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ORIGINAL ARTICLE

Red cell parameters, iron, vitamin B12 and folate levels of pulmonary tuberculosis patients attending clinic at General Hospital, Akamkpa, Cross River State, Nigeria

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Abstract

Introduction: Tuberculosis caused by Mycobacterium tuberculosis, is a disease of public health importance, characterized by a chronic granulomatous inflammation in humans. In this study, red cell and iron parameters as well as vitamin B12 and folate levels were assessed with a view to investigate the presence or absence of iron deficiency and/or vitamin B12 and folate deficiency in tuberculosis disease

Methods: Fifty (50) male and female tuberculosis patients within the ages of 15-60 years and attending clinic at the General Hospital, Akamkpa were enrolled in this study. Fifty apparently healthy and mantoux-negative subjects who were age and gender matched and resident in Akamkpa served as control. Ethical approval and informed consent were obtained from the Cross-River State Ministry of Health and all participants. Demographic information was obtained by face to face interview. Diagnosis of TB was by the Ziehl-Neelsen technique. Red cell and iron parameters, vitamin B12 and folate levels were determined by standard methods. Data were analyzed using student t-test on statistical package for social sciences version 21and a P value less than or equal to 0.05 is considered significant.

Results: The mean age of TB patients (34.7±10.4 years) is comparable to that of the control (32.4±8.6 years) with more males (56%) affected than females (44%). Fifteen (30%) of the TB patients have primary level of education with 31 (62%) and 4 (8%) having attained secondary and tertiary levels. The level of education for the control was 12 (24%), 22 (44%) and 16 (32%) for primary, secondary and tertiary respectively. The TB patients were farmers 20 (40%), traders 15 (30%), students 9 (18%) and civil servants 6 (12%) while the controls consisted of 14 (28%) farmers, 16 (32%) traders, 12 (24%) students and 8 (16%) civil servants. The packed cell volume and haemoglobin concentration of TB patients (0.34±0.05 L/L and 121.4±11.3 g/L) was significantly lower ($p < 0.05$) than 0.41±0.04 L/L and 145.2±13.1 g/L for the control. The red cell count and mean cell volume of TB patients (4.55±0.73 x 10 12/L and 79.21±2.40 fl) was comparable

(p > 0.05) to control values (4.84±0.54 x 10 12/L and 80.18±1.30 fl). Mean cell haemoglobin and mean cell haemoglobin concentration of TB patients was 27.58±2.12 pg and 34.37±1.43 g/dl, which was significantly lower than 29.72±2.24 pg and 35.73±1.38 g/dl obtained for control. The serum iron of TB patients (47.90±6.25µg/dl) was significantly lower (p = 0.001) than control value 109.03±8.56µg/dl. The total iron binding capacity (188.05±33.01µg/dl) and transferrin saturation with iron (28.00±4.54 %) were significantly lower (p = 0.001) for TB patients versus control (252.28±36.30µg/dl and 42.46±6.23%). Serum ferritin of TB patients (345.30±82.61 ng/ml) was significantly higher (p = 0.001) when compared to the value for control (108.62±28.50 ng/ml). Vitamin B12 and folate levels (245.37±39.62 ng/L and 353.34±57.06 µg/l) were significantly lower (p = 0.001) for TB patients when compared with control (550.20±82.33 ng/L and 681.93±97.36µg/l) though within the reference values.

Conclusions: This study has shown a lower packed cell volume and haemoglobin concentration in tuberculosis disease indicating the presence of anaemia. An alteration in iron metabolism and increased iron stores has also been demonstrated ruling out iron deficiency anaemia. Vitamin B12 and folate levels though lower for TB patients, are within the reference range thus excluding megaloblastic anaemia. Tuberculosis shows a normocytic normochromic anaemia which is typical of anaemia of chronic disease and inflammation.

Keywords: Tuberculosis, iron, vitamin B12, folate, Akamkpa.

Introduction

Tuberculosis (TB) is an age-long infectious disease caused by Mycobacterium tuberculosis. It is acquired through inhalation of airborne droplets released when an infected person coughs or sneezes. The disease is characterized by a protracted cough, fatigue, low-grade fever, loss of weight and appetite and night sweats. According to the WHO, the TB incidence which experienced a steady decline of 2% per year for two decades, suddenly reversed and rose by 3.6% between 2020 and 2021, a trend attributed to the Covid 19 pandemic. A disease of public health importance, tuberculosis is the 12th leading cause of death globally and is still of enormous public health challenge in Nigeria as the country is ranked among the first eight countries with the highest burden of TB worldwide and one of the countries that is yet to meet targets for the WHO's END-TB strategy (1). Tissue response in tuberculosis infection represents a classical example of chronic granulomatous inflammation in humans. Infection with M. tuberculosis typically results in formation of granuloma; the process being a cell-mediated type IV hypersensitivity reaction. This is meant to be a protective defense reaction by the host but eventually causes tissue destruction because of persistence of the poorly digestible antigen. At first, neutrophils try to engulf the organisms, but they fail. Macrophages present the antigen to CD4+ T lymphocytes. The lymphocytes are activated and express lymphokines such as

interferon γ (IFN-γ) and tissue necrotic factor α (TNF-α) (2,3,4). Indeed, an increase in the levels of inflammatory cytokines has been reported in tuberculosis disease (5).

Anaemia has been reported to occur in tuberculosis disease (6). Anaemia is a condition in which the haemoglobin concentration which represents the oxygen-carrying capacity of the red cell, is insufficient to meet the physiologic needs of the body; this may vary by age, gender, altitude, smoking, and pregnancy status. Globally, anaemia is the most common public health problem (7). Anaemia of chronic inflammation (ACI) or anaemia of chronic disease is a common cause of anaemia and the second most prevalent cause of anaemia, after iron deficiency anaemia (IDA) (8). The common conditions associated with ACI are acute and chronic infections (viral including HIV infection, bacterial, parasitic, fungal), cancers (haematological and solid tumours), autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus and connective-tissue diseases, vasculitis, sarcoidosis, inflammatory bowel disease), chronic kidney diseases and other chronic inflammatory conditions such as rejection following solid organ transplantation. The estimated prevalence of anaemia due to chronic inflammation accounts for 23-50%. It may be difficult to delineate the prevalence of this condition as it is often confused with iron deficiency anaemia thus requiring a diagnosis of exclusion (9). Therefore, this study seeks to assess red cell and iron parameters as well as vitamin B12 and folate levels with a view to investigate the presence or absence of iron deficiency anaemia and B12 and folate deficiencies in tuberculosis disease.

Methods

Study site

The study site for this research work is Akamkpa, located in the southern part of Cross River State, in Nigeria's south-south geo-political zone. Sampling was done at the tuberculosis clinic of the General Hospital, Akamkpa.

Ethical consideration and Informed consent

Approval was obtained from the Health Research Ethics Committee, Ministry of Health, Cross River State. Informed consent was sought and obtained from all participants.

Study design

Case-control experimental study design was used involving the comparison of two groups (TB patients and non-patients) of similar age. Follow-up is not required.

Selection of subjects and inclusion/exclusion criteria

A total of one hundred (100) subjects including males and females within the age range of 15-60 years were recruited for the study. This comprised of fifty (50) newly diagnosed pulmonary tuberculosis patients attending clinic at the General Hospital, Akamkpa, Cross River State, Nigeria. Fifty (50) apparently healthy subjects with a negative tuberculin skin test (mantoux) in the preceding six months and no history of tuberculosis were recruited from among residents of Akamkpa to serve as control. Tuberculosis patients with other disease conditions and subjects below 15 and above 60 years of age and controls who had not done the mantoux test were excluded from the study. Also, subjects who objected to participation in the study were excluded.

Collection, handling and storage of sample

Venous blood (6 ml) was withdrawn aseptically and with minimum stasis from each subject. Two ml was dispensed into ethylene diamine tetra-acetic acid (EDTA) to a final concentration of 2mg/ml. Four ml was dispensed into plain sample containers. The samples were transported in cold chain to Medical Laboratory Science Research laboratory, University of Calabar, for analysis. The EDTA sample was analyzed within four hours of collection while the clotted sample was centrifuged and serum was separated into fresh containers and stored frozen until needed for analysis. Serum samples were transported in cold chain to the University

College Hospital, Ibadan for the determination of vitamin B12 and folate by absorption spectrophotometry.

Diagnosis of TB

Subjects were diagnosed as TB patients based on the microscopic detection of acid-fast bacilli in their sputum using the Ziehl-Neelsen technique (10).

Principle: Dried sputum smear is heat-fixed and covered with carbol fuchsin stain. The stain is heated to 60oC and allowed on the slide for 5 minutes. After washing with clean water, the smear is decolorized with 3% v/v acid alcohol for 5 minutes, washed and counter stained with malachite green for 1 minute. The smear is then washed, air-dried and examined microscopically using the X100 objective. Detection of slender rod-like red bacilli on a green background indicates positivity for acid fast bacilli. The bacilli load is graded based on standard guidelines.

Determination of red cell parameters

Red cell parameters namely packed cell volume (PCV), haemoglobin (Hb), red blood cell count (RBC), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were determined using automatic haemoanalyzer (ERMA INC model PCE-210N).

The principle depends on the fact that red cells are poor conductors of electricity while certain diluents are good conductors. Blood is highly diluted in a buffered electrolyte solution. The flow rate of this diluted sample is controlled by displacement of a tightly fitting piston. This results in a measured volume of the sample passing through an aperture tube of specific dimensions. By means of a constant source of electricity, a direct current is maintained between two electrodes, one in the sample beaker and the other in the aperture tube. As a blood cell is carried through the aperture, it displaces some of the conducting fluid and increases the electrical resistance. This produces a corresponding change in potential between the electrodes. The height of the pulses produced indicates the volume of the cells passing through. The pulses are led to a threshold circuit provided with an amplitude discriminator for selecting the pulse height which will be counted.

Procedure

The anti-coagulated sample was mixed properly by gentle inversion and 100µl was sucked into the machine through a probe. The blood was diluted and the various cell types were counted. The result was displayed on the computerized screen in standard units.

Determination of serum iron (SI) and total iron binding capacity (TIBC)

Serum iron and TIBC were determined using the Modified Automated AAII-25 Colorimetric Method (11).

Principle for serum iron: the serum iron bound to transferrin is released in an acid environment. Iron ions react with the chromazurol -p and cetyltrimethylammoniurn bromide (CTMA) forming a ternary complex coloured in blue. The color intensity is directly proportional to the amount of iron present in the sample.

Procedure

Pipette	Blank	Sample	Standard
Reagent A Water	1000µl 50µl	1000µl	1000µl
Sample		50µl	
Standard			50µl

It was well mixed and incubated for 4 minutes at 37oC. The Test (A) and the standard (AS) was read in a colorimeter at 623nm.

Principle for total iron binding capacity: The serum is treated with excess of Fe (I) to saturate the iron binding sites on transferrin. The excess Fe (II) is absorbed and precipitated and the iron content in the supernatant is measured to give the TIBC.

Procedure

Wave length: 660nm; Light path: 1cm; Temperature: 37°C

	Blank		Calibrator/sample
Standard/sample	-		80µl
Acidic reagent	1000µl		1000µl
Mix and incubate for 6 minutes at assay temperature			
Neutral buffer	300µl		300µl
Mix and measure A1 immediately and A2 after an incubation of 7.5 minutes at assay temperature. The reaction is a decreasing reaction. ΔA = (A2-A1)			

Table 1. Demographic data of tuberculosis patients and control subjects

	TB Patients (n=50)	Control (n=50)
Mean age (years)	34.7±10.4	32.4±8.6
Gender		
Males n (%)	28 (56)	30 (60)

Females n (%)	22 (44)	20 (40)
Education		
Primary n (%)	15 (30)	12 (24)
Secondary n (%)	31 (62)	22 (44)
Tertiary n (%)	4 (8)	16 (32)
Occupation		
Farmers n (%)	20 (40)	14 (28)
Traders n (%)	15 (30)	16 (32)
Students n (%)	9 (18)	12 (24)
Civil servants n (%)	6 (12)	8 (16)

Table 2. Red cell parameters of tuberculosis patients and control

Parameters	TB Patients (n=50)	Control (n=50)
Packed cell volume (L/L)*	0.34±0.05	0.41±0.04
Hb (g/L)*	121.4±11.3	145.2±13.1
RBC ($x10^{12}$/L)	4.55±0.73	4.84±0.54
Mean cell volume (fl)	79.21±2.40	80.18±1.30
Mean cell haemoglobin (pg)*	27.58±2.12	29.72±2.24
Mean cell haemoglobin concentration (g/dl)*	34.37±1.43	35.73±1.38

= p < 0.05

Table 3. Iron parameters of tuberculosis patients and control

Parameters	TB patients (n=50)	Control (n=50)
Serum Iron (µg/dl)*	47.90±6.25	109.03±8.56
Total iron binding capacity (µg/dl)*	188.05±33.01	252.28±36.30
Transferrin saturation (%)*	28.00±4.54	42.46±6.23
Serum ferritin (ng/ml)*	345.30±82.61	108.62±28.50

***p = 0.001**

Table 4. Vitamin B12 and folate levels of tuberculosis patients and control subjects

Parameters	TB patients (n=50)	Control	(n=50)
Vitamin B12 (ng/ml)*	245.37±39.62	550.20±82.33	
Folate (µg/L)*	353.34±57.06	681.93±97.36	

***p = 0.001**

Determination of serum ferritin by Enzyme Linked Immunosorbent Assay (ELISA)

Principle: The ferritin ELISA kit is a solid phase sandwich assay method. Designated wells coated with biotinylated anti-ferritin antibody binds to ferritin in the patient's serum. The addition of the horseradish peroxidase (HRP) conjugated anti-ferritin antibody reagent forms a sandwich complex with the analyte of interest being in between the two highly specific antibodies, labeled with Biotin and HRP. Upon the addition of the substrate, the intensity of the colour developed is directly proportional to the concentration of ferritin in the sample.

Procedure:

25 µl of ferritin standards, controls and samples was pipetted into appropriate wells already coated with anti-ferritin antibody and the wells were incubated for 30 minutes at room temperature. Unbound protein conjugated antibody was washed off by wash buffer. 100 µl of enzyme reagent was added into each well, covered and incubated at room temperature for 30 minutes. Unbound excess enzyme was washed off using wash buffer. 100 µl of Tetramethylbenzidine substrate was then added to all wells and incubated at room temperature for 15 minutes after which 50 µl of stop solution was added to all wells and absorbance was read on ELISA microplate reader at 450nm.

Determination of vitamin B12 and folate in serum

Vitamin B12 and folate were measured in serum using absorption spectrophotometric method.

Principle: The light source is a hollow - cathode lamp which consists of an evacuated gas-tight chamber containing an anode, a cylindrical cathode and an inert gas (helium or algon). When current is applied between the two electrodes inside the hollow - cathode lamp, the filter is ionized. Ions attracted to the cathode collide with the analyte and knocks atoms off into the gases inside the glass envelope; the atoms lose energy and emit light (radiation) energy which is characteristic of the analyte. The intensity of emitted light is directly proportional to the amount of analyte in the patient sample.

Procedure

One 1ml of serum was deproteinized with 9ml of 10% (w/v) tetrachloroacetic acid (TCA) in 0.1% lanthanium solution. The resultant solution was transferred into a centrifuge tube and spun for five (5) minutes at 250rpm. The resulting supernatant was then diluted with water and aspirated into the atomic absorption spectrophotometer (AAS) for reading at 460 nanometers.

Reference range: Vitamin B12: 180-914ng/L; Folate: 200-1400µg/L.

Results

In this study, red cell and iron parameters as well as vitamin B12 and Folate levels of TB patients were assessed. The demographic data of TB patients and their control is presented in Table 1. The mean age of TB patients (34.7±10.4 years) is comparable to that of the control (32.4±8.6 years). The tuberculosis patients consist of 28 (56%) males and 22 (44%) females while the controls were 30 (60%) males and 20 (40%) females. Fifteen (30%) of the TB patients have primary level of education with 31 (62%) and 4 (8%) having attained secondary and tertiary levels respectively. The control subjects' level of education was 12 (24%) for primary, 22 (44%) for secondary and 16 (32%) for tertiary. In terms of occupation, TB patients were made of up farmers 20 (40%), traders 15 (30%), students 9 (18%) and civil servants 6 (12%) while the controls consisted of 14 (28%) farmers, 16 (32%) traders, 12 (24%) students and 8 (16%) civil servants.

The red cell parameters of TB patients and control subjects is presented in table 2. The packed cell volume and haemoglobin concentration of TB patients was observed to be 0.34±0.05 L/L and 121.4±11.3 g/L and this was significantly lower ($p < 0.05$) than 0.41±0.04 L/L and 145.2±13.1 g/L obtained for the control. The red cell count and mean cell volume of TB patients (4.55±0.73 x 10 12/L and 79.21±2.40 fl) was comparable ($p > 0.05$) to the value obtained for the control (4.84±0.54 x 10 12/L and 80.18±1.30 fl). It was also observed that the mean cell haemoglobin and mean cell haemoglobin concentration of TB patients was 27.58±2.12 pg and 34.37±1.43 g/dl which was significantly lower than 29.72±2.24 pg and 35.73±1.38 g/dl obtained for the control. Table 3 shows some iron parameters of TB patients versus the control. The serum iron of TB patients was 47.90±6.25 µg/dl and this was significantly lower ($p = 0.001$) than the control value 109.03±8.56 µg/dl. Similarly, the total iron binding capacity (188.05±33.01 µg/dl) and transferrin saturation with iron (28.00±4.54 %) of TB patients was found to be significantly lower ($p = 0.001$) than the control values (252.28±36.30 µg/dl and 42.46±6.23 %). Conversely, the serum ferritin of TB patients (345.30±82.61 ng/ml) was significantly higher ($p = 0.001$) when compared to the value for control (108.62±28.50 ng/ml). Table 4 shows the vitamin B12 and folate levels of tuberculosis patients and control. It was observed that the vitamin B12 of TB patients (245.37±39.62 ng/L) was significantly lower ($p = 0.001$) when compared with control value (550.20±82.33 ng/L). Similarly, the folate level of TB patients (353.34±57.06 µg/l) was significantly lower ($p = 0.001$) when compared to control (681.93±97.36µg/l).

Discussions

In this study, red cell and iron parameters as well as vitamin B12 and folate levels were investigated in pulmonary TB patients. The mean age of the TB patients was observed to be 34.7±10.4 years which implies that the disease affects the economically productive age group. Considering the debilitating nature of tuberculosis disease as well as its chronic course, this has implications in terms of loss of man-hours and job effectiveness for the affected persons. Seventy five percent of TB cases has been reported to occur in the economically active age group and the disease has been known to reduce productivity of the affected persons (12). More males (56%) were affected than females, this trend has been observed in several studies and also reported by the WHO (13,14,1) and is mostly attributed to the social behavior of males as they interact more with friends and neighbors in addition to indulging in habits such as smoking and alcoholism hence increasing the chance of exposure to the airborne Mycobacterium tuberculosis bacilli. The educational level of majority (92%) of the TB patients was secondary school and below.

Also, their occupation is mostly farming and trading thus implying that they belong to a low socio-economic class. Tuberculosis has been identified as a disease of the poor as factors that encourage the transmission

and acquisition include poor feeding and overcrowded living conditions (15,16,17).

The packed cell volume and haemoglobin concentration of TB patients was observed to be significantly lower when compared to the control. Anaemia is defined by haemoglobin values below the reference range for age and gender. In this study, the mean haemoglobin value for the TB patients 121.4 g/L just falls at the lower limit of the reference range for females whereas most of the patients were males. This suggests the presence of a mild to moderate anaemia in tuberculosis disease. On the other hand, the red cell count and the mean cell volume was comparable for TB patients when compared to the control although the MCV value was slightly lower than the haemoanalyzer reference value of 80-95 fl. The MCV value suggest that the red cells are normocytic. Normal red cell size has been observed previously in anaemia of chronic disease or inflammation (18,19). The mean cell haemoglobin and mean cell haemoglobin concentration of TB patients was found to be significantly lower than for the control. However, the values are within the reference ranges of 27-29 pg for MCV and 32-36g/dl for MCHC. This suggests that the red cells of the TB patients are normochromic in terms of haemoglobin content. The MCH is a measure of the average amount of haemoglobin found in each red cell while MCHC measures the amount of haemoglobin relative to the size of a red cell. The MCH and MCHC are useful indices in the identification of hypochromic anaemias such as in iron deficiency where their values fall below the reference ranges (20). The serum iron of the TB patients was lower than the control value and also found to be below the reference value of 60 -170 µg/dl. Again, the total iron binding capacity was lower for TB patients versus the control as well as the reference value of 240-450 µg/dl. The lower SI and TIBC suggests the presence of iron depletion or a mild iron deficiency. However, the transferrin saturation with iron of TB patients though observed to be lower than the control value, falls within the reference range of 15-50% thus suggesting an ongoing transportation of iron in blood to the tissues and organs. Hence, it can be deduced that there is no problem with iron availability or transportation in tuberculosis disease. It has been observed that iron metabolism is altered in infectious diseases due to an increase in hepcidin levels which causes inhibition of iron absorption and recycling and results in iron sequestration (21). This is further confirmed by the observation that the serum ferritin levels of the TB patients (345.30±82.61 ng/ml) was found to be higher than the control with the lower limit (263ng/ml) being within and the upper limit (427ng/ml) being far in excess of the reference value of 24 – 336 ng/ml. It appears that there is enough iron in the stores and transferrin is adequately saturated with iron yet, the circulating iron (SI and TIBC) is lacking. There appears to be a withholding of iron by the body, making it unavailable for the pathogen M. tuberculosis. This has been reported severally as a mechanism used to curtail pathogen growth and spread in chronic infections and inflammatory states and is a typical picture in anaemia of chronic inflammation (22). The pathogenetic processes are thought to be mediated through the actions of tumour necrosis factor (TNF) and interleukins (IL)-1 and -6, and interferon (IFN). Also, serum ferritin is an acute phase reactant and its level is expected to increase where there is an overwhelming infection such as in tuberculosis disease (23, 21).

The vitamin B12 level of tuberculosis patients was significantly lower when compared with control values. Similar finding has been reported (24). The M. tuberculosis has been known to synthesize vitamin B12 and also import it from the environment as the bacterium uses B12 for its proliferation hence one would expect an increase in B12 levels (25,26). However, it has been reported that the immune system produces a molecule called itaconate. Itaconate blocks the vitamin B12 pathway in order to neutralize and stop the

progression of the tubercle bacilli (27). This may account for the lower B12 level observed for TB patients in this study. Again, the folate level of tuberculosis patients was significantly lower when compared with control values. Low serum folate in TB has been reported previously and attributed to low nutrient intake due to loss of appetite (28,29). Although the B12 and folate levels were lower in TB, the values were all within the reference range. It is deduced that there is no deficiency in vitamin B12 and folate in tuberculosis disease therefore macrocytic or megaloblastic anemia is ruled out.

Conclusions

This study has shown a lower packed cell volume and haemoglobin concentration in tuberculosis disease indicating the presence of anaemia. An alteration in iron metabolism and increased iron stores has also been demonstrated ruling out iron deficiency anaemia. Vitamin B12 and folate levels though lower for TB patients, are within the reference range thus excluding megaloblastic anaemia. Tuberculosis shows a normocytic normochromic anaemia which is typical of anaemia of chronic disease and inflammation.

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