscopy and confirmed positive using gene Xpert.

Diagnostic Assessments

The HIV status of study subjects was determined using the Determine HIV-1/2 (Abbott laboratories, Japan) as the screening test, the Capillus HIV-1/2 (Trinity Biotech, Ireland) as the confirmatory test and Uni-Gold HIV-1/2 recombinant (Trinity Biotech, Ireland) as a tie breaker test.

Diagnosis of Malaria

P. falciparum malaria was detected using thick and thin blood smears for microscope detection (employing Giemsa staining technique) and malaria plasmodium falciparum rapid test device (CARESTART^{TM} Malaria HRP2 (Pf) by ACCESS BIO,INC. USA) which is a chromatographic immunoassay for the qualitative detection of circulating *p. falciparum* antigen in whole blood. It contains a membrane strip, which is pre-coated with a monoclonal antibody as a single line across a test strip. The monoclonal antibody is specific to the HRP2 (histidine-rich protein 2) of the P. *falciparium.* The conjugate pad is dispensed with antibody absorbed on gold particles, which are specific to HRP2 of P. *falciparium.*

Mantoux test[14]

Tuberculin purified protein derivative (PPD) was utilized for the mantoux test to assist in clinical diagnosis of tuberculosis for diagnosis and differential diagnosis of tuberculosis, early detection of tuberculosis and screening for infection by *M. tuberculosis* (latent tuberculosis infection). This test is known as the tuberculin skin test. The PPD used was obtained from BB – NCIPD Ltd, sofia, Bulgaria. Each vial contained 1ml (10 doses) containing 50TU of PPD = 5TU/0.1ml per dose.

Methods for the estimation of FFA and lipid Profile

Study Designs and Populations

Estimation of Free Fatty Acid Quantification Colorimetric/Fluorometric Kit.

Principle

Fatty Acids are converted to their CoA derivatives, which are subsequently oxidized with the concomitant generation of color or fluorescence. C-8 (octanoate) and longer fatty acids can then be easily quantified by either colorimetric (spectrophotometry at 570 nm wavelenght) or fluorometric (at Ex/Em = 535/587 nm) methods with detection limit 2 µM free fatty acid in variety samples.

Procedure

All reagents, standard solutions, and samples were prepared as instructed and brought to room temperature before use.

Standard Curve Preparation

For the colorimetric assay, 0, 2, 4, 6, 8, 10 µl Palmitic Acid Standard was added into 96-well plate individually and volume was adjusted to 50 µl/well with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of the Fatty Acid Standard.

Sample Preparation: different volume of samples was added to each well in a 96-well plate and the volume was brought up to 50 µl/well with Assay Buffer.

Acyl-CoA Synthesis

2 µl ACS Reagent was added into all the standard and sample wells. Mixed well; the reaction was incubated at 37°C for 30 min. 50 µl of the Reaction Mix was added to each well containing the Standard or test samples. The reaction was incubated for 30 min at 37°C, avoiding exposure to light. O.D. was measured at 570 nm for colorimetric assay in a micro-plate reader. The Fatty Acid amount in the sample wells were calculated from the standard curve. Fatty Acid Concentration = Fa/Sv (nmol/µl or mM) Fa is the Fatty Acid amount (nmol) in the well obtained from standard curve. Sv is the sample volume (µl) added to the sample well.

(a) Total Cholesterol

(a) Total Cholesterol was estimated by Cholesterol enzymatic End-point Method.[15] The kit was purchased from Randox Diagnostic LTD, Cat. No. CH 200.

Principle

Cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4- amino antipyrine in the presence of phenol and peroxidase.[16]

Cholesterol ester + $H_2O \xrightarrow{\text{cholesterolesterase}}$ Cholesterol + fatty acids

Cholesterol + $O_2 \xrightarrow{\text{Cholesterol oxidase}}$ Cholestene-3-one + H_2O_2

$2H_2O$ + phenol + 4-Aminoantipyrine $\xrightarrow{\text{peroxidase}}$ quinoneimine + $4H_2O$

Procedure

10(ul) of serum, distilled water, control and standard cholesterol was collected and transferred into four test tubes labelled sample, reagent blank, control and standard respectively. 1000(ul) of reagent(R1) was added into all the test tubes, mixed and incubated for 5 minutes at 37^OC. The absorbance of the sample (A_{sample}) against the reagent blank was read spectrophotometrically within 60 minutes at 500nm.

(b) Triglycerides

Triglycerides was estimated by enzymatic method[17,16]

The kit was purchased from Randox Diagnostic LTD, Cat. No. TR 210.

Principle

The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.(Tietz, 1990 and Trinder, 1969).

Triglycerides + $H_20 \xrightarrow{\text{lipases}}$ glycerol + fatty acids

Glycerol +ATP $\xrightarrow{\text{GK}}$ glycerol-3-phosphate +ADP

Glycerol-3-phosphate + $O_2 \xrightarrow{\text{GPO}}$ dihydroxyacetone + phosphate + H_2O_2

$2H_2O_2$ +4-aminophenazone +4 chlorophenol $\xrightarrow{\text{POD}}$ quinoneimine +HCl +$4H_2O$

Procedure

10(ul) of serum, distilled water, control and standard cholesterol was collected and transferred into four test tubes labelled sample, reagent blank, control and standard respectively. 1000(ul) of reagent(R1=reagent mixture R1a +R1b) was added into all the test tubes, mixed and incubated for 5 min at 37^OC. The absorbance of the sample (A_{sample}) against the reagent blank was read spectophotometrically within 60 minutes at 500nm.

(c) High Density Lipoproteins-cholesterol (HDL-C)

The kit was purchased from Randox Diagnostic LTD, Cat. No. CH 203.

Principle

Low Density Lipoprotein (LDL AND VLDL) and chylomicron fractions are precipitated quantitatively from serum by the addition of phosphotungstic acid in the presence of magnessium ion. After centrifugation, the cholesterol concentration in the HDL (High Density Lipoprotein) fraction which remains in the supernantant is determined.

Procedure

(i) Precipitation reaction, 500(ul) of sample and standard was transferred into two test tubes, each containing 1000(ul) of precipitant (R1), and labelled, sample and standard respectively. The tubes were mixed and allowed sitting for 10minutes at room temperature. Then centrifuged for 10 minutes at 4000 rpm, or 2 minutes at 12000rpm. The cleared supernatant was separated off within two hours and was used for determining the cholesterol content as follows.

(ii) 100(ul) of supernatant, distilled water and standard supernatant was collected and transferred into three test tubes labelled sample, reagent blank and standard respectively.1000(ul) of reagent (R1) was added into all the test tubes, mixed and incubated for 5 min at 37^OC. The absorbance of the sample (A_{sample}) against the reagent blank was read spectophotometrically within 60 minutes at 500nm.

(d) Low Density Lipoprotein Cholesterol (LDL-C) in mmol/l was estimated by a clearance method for the direct measurement of LDL cholesterol using Randox Diagnostic LTD kit.

Principle

The assay consists of two distinct reaction steps

Elimination of chylomicrons, VLDL-Cholesterol and HDL-Cholesterol by cholesterol esterase, cholesterol oxidase and subsequently catalase.

Specific measurement of LDL-Cholesterol after release of LDL-Cholesterol by Cholesterol enzymatic End-point Method.[15]

Statistical Analysis

Effects of TB on the biochemical parameters under study of NHC,LTBI,TB+, TB+ and HIV+, and TB+ and MP+ were analyzed using one way ANOVA with Turkey's post test to compare all pairs of columns. The IBM Statistical Package for Social Sciences (SPSS) VERSION 21 was used for the statistical analysis. The results were presented as mean ± standard deviation. Significant levels were considered at $P< 0.05$.

RESULT

There was a significant difference in the mean serum levels of FFA (nmol/µl), TG(mmol/l), TC (mmol/l), LDL-C(mmol/l), HDL-C (mmol/l) and VLDL-C(mmol/l) in NHC, LTBI, TB, TB & HIV and TB and MP ($F=15.35$, $P<0.05$; $F=39.95$, $P<0.05$; $F=24.12$, $P<0.05$; $F=67.51$, $P<0.05$; $F=2.36$,$P<0.05$ and $F=5.52$, $P<0.05$)

respectively. The levels of FFA, total cholesterol (TC), triglyceride(TG), low-density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C) and very low-density lipoprotein cholesterol (VLDL-C) in TB+, TB+HIV+ and TB+MP+ subjects were significantly lower compared to normal healthy control and LTBI subjects (P<0.05). Further, there was no significantly lower difference in the levels of FFA,TG,TC,HDL-C, LDL-C, and VLDL-C in LTBI subjects compared to healthy control subjects (P>0.05). Also, there was no significant difference in the levels of FFA,TG,TC, LDL-C,VLDL-C and HDL-C in TB subjects compared to TB+ HIV+ and TB+MP+ subjects (P>0.05) respectively (Table 1).

Table 1: Serum Levels of Baseline Free Fatty Acid and Lipid Profile.

GROUP	FFA (nmol/µl)	TG (mmol/l)	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	VLDL-C (mmol/l)
1.NHC(105)	0.32±0.15	1.55±0.87	5.12±1.11	2.71±1.30	1.71±1.00	0.71 ±0.39
2.LTBI(26)	0.29±0.11	1.12±0.49	4.98±1.80	2.47±1.53	2.00±1.20	0.51±0.22
3.TB (82)	0.19±0.08	0.56±0.27	2.44±1.50	0.51±0.48	1.68±1.32	0.25±0.12
4.TB & HIV(26)	0.23±0.07	0.44±0.26	2.35±1.64	0.48±0.53	1.36±1.10	0.20±0.12
5.TB & MP(12)	0.20±0.07	0.41±0.21	2.46±1.84	0.67±0.46	1.60±1.48	0.19±0.10
F-VALUE	15.35	39.95	24.12	67.51	2.36	5.52
P-VALUE	0.00	0.00	0.00	0.00	0.05	0.00
1 VS 2	0.19	0.09	0.54	0.13	0.30	0.03
1 VS 3	0.00	0.00	0.00	0.00	0.03	0.00
1 VS 4	0.00	0.00	0.00	0.00	0.00	0.00
1 VS 5	0.00	0.00	0.00	0.00	0.08	0.00
2 VS 3	0.00	0.00	0.00	0.00	0.04	0.00
2 VS 4	0.03	0.00	0.00	0.00	0.00	0.00
2 VS 5	0.04	0.00	0.00	0.00	0.03	0.00
3 VS 4	0.10	0.32	0.53	0.86	0.34	0.08
3VS 5	0.76	0.42	0.31	0.63	0.50	0.36
4 VS 5	0.43	0.88	0.19	0.59	0.22	0.06

DISCUSSION

This study was done to assess any difference in concentrations of FFA and lipid profile (FFA, TC, TG, LDL-C, VLDL-C and HDL-C) between TB subjects with and without HIV infection, TB subjects with or without MP infection, latent TB infected and normal healthy control subjects. The result shows that the levels of FFA, total cholesterol (TC), triglyceride(TG), low-density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C) and very low-density lipoprotein cholesterol (VLDL-C) in TB+, TB+HIV+ and TB+MP+ subjects were significantly lower compared to normal healthy control and LTBI subjects (P<0.05). These findings agree with the study in Ethiopia by[18] whose study showed significantly lower levels of TC, LDL-C, and HDL-C concentrations at baseline in active TB subjects compared to NHC and LTBI subjects. It also collaborates with the work in Benin Republic by[10], who observed statistically significant lower levels of TC, HDL-C, LDL-C and TG in pulmonary TB patients than normal subjects while investigating whether tuberculosis (TB) treatment normalizes the lipid profile strongly affected by pulmonary TB. In the studies by[10,18], lipid profiles were raised in TB subjects compared to the normal healthy control after treatment. Although, none of these studies included free fatty acids but this study considered it because it plays very important roles in normal metabolism and many disease developments. Fatty acids are precursors to a number of bioactive classes of compounds such as prostaglandins, leucotrienes and others and have been implicated in diverse functions such as autism, immune system and inflammation response (Burtis, 2008). The statistically significant lower level of FFA in LTBI and especially active TB subjects with other lipids compared to normal healthy control observed in this study was not surprising since triglycerides are chemically tri esters of fatty acids and glycerol. Triglycerides are formed by combining glycerol with three fatty acid molecules, and TG was also reduced in this study. Moreover,[19] from Southern West, Nigeria and[20] in their studies from India, had similar results where all lipid parameters(TC,TG,HDL-C,LDL-C,VLDL-C) were significantly (p<0.05) low at baseline in TB subjects than controls. Furthermore, according to this study, it was found that subjects with pulmonary TB have low lipid profiles (FFA,TC, TG, LDL-C, VLDL-C and HDL-C) that probably indicate TB disease might be a risk factor for low lipid profiles at diagnosis time point.

CONCLUSION

Low level of free fatty acid and hypolipidaemia in TB subjects could be attributed to tuberculosis especially in active TB infection.

Recommendation

Further research is needed with larger number of patients with individual-level dietary history and longer follow-up periods in order to provide additional support to this finding.

Ethical Approval

The study obtained ethical clearance from Nnamdi Azikiwe University Teaching Hospital Ethics Committee

(NAUTHEC,NAUTH/CS/66/VOL.7/79) Nnewi, Immaculate Heart Hospital Ethics Committee (IHHEC,06/14/2016) and St. Charles Borromeo Hospital, (07/24/2017) Nkpor.

Consent

All study participants provided written, informed consent at enrollment.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ihim A.C., Onyenekwe, C.C. and Meludu, S.C were lead authors in planning, implementation of the study, data analysis, writing of the draft, and final version of the manuscript; Ihim, A.C. and Obi. P.C. participated in laboratory tests; Anyabolu, A.E. and Njoku, Chidiadi mary Ann were involved during data analysis and in writing during the draft interim and final version of the manuscript. All authors have seen and approved the final manuscript.

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