



Evaluation of Some Cytokines, CD4, Hepcidin, Iron Profile and Some Haematological Parameters of Pulmonary Tuberculosis Patients Coinfected with HIV in Southeast of Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author OEI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EBN and OGU managed the analyses of the study. Authors OOMTB, CEF, EIS and OKC managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The study was done to determine the levels of interferon-gamma, interleukin 6, interleukin 10, iron status, hepcidin and haematological parameters of patients with pulmonary tuberculosis co-infected with human immunodeficiency virus in Southeast, Nigeria. This study was carried out at the directly

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observed treatment-short course Tuberculosis (TB DOTS) centre of Federal Medical Centre, Umuahia, located in South-Eastern Nigeria. Therefore, sample size of 240 was used to give room for attrition. A total of two hundred and forty (240) subjects aged 18-60 years were enlisted for this study. Seven milliliters (7ml) of venous blood was collected from each subject and 2.5 ml was dispensed into bottles containing di-potassium salt of ethylenediamine tetra-acetic acid (K₂-EDTA) and was used for full blood count, CD4 count and HIV screening. Also, 4.5ml was dispensed into plain tubes. Serum was obtained after clotting by spinning at 3000 RPM for 10 minutes and was used for interferon gamma, interleukin-6, and interleukin-10, iron and hepcidin determination. Data was analysed using statistical package for social science (SPSS) version 20. Student t-test, ANOVA (Analysis of Variance), Pearson Product Moment and Chi-Square were the tools employed. Results were expressed as mean \pm standard deviation and are presented in table and significance level was set at $P < 0.05$. The results showed difference that was statistically significant ($P < 0.05$) in IFN- γ ($P = 0.000$), IL-6 ($P = 0.000$) IL-10 ($P = 0.000$), CD4 ($P = 0.000$), hepcidin ($P = 0.000$), Iron ($P = 0.000$), TIBC ($P = 0.000$), %TSA ($P = 0.001$), WBC ($P = 0.000$), Neutrophils ($P = 0.000$), Lymphocytes ($P = 0.000$), Monocytes ($P = 0.000$), Eosinophils ($P = 0.000$), Basophils ($P = 0.018$), RBC ($P = 0.000$), haemoglobin ($P = 0.000$), PCV ($P = 0.000$), MCV ($P = 0.000$), MCH ($P = 0.000$), MCHC ($P = 0.000$), Platelets ($P = 0.000$), ESR ($P = 0.000$) when compared among control, TB, HIV and TB-HIV subjects respectively. The co infection of HIV on pulmonary TB patients increases the levels of the cytokines. The cytokines and hepcidin can be used as adjunct to prognostic and diagnostic markers as their levels decreased with increased duration of treatment of the patients. The study has shown wide variations in the haematological indices studied.

Keywords: Interferon-gamma; IL-6; IL-10; CD4; hepcidin; iron; haematological parameters; pulmonary tuberculosis patients; HIV.

1. INTRODUCTION

Pulmonary tuberculosis (TB) is a recurring bacterial illness triggered by *Mycobacterium tuberculosis* (MTB) complex which frequently affects the lungs; (pulmonary TB (PTB), but can affect other parts as well; (extra-pulmonary TB (EPTB) as opined by Thumamo et al. [1,2]. *Mycobacterium tuberculosis*, the bacterium that leads to human pulmonary tuberculosis illness, is an ancient foe. Chronologically; pulmonary tuberculosis (PTB) has a pedigree that could be linked to the earliest origin of humans having been in life as 150,000-200,000 years ago [3]. It is known that pulmonary tuberculosis first made its damaging existence felt in Europe and later got to the US, Africa and Asia via travelers and old settlers [3]. *Mycobacterium tuberculosis* is an acid fast facultative intracellular rod shaped bacterium. It is non- motile, obligate aerobe with extended generation period and favours especially to localize in macrophages [4,5].

Pulmonary tuberculosis (PTB) is a world public health challenge and is the second major cause of loss of life. All inclusive, the illness causes death every 20 seconds [6,7]. Though much improvement has been done in line to the control measures, the World Health Organization approximated that 9 million people had tuberculosis in 2013 and that 1.5 million died, involving 360,000 people who were infected with

human immunodeficiency virus [8]. Pulmonary tuberculosis is a major global public health problem in Nigeria with an approximated prevalence of 616 cases per 100,000. Nigeria ranks first in Africa, and fourth among the 22 high pulmonary TB burden countries in the world, and not less than 460,000 cases of pulmonary TB are reported annually in Nigeria [9]. Ita and Udofia [10] reported the prevalence rate of 38.5% pulmonary TB in Ikot Ekpene and 17.6% in Itu Local Government area of Akwa Ibom State; they reported that male subjects had a higher incidence rate of pulmonary TB (35.6%) compared to 29.6% in female. Similarly, Nwanta et al. [11] reported an overall prevalence rate of 37.9% pulmonary TB in Enugu state, Nigeria.

Human immunodeficiency virus infection is the single most relevant factor for the reactivation of pulmonary TB worldwide and the great cause for letdown to reach set pulmonary tuberculosis control targets like in areas with high prevalence [3]. Hospital document show that pulmonary tuberculosis and HIV have synergistic relationships that greatly accelerate the reduction of the host immune status, accentuating the advance of each other. Pulmonary tuberculosis - HIV co-infection, the occurrence of the 2 illnesses at the same point in a patient, at hand creates severe and great public health problems like in the African region, including Nigeria. Worldwide, some 14 million people are approximated to have

pulmonary TB- HIV co-infection with the double epidemics being particularly pervasive in Africa due to the high incidence of HIV in this region [12]. In Africa, 44% of pulmonary TB patients were infected with the Human Immunodeficiency virus (HIV) according to WHO [13].

The co infection has major effects on the immune system, as it is able to remove the host's immune reactions [12]. Human Immunodeficiency virus co-infection is the most prevailing known risk factor for advancement of *Mycobacterium tuberculosis* infection to active illness, elevating the risk of dormant pulmonary tuberculosis resurgence 20-fold [12] and that is why this study was done to consider some of these major cytokines to understand what happens to immunological system which has direct or indirect effects on the haematological parameters.

There are few published works on host iron status at the time of pulmonary tuberculosis diagnosis [14]. Friis et al. [15] in their study opined that iron limited erythropoiesis and anaemia of inflammation during infections. According to them, infection often precipitates a substantial acute protein which causes sequestration of iron. In this study, iron status was determined to find out the impact of pulmonary tuberculosis with HIV co infection on iron status which may have a role in the pathogenesis of the infection. The alarming increase in the incidence of pulmonary tuberculosis in our country has been made worse by elevated occurrence of HIV/AIDS [16]. Cytokines are important immunomodulating agents of immune system. Human immunodeficiency virus co infection has been suggested to alter blood cell populations and change Th1/Th2 balance [17], which affects the course of pulmonary tuberculosis, clinical presentation, signs and symptoms [18], resulting to misdiagnosis or delay in diagnosis of pulmonary tuberculosis [19].

Peptide hepcidin, is a key iron-regulatory hormone [20], which is released from hepatocytes in response to inflammation via iron and oxygen. Interestingly, inflammation induces hepcidin production, mediated by the inflammatory cytokine IL-6. This results in sequestration of Fe in the stores and Fe-limited erythropoiesis, and eventually anaemia of inflammation [20]. The study will determine hepcidin level and IL-6 and correlate them to haemoglobin and PCV. This will help to discover the role of the co infection on these parameters

which may be the major cause of anaemia in the patients.

Interleukin 6 (IL-6) is a proinflammatory cytokine that regulates various physiological processes [21]. It performs a major function in the acute phase response and in the change from acute to chronic inflammation [22]. Evidence has accrued to suggest that dysregulation of IL-6 production is a great contributor to the pathogenesis of chronic inflammatory diseases [21,23]. Human immunodeficiency virus (HIV) infection has long been shown to induce expression and secretion of IL-6 [24,25]. This study will find out the changes that may be associated to the IL-6 levels in pulmonary tuberculosis patients with HIV coinfection. Interleukin 6 is known to exhibit multifactorial function. It will be important to determine the changes the co infection could cause to this cytokine. The lack of reliable biomarkers to indicate or predict the different clinical outcomes of *M. tuberculosis* infection has been given as a key reason for the failure of developing new diagnostic and prognostic tools, drugs and vaccines against tuberculosis [26].

A research by Akpan et al. [27] reported that the mean of the total WBC count in pulmonary tuberculosis patients is usually normal or not significantly increased as compared to apparently healthy persons. This study determined the levels of haematological parameters at the point of diagnosis. Platelets are effector cells that play an important role in the inflammatory and immunological response and have the capacity to release cytokines and, thus acting as an immune regulator; therefore this direct relationship between platelet and WBC is logical because when there is an immune response at pulmonary tuberculosis infection, platelets tend to increase [28].

The study was done to determine the levels of interferon-gamma, interleukin 6, interleukin 10, iron status, hepcidin and haematological parameters of patients with pulmonary tuberculosis co-infected with human immunodeficiency virus in Southeastern part of Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

This study was carried out at the directly observed treatment-short course Tuberculosis (TB DOTS) centre of Federal Medical Centre, Umuahia, located in South-Eastern Nigeria and

serve patients of high, middle and lower socio-economic status and with Igbo as the dominant tribe.

2.2 Study Population and Enrolments

Sample size was calculated using the formular by Araoye [1]. Therefore, sample size of 240 was used to give room for attrition

A total of two hundred and forty (240) subjects aged 18-60 years were enlisted for this study. The participants were recruited by purposive sampling technique. The HIV and pulmonary tuberculosis subjects were recruited from the tuberculosis directly observed treatment, short course (TB-DOTS) clinic and HIV clinic based on sputum smear acid fast bacilli by Ziehl Neelsen's stain and GeneXpert MTB/RIF assay and HIV screening tests, while apparently healthy age and sex matched subjects were recruited as controls. The subjects were grouped into:

- Group A: 50 control subjects.
- Group B: *Mycobacterium tuberculosis* positive subjects (n=100)
- Group C: HIV positive subjects (n=50)
- Group D: PTB-HIV subjects (n=40)

2.3 Selection Criteria

2.3.1 Inclusion criteria

- (i) Subjects of both sexes aged 18-60 years positive for *Mycobacterium tuberculosis* and HIV were included in the study.
- (ii) Those that gave consent were included.

2.3.2 Exclusion criteria

The following subjects were excluded

- a. Those that tested negative for pulmonary tuberculosis and HIV
- b. Pregnant women
- c. Diabetes mellitus patients
- d. Persons below 18 years and above 60 years
- e. Those that did not give consent.

2.4 Sample Collection

Seven milliliters (7ml) of venous blood was collected from each subject and 2.5ml was dispensed into bottles containing di-potassium salt of ethylenediamine tetra-acetic acid (K₂-EDTA) at a concentration of 1.5mg/ml of blood and was used for full blood count, CD4 count and HIV screening.

Also, 4.5ml was dispensed into plain tubes. Serum was obtained after clotting by spinning at 3000 RPM for 10 minutes and was used for interferon gamma, interleukin-6, and interleukin-10, iron and hepcidin determination.

Three separate sputum samples (consisting of one early morning sample and two spot samples) were collected in a wide mouth container from the subjects for pulmonary tuberculosis diagnosis.

The whole samples was analysed in Links Laboratory, Owerri by Sandwich ELISA method for interferon gamma, interleukins (6 and 10) and hepcidin and and HIV tests CD4 count, Full Blood count analysed in the Diagnostic Laboratory Unit, University Health Services Department of Michael Okpara University of Agriculture, Umudike, Abia State. Ziel Nelosn and GeneXpert were done in Federal Medical Centre, Umuahia, Abia State, Nigeria.

3. LABORATORY PROCEDURES

All reagents were commercially purchased and the manufacturer's standard operating procedures were strictly adhered to.

3.1 Determinations

3.1.1 Ziehl-Nelson Technique for *Mycobacterium tuberculosis* diagnosis [29]

Procedure:

Smear preparation: A piece of clean stick was used to transfer and spread sputum materials evenly covering an area of about 15-20mm diameter on a glass slide. The smear was air dried and labeled.

Heat fixation: The slide with the smear uppermost was rapidly passed three times through the flame of a Bunsen burner and was allowed to cool.

Ziehl-Nelson Staining: The slide containing the smear was placed on a slide rack and the smear covered with carbol fuschin stain. The stain was heated until vapour just begins to rise. The heated stain was allowed to remain on the slide for 5 minutes. The stain was washed off with clean water and then covered with 3% v/v acid alcohol for 5 minutes or until smear is sufficiently decolourised, that is pale pink. The slide was washed off with clean water. The smear was

covered with Methylene blue stain for 2 minutes and then washed off with clean water. The back of the slide was wiped clean and placed in a draining rack for the smear to air dry.

3.1.2 *Mycobacterium tuberculosis* diagnosis

The smear was examined microscopically using the X100 oil immersion objective. Scanning of the smear was done systematically and when any definite red bacillus is seen, it was reported as AFB positive.

3.1.3 GeneXpert method for detection of *Mycobacterium tuberculosis* and Rifampicin resistance (GeneXpert MTB/FIF)

Procedure: The assay consists of a single-use multi-chambered plastic cartridge pre-loaded with the liquid buffers and lyophilised reagent beads necessary for sample processing.

3.1.4 DNA extraction and hemi-nested real-time PCR

Sputum samples were treated with the sample reagent (containing NaOH and isopropanol). The sample reagent was added in the ratio of 2:1 to the sputum sample and the closed specimen container was manually agitated twice during 15 minutes of incubation at room temperature. 2ml of the treated sample was transferred into the cartridge, the cartridge was loaded into the GeneXpert instrument and automatic step completed the remaining assay steps.

The assay cartridge also contained lyophilized *Bacillus globigii* spores which served as an internal sample processing step and the resulting *B.globigii* DNA was amplified during PCR step. The standard user interface indicates the presence or absence of *Mycobacterium tuberculosis*, the presence or absence of rifampicin resistance and semi quantitative estimate of *Mycobacterium tuberculosis* concentration (high, medium, low and very low). Assays that are negative for *Mycobacterium tuberculosis* and also negative for *B.globigii* internal control was reported as invalid.

3.1.5 Determination of CD4 count by flowcytometry (Partec Cyflow counter), Germany

Procedure: All required reagents was brought to room temperature and 850µl of the count check bead green will be analysed to ensure that the cyflow machine is working properly. The desired

numbers of rohren test tubes was placed in a test tube rack. 20µl of CD4 easy count kits (CD4 Mab-PE) were pipetted into different test tubes labeled appropriately for the assay. Then, 20µl of blood sample was also pipette into each respective test tube and incubated in the dark for 15 minutes at room temperature after mixing properly. This was followed by the addition of 850 µl easy count. No lyse buffer was added to each test tube. This was mixed properly to avoid air bubbles and analysed on the Partec Cyflow. The result was displayed and copied from the screen.

3.1.6 Full blood count by automation using Mindray BC-5300, China

Procedure: The sample is EDTA bottle was placed in the spiral mixer and allowed to mix very well. Whole blood mode was activated in the LCD screen, the sample no (code) was inputted via key board and then the key will be selected. Then the sample was mixed very well again and the cap was removed and inserted into the probe and the SART button was pressed. When the LCD screen displays ANALYSING; the sample was removed and recapped. The analyser was executed automatic analysis and displays the result on LCD screen.

3.1.7 Determination of serum iron concentration by Ferozine method Teco Diagnostics (Iron/TIBC) Laketiew Ave, Acashein, CA 92807

Procedure: Iron free clean tubes were labeled as test, blank and standard. The 2.5ml of iron buffer reagent was added to all the labeled tubes. Also, 0.5ml of the samples was added to the respective tubes and was mixed. The reagent blank was used to zero the spectrophotometer at 560nm. The absorbance of all tubes was read and value will be recorded (A1 reading). Then, 0.5ml of iron reagent was added to all the tubes and was mixed properly. The tubes were palced in a heating bath at 37⁰C for 10 minutes. The reagent blank was also used to zero the spectrophotometer at 560nm and another absorbance of all the tubes was read and the value obtained was recorded (A2 reading).

Calculation

Serum iron (µg/dl) = $\frac{A2 \text{ Test} - A1 \text{ Test} \times \text{Con of A2 std}}{A2 \text{ std} - A1 \text{ std}}$

Where A1 Test= Absorbance of first reading of the test

A2= Absorbance of the second reading of the test

A1 std= Absorbance of the first reading of the standard

A2 std= Absorbance of the second reading of the standard

3.1.8 Determination of total iron binding capacity by Ferozine method of TECO Diagnostics (Iron/TIBC) Laketiew Ave, Acashein, CA 92807

Procedure: Iron free clean test tubes were labeled as test, blank and standard and 0.2ml of unsaturated iron binding capacity buffer reagent was added to all the tubes according to the sample number, while 10ml of iron free water was added to standard tube and was properly mixed. To the test 0.5ml of sample and 0.5ml iron standard were added to the test, and was properly mixed. The reagent blank was used to zero the spectrophotometer at 560nm wavelength. The absorbance of the samples was read and recorded as A1 reading. Also 0.5ml iron standard tube and was properly mixed. To the test, 0.5ml of sample and 0.5ml iron standard were added to the test, and was properly mixed. The reagent blank was used to zero the spectrophotometer at 560nm wavelength. The absorbance of the samples was added to the tubes and was mixed properly and was placed in a heating bath at 37°C for 10 minutes. The reagent blank was used to zero the spectrophotometer at 560nm and another reading was taken as the A2 reading.

Calculation

$$\text{UIBC } (\mu\text{g/dl}) = (\text{Conc. Of std-A2 Test-A1 Test} / \text{A2 std-A1 std}) \times \text{Conc. of std}$$

$$\text{TIBC } (\mu\text{g/dl}) = \text{Iron} + \text{UIBC}$$

Where A1 Test= Absorbance of first reading of the test

A2= Absorbance of the second reading of the test

A1 std= Absorbance of the first reading of the standard

A2 std= Absorbance of the second reading of the standard

3.1.9 Alere Determine HIV-1/2 Kit (Japan, Lot No: 84904k100a) for first Line HIV Screening test

Procedure: The desired numbers of test units from the test card were removed by bending and

tearing at the perforation. The protective cover from each test was removed. About 50µl of sample (serum) was added to the sample pad and allowed to flow through the solid phase. The result was read within a 15 minutes.

3.1.10 Uni-Gold™ HIV (Trinity Biotech, Lot No: HIV7110042) for second line HIV Screening test

Procedure: Two drops of whole blood were applied to the sample port, followed by 2 drops of wash solution and was allowed to react. Antibodies of any immunoglobulin class, specific to the recombinant HIV-1 or HIV-2 proteins reacted with the colloidal gold linked antigens. The antibody protein colloidal gold complex moves chromatographically along the membrane to the test and control regions of the test device.

3.1.11 Chembio HIV ½ Stat-Pak (USA, Lot No: 33020516) used as tie breaker

Procedure: With the sample loop provided, 5µl of the sample was taken and applied on the sample pad of the device. Then 3 drops (105µl) of the running buffer were added on the sample well also. The result was then read after 10 minutes.

3.1.12 Human Interferon-gamma (IFN-γ) ELISA Kit by Melsin Medical Co Limited, Catalogue Number: EKHU-0162

Procedure: Dilutions of standard was prepared to get a concentration of 240ng/l, 160ng/l, 80ng/l, 40ng/l and 20ng/l. 50µl of standards were pipette into the standard wells. 10µl of test serum were added into each well. 40µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50µl of chromogen solution A and 50µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

3.1.13 Interleukin 6 (IL-6) assay

Human Interleukin 6 commercial ELISA Kit by MELSIN Medical Co Limited was used. Catalogue Number: EKHU-0140.

Procedure: Dilutions of standard was prepared to get a concentration of 240ng/l, 160ng/l, 80ng/l, 40ng/l and 20ng/l. 50µl of standards were pipette into the standard wells. 10µ of test serum were added into each well. 40µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50µl of chromogen solution A and 50µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Human Interleukin 10 (IL-10) Assay by commercial ELISA Kit by MELSIN Medical Co Limited was used. Catalogue Number: EKHU-0155

Procedure: Dilutions of standard was prepared to get a concentration of 240ng/l, 160ng/l, 80ng/l, 40ng/l and 20ng/l. 50µl of standards were pipette into the standard wells. 10µ of test serum were added into each well. 40µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50µl of chromogen solution A and 50µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Human Hepcidin (Hepc) ELISA Kit by MELSIN Medical Co Limited was used with Catalogue Number: EKHU-1674.

Procedure: Dilutions of standard was prepared to get a concentration of 240ng/l, 160ng/l, 80ng/l, 40ng/l and 20ng/l. 50µl of standards were pipette into the standard wells. 10µ of test serum were added into each well. 40µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50µl of chromogen solution A and 50µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there

3.2 Statistical Analysis

Data was analysed using statistical package for social science (SPSS) version 20. Student t-test, ANOVA (Analysis of Variance), Pearson Product Moment and Chi-Square were the tools employed. Results were expressed as mean ± standard deviation and are presented in table and significance level was set at P<0.05.

4. RESULTS

The results of the study are presented here in table as mean ± standard deviation (SD).

The results showed difference that was statistically significant (P<0.05) in IFN-γ (16.25±0.87pg/ml, 40.28±8.99pg/ml, 23.90±5.65pg/ml, 34.68±13.34pg/ml, P=0.000), IL-6 (7.98±0.22pg/ml, 12.46±3.29pg/ml, 9.93±1.53pg/ml, 14.72 ±4.72pg/ml, P=0.000) IL-10 (8.52±0.62pg/ml, 16.42±4.36pg/ml,

Table 1. Mean± SD values of interferon-gamma, IL-6, IL -10, CD4, hepcidin, iron, and some haematological parameters of control, PTB, HIV and PTB-HIV subjects

Parameters	Control	TB	HIV	TB-HIV	F-value	P-value
IFN-γ(pg/ml)	16.25±0.87	40.28±8.99	23.90±5.65	34.68±13.34	107.184	0.000
IL-6(pg/ml)	7.98±0.22	12.46±3.29	9.93±1.53	14.72 ±4.72	47.506	0.000
IL-10(pg/ml)	8.52±0.62	16.42±4.36	13.51±3.40	20.74 ±7.80	60.559	0.000
CD4(cells/l)	1045.54±247.24	264.24 ±49.74	292.39±123.49	231.43 ±47.17	467.496	0.000
Hepcidin(ng/ml)	6.03±1.38	35.59 ±10.68	30.23±10.25	49.16±15.01	144.277	0.000
Iron(µg/dl)	86.29±7.27	77.19±12.94	79.43±9.29	65.47±13.21	17.037	0.000
TIBC(µg/dl)	345.56±28.40	313.48 ±30.53	296.33±17.08	269.13±38.96	53.829	0.000
%TSA(%)	25.16±3.18	24.52±4.41	26.85 ±3.11	24.21±4.21	5.519	0.001
WBC(X 10 ⁹ /L)	5.87 ±0.88	5.40±0.89	4.75±0.59	4.69±0.75	23.481	0.000
Neu(%)	60.57±2.83	58.77±4.36	72.10±4.51	65.22±3.57	134.183	0.000
Lym(%)	30.69 ±2.84	30.33±7.49	20.85±4.43	22.15 ±6.38	42.762	0.000
Mon(%)	5.59 ±1.2	8.54±2.64	4.08±1.02	8.00±3.02	53.833	0.000
Eos(%)	2.30 ±1.05	1.19±0.69	1.92±0.74	5.15±0.66	9.443	0.000
Bas(%)	0.86 ±0.39	1.17±0.54	1.09±0.76	0.99±0.62	3.404	0.018
RBC(X 10 ¹² /L)	4.92±0.30	4.10±0.49	3.47±0.23	3.68±0.5	105.259	0.000
Hb(g/dl)	14.75±0.90	12.01±1.49	10.42±0.69	10.81±2.46	84.571	0.000
PCV(%)	44.25±2.70	36.0±4.46	31.35±2.11	33.08±5.30	103.884	0.000
MCV(fl)	89.92±2.3	80.20±3.23	83.82±4.69	75.93 ±2.3	116.976	0.000
MCH(pg)	36.12±1.53	29.70±2.54	27.59±1.63	26.10±4.54	129.674	0.000
MCHC(g/l)	368.46±12.28	196.22±62.39	326.24±18.20	338.28±27.25	39.402	0.000
Plt(X 10 ⁹ /L)	261.75±22.71	169.20±26.45	217.74±31.84	145.45±36.48	165.842	0.000
ESR(mm/hr)	7.03 ±1.38	34.28±10.29	51.43±9.92	44.98±14.49	187.950	0.000

Significant level - *P < 0.05
ns - Not significant (P > 0.05)

13.51±3.40Pg/ml, 20.74 ±7.80pg/ml, P=0.000), CD4 (1045.54 ±247.24Cells/L, 264.24 ±49.74Cells/L, 292.39±123.49cells/L, 231.43 ±47.17/L, P=0.000), hepcidin (6.03±1.38ng/ml 35.59 ±10.68ng/ml, 30.23±10.25 ng/ml, 49.16±15.01ng/ml, P=0.000), Iron (86.29±7.27 µg/dl, 77.19±12.94 µg/dl, 79.43±9.29 µg/dl, 65.47±13.21 µg/dl, P=0.000), TIBC (345.56±28.40 µg/dl, 313.48 ±30.53µg/dl, 296.33±17.08µg/dl, 269.13±38.96 µg/dl, P=0.000), %TSA (25.16±3.18%, 24.52±4.41%, 26.85 ±3.11%, 24.21±4.21%, P=0.001), WBC (5.87 ±0.88X 10⁹/L, 5.40±0.89X 10⁹/L, 4.75±0.59 X 10⁹/L, 4.69±0.75 X 10⁹/L, P=0.000), Neutrophils (60.57±2.83%, 58.77±4.36%, 72.10±4.51%, 65.22±3.57%, P=0.000), Lymphocytes (30.69 ±2.84%, 30.33±7.49%, 20.85±4.43%, 22.15 ±6.38%, P=0.000), Monocytes (5.59 ±1.2%, 8.54±2.64%, 4.08±1.02%, 8.00±3.02%, P=0.000), Eosinophils (2.30 ±1.05%, 1.19±0.69%, 1.92±0.74%, 5.15±0.66%, P=0.000), Basophils (0.86 ±0.39%, 1.17±0.54%, 1.09±0.76%, 0.99±0.62%, P=0.018), RBC (4.92±0.30 X 10¹²/L, 4.10±0.49 X 10¹²/L, 3.47±0.23 X 10¹²/L, 3.68±0.5 X 10¹²/L, P=0.000), haemoglobin (14.75±0.90g/dl, 12.01±1.49g/dl, 10.42±0.69g/dl, 10.81±2.46g/dl, P=0.000), PCV (44.25±2.70%, 36.0±4.46%, 31.35±2.11%, 33.08±5.30%, P=0.000), MCV (89.92±2.3fl, 80.20±3.23fl, 83.82±4.694fl, 75.93 ±2.3fl,

P=0.000), MCH (36.12±1.53pg, 29.70±2.54Pg, 27.59±1.63pg, 26.10±4.54pg, P=0.000), MCHC (368.46±12.28g/l, 196.22±62.39g/l, 326.24±18.20g/l, 338.28±27.25g/l, P=0.000), Platelets (261.75±22.71 X 10⁹/L, 169.20±26.45 X 10⁹/L, 217.74±31.84 X 10⁹/L, 145.45±36.48 X 10⁹/L, P=0.000), ESR (7.03 ±1.38mm/hr, 34.28±10.29mm/hr, 51.43±9.92mm/hr, 44.98±14.49mm/hr, P=0.000) when compared among control, TB, HIV and TB-HIV subjects respectively.

5. DISCUSSION

The results showed that interferon gamma increased in pulmonary TB, HIV, pulmonary TB-HIV subjects than the control which was statistically significant. This shows that interferon gamma is seriously involved in the pathogenesis of tuberculosis, HIV and in pulmonary TB co infected with HIV but higher in pulmonary TB than in HIV unlike the apparently healthy individuals. This could be linked to inflammation and oxidation involved in these synthesis and release of interferon gamma as a defective mechanism by the body. Interferon gamma (IFN-γ) has major functions in pulmonary TB-HIV co infection and is related to inflammatory granuloma, organization, immune protection against intracellular mycobacterium and cell mediated immunity [30]. This shows that

interferon gamma is more involved in pulmonary TB infection than in HIV infection as well as in pulmonary TB-HIV infection. It implies that HIV infection has a less increasing effect on the interferon gamma even though interferon gamma was raised in HIV and pulmonary TB-HIV. Interferon gamma should be monitored in pulmonary TB infection and should be targeted in the course of treatment. The level was higher than in HIV monoinfection. This shows that pulmonary TB has more pronounced effect on interferon gamma than HIV infection. In the management of pulmonary TB, interferon gamma should be highly regulated to ensure better prognostic effect in those with tuberculosis.

Some works have revealed that when tuberculosis infection occurs, a variety of pro and anti-inflammatory cytokines are produced at disease sites and then released into circulation [31,32]. Interleukin 10 (IL-10) is one of the most important anti-inflammatory cytokines reported to affect multiple cell types, including macrophages, monocytes, dendritic cells, CD4 T cells and CD 8 T cells [33]. The dominant function of IL-10 is to down-regulate the immune reaction and reduce tissue injury. Meanwhile, the elevated synthesis of this cytokine directly inhibits CD4+ T cells responses which may result in a failure to control the infection [34]. Interleukin 10 is one of the most relevant anti-inflammatory cytokines documented to inhibit CD4 + T cell reactions by hindering APC role of cells infected with mycobacteria [35]. The results showed that the levels of IL-10 were significantly higher in pulmonary TB patients compared to healthy subjects ($P < 0.05$). The findings are similar with previous studies that have shown higher levels of IL-10 in the active pulmonary TB group than in the control group [36-38]. Interleukin 10 can be seen in the serum, plasma and bronchoalveolar lavage fluid of active pulmonary TB patients and may lead to the anergy and collapse of lymphocytes to proliferate in reaction to pulmonary TB [39,40]. It is the equilibrium between the inflammatory and protective immune reaction that determines the result of tuberculosis infection [34]. Also, the study showed higher levels of IL-10 among pulmonary TB, HIV and pulmonary TB-HIV than in control group. This still points to the fact that IL-10 is raised in pulmonary TB, HIV and pulmonary TB-HIV co infection. Interleukin 10 could be an indicator of the presence of these infections. Interleukin 10 was higher in pulmonary TB-HIV than pulmonary TB monoinfection and HIV monoinfection. This shows that pulmonary TB associated with HIV

infection has stimulatory effect on IL-10 which could be as a result of oxidative stress and hypermetabolic processes in the patients. This elevation of IL-10 helps to control the raised inflammatory cytokines to prevent tissue damage in the patients. The free radicals released in the course of these infections may affect the release of these cytokines and in turn affect the well-being of the patients.

CD4 is very important in accessing the immune level especially in HIV infection because HIV attacks CD4 exposing the body to a lot of opportunistic infections. CD4 was higher in control group than in pulmonary TB, HIV and pulmonary TB-HIV groups but there was no significant difference between pulmonary TB, HIV levels of all the test groups are suppressed unlike the control group. The CD4 count in pulmonary TB and HIV and pulmonary TB-HIV was reduced which could be attributed to the infection. CD4 is a major immune arm that the body uses in defending itself against these agents. The level of CD4 was higher in HIV group than pulmonary TB monoinfection and pulmonary TB-HIV has the lowest level of CD4 but was not significant.

Hepcidin is the main hormone that regulates the synthesis and release of iron in the body. Hepcidin is an acute phase reactant peptide that is the central regulator of iron homeostasis, and its expression is regulated by many variables, involving body iron status and hypoxia [41]. Similarly, infections and inflammation may stimulate hepcidin expression by hepatocytes, a process that is mediated via proinflammatory cytokines, usually interleukin 6 (IL-6), and signaling through the STAT-3 pathway [42,43]. Hepcidin causes the means of ACD by causing iron to be diverted from the circulation and sequestered within cells of the reticuloendothelial system and by inhibiting duodenal absorption of iron. Thus, as a consequence of inflammation, hepcidin limits the presence of iron for addition into erythroid progenitor cells [44]. There was increase of hepcidin in pulmonary TB, HIV and pulmonary TB-HIV compared to control. This shows that hepcidin is involved in the pathogenesis of pulmonary TB and HIV as well as in pulmonary TB-HIV infection. In conjunction to its major function in iron modulation, hepcidin has antimicrobial functions and shows to have a relevant function in the innate immune reaction against *Mycobacterium tuberculosis* [45]. While a small numbers of clinical researches have shown a relationship between increased hepcidin levels

and tuberculosis [46,47]. The elevation in hepcidin in these patients may be linked to anaemia observed in the body and this may be a mechanism the body uses to counteract the anaemia seen in them by challenging the body to produce more iron to couple with globin to form haemoglobin to transport oxygen needed in the metabolism of the body. The level of hepcidin was higher in pulmonary TB-HIV than in pulmonary TB monoinfection and HIV monoinfection. Emerging evidence suggests a key role for the iron regulator hepcidin in the innate immune response to *M. tuberculosis* infection [48]. In patients with tuberculosis, higher hepcidin concentrations were strongly associated with more severe anaemia. Since hepcidin has a well described, central role in anaemia of chronic diseases (ACD) as opined by Weiss and Goodnough [44], in which its expression is upregulated predominantly by IL-6 in response to infections such as tuberculosis [42], these results provide further evidence to suggest that ACD is the predominant mechanism underlying anemia in patients with tuberculosis-associated HIV [49]. This shows that the impact of HIV on patients with pulmonary TB infection increases the level of hepcidin and may be part of the challenges faced by these patients especially during treatment.

The study showed that the level of %TSA showed no significant difference between control and the test groups but HIV positive subjects showed higher level than pulmonary TB and pulmonary TB-HIV subjects. This shows that HIV group has more reserve of iron than pulmonary TB and pulmonary TB-HIV. HIV group may suffer less of non deficiency anaemia unlike pulmonary TB and pulmonary TB-HIV. Iron supplement should be given more to pulmonary TB and pulmonary TB-HIV group than the HIV group. Distortions in iron availability are common in infectious diseases and most of these alterations may be associated to actions of the iron-regulatory hormone hepcidin [50,51].

6. CONCLUSION

The study shows that interferon gamma, interleukin 6, interleukin 10 and hepcidin are adjuncts to biomarkers in the pathogenesis of pulmonary TB and HIV but may be utilized more in pulmonary TB infection than in HIV infection. The co infection increases the levels of the cytokines. The cytokines and hepcidin can be used as adjuncts to prognostic and diagnostic markers as their levels decreased

with increased duration of treatment of the patients.

The study has shown wide variations in the haemological indices studied. The red blood cell, packed cell volume and haemoglobin were suppressed but improved with the course of treatment. Anaemia is a major factor causing morbidity and mortality in the patients especially pulmonary TB patients co infected with HIV. This will help the Physicians and all health care providers handling pulmonary TB patients in tackling the challenges of drug failure and enlighten the world on the level of improvement associated to the duration of treatment that are expected to occur in the patients.

The haemological parameters like haemoglobin, RBC and PCV increased significantly with increased duration of treatment showing improvement in health status of the patients and monocytes decreased significantly in pulmonary TB patients.

CONSENT AND ETHICAL APPROVAL

With a well detailed research proposal and a letter of introduction from the Head of Department, Consent form and an application letter were submitted to the Head, Health Research and Ethics Committee of the Institution was met. After their meetings and thorough perusal of the protocols of the research, an ethical approval was given for the study. Participants' written consent has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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