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Haemostatic Indices as Markers for Monitoring Pulmonary Tuberculosis Treatment

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Summary: Tuberculosis (TB) is an infectious disease inducing a state of chronic inflammation which could affect the haemostatic mechanism as part of host defences against infection. Proper diagnosis and monitoring of tuberculosis patients undergoing therapy is still a challenge especially in a poor resource country such as Nigeria. This study aims to assess some haemostatic indices of tuberculosis patients and their possible use as markers in monitoring response to anti-tuberculosis treatment. One hundred and twenty TB patients aged 15-60 years and 120 apparently healthy (control) subjects age and gender-matched were studied. Demographic/bio data was compiled by interview and from patients' case notes. Diagnosis of TB was by sputum smear microscopy, radiography and clinical assessment. Platelet count (PLT), platelet factor 4 (PF4), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TCT) and fibrinogen (FIB) were determined using standard techniques. The platelet factor 4, prothrombin time, activated partial thromboplastin time and fibrinogen levels of TB patients were significantly higher while the thrombin clotting time was significantly lower ($P<0.05$) when compared with healthy subjects. While PF4, TCT and FIB improved significantly ($P<0.05$) as anti-tuberculosis therapy progressed, PLT, PT and APTT remained the same. It is concluded that abnormal activation of haemostasis occurs in TB condition thus pre-disposing TB patients to bleeding complications**.** Furthermore, platelet factor 4, thrombin clotting time and fibrinogen improved as therapy progressed and therefore may be used as markers for monitoring response to anti-tuberculosis therapy.

Keywords*:* Tuberculosis, Haemostasis, Inflammation, Anti-tuberculosis therapy, Infection

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INTRODUCTION

Haemostasis is an efficient system in humans with the ability to stop bleeding from sites of blood vessel injury through a series of enzymatic reactions. It describes a fragile balance between procoagulant as well as anticoagulant mechanisms involving an intricate series of events (Hoffbrand *et al*., 2011). When there is tissue injury due to infection, the body reacts in a process known as inflammation. This defense reaction attempts to remove or at least limit the spread of the offending agent, and in addition clear necrosed cells and tissues from the affected area (Mohan, 2010). Tuberculosis (TB) as a disease is a state of chronic granulomatous inflammation arising from infection by a family of organisms collectively called the *Mycobacterium tuberculosis* complex (Iseman, 2000). It has been reported that inflammation results in activation of the haemostatic system, with the latter also affecting the activity of the former (Verhamme and Hoylaerts, 2009). Although haemostatic function is geared towards preserving the integrity of the circulatory system, it can become an imbalanced process with morbidity and mortality as possible sequel. Haemostatic activity that is poorly controlled as a consequence of inflammation may be a significant contributor to the development and course of disease as well as its progression. This is clearly expressed in systemic inflammatory response to infection as may be the case in tuberculosis (Margetic, 2012). Tuberculosis (TB) is still a significant public health problem in Nigeria with a prevalence of 590,000 and 170,000 deaths recorded in 2014 (Federal Ministry of Health Nigeria, 2008; World Health Organisation, 2012; WHO, 2015). The high death rate due to TB is unacceptable since diagnosis in good time and correct treatment can ensure that virtually all TB patients are cured of the disease. Proper diagnosis and monitoring of tuberculosis patients is still a challenge especially in a poor resource country such as Nigeria. This has led to continuous spread, non-compliance and development of multi-drug resistant TB. This study therefore aims to assess some haemostatic parameters

of tuberculosis patients and their possible use in monitoring response to anti-tuberculosis therapy.

MATERIALS AND METHODS

Study design/ethical considerations: The study area for this research work is Calabar, located in the southern part of Cross River State in Nigeria's south-south geo-political zone. Case-control experimental study design was used involving TB patients and non-patients of similar age. Approval was obtained from the Health Research Ethics Committee, Ministry of Health, Cross River State. Informed consent was sought and obtained from all participants.

Subject selection: Subjects comprised of 120 male and female tuberculosis patients within the age range of 15-60 years including newly diagnosed patients and those on anti-tuberculosis therapy, attending tuberculosis treatment centres at Dr Lawrence Henshaw Memorial Hospital, General Hospital, Ekpo-Abasi and Adazi health centres, Calabar. Diagnosis of TB was by sputum smear microscopy, radiology or clinical assessment. One hundred and twenty apparently healthy subjects, age and gender matched with a negative tuberculin skin test (mantoux) in the preceding six months and no history of tuberculosis, selected from residents of Calabar metropolis served as controls. Tuberculosis patients with other disease conditions and subjects who objected to participation in the study were excluded.

Demographic data/sample collection: Demographic data were compiled by interview while treatment duration was obtained from patients' case notes. Two sputum samples were collected on consecutive days for the diagnosis of pulmonary TB. Six milliliters of venous blood was collected asceptically; 2ml was dispensed into ethylene diamine tetra acetic acid (EDTA) to a final concentration of 2mg/ml and used for platelet count within 4 hours of collection; 2ml was dispensed into 0.2ml of 3.13% trisodium citrate, centrifuged at 3000rpm for 10 minutes to obtain platelet poor plasma for the determination of prothrombin time, activated partial thromboplastin time, thrombin clotting time and fibrinogen; the remaining 2ml was dispensed into a plain container, allowed to clot to obtain serum which was stored frozen until used for determination of platelet factor 4.

Sample analysis: Sputum samples were mixed and smeared thinly on a microscope slide, the smear was heat fixed and stained using the Ziehl-Neelsen technique (Brooks *et al*., 2013). The slides were examined microscopically using x 100 objective for the presence of acid fast bacilli which appear as red rods (bacilli) on a green background. The bacilli load was determined using the WHO/International Union against tuberculosis and lung disease (IUATLD) Grading Guidelines (1998). Platelet count (PLT) was determined using an automatic cell counter. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were by the Quick's one stage technique (Dacie and Lewis, 2010) using kit purchased from Giesse Diagnostics, Italy. Thrombin clotting time (TCT) and fibrinogen concentration (FIB) were measured by Clauss technique (Clauss, 1999) with kit purchased from Giesse Diagnostics, Italy. Determination of PT, APTT, TCT and FIB was by addition of respective reagents to platelet poor plasma in glass tubes at 37^{o}C in a water bath and recording the time of clot formation using a stop watch. Platelet factor 4 (PF4) was measured based on sandwich enzyme immunoassay (ELISA) using kit purchased from Cloud-Clone Corporation, USA. The manufacturers' instructions for storage and assay were followed strictly.

Statistical analysis: Data obtained from this study are presented as chart and in tables as mean ± standard deviation. Chi-square analysis, one way analysis of variance (ANOVA) and Games-Howell post hoc test and Student's T-test were used to test hypotheses on statistical package for social sciences (SPSS) version 20 software. A P-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Haemostatic and inflammatory parameters of one hundred and twenty tuberculosis (TB) patients and one hundred and twenty apparently healthy subjects (control) were assessed in this research. In table 1, the demographic parameters of TB patients were compared with their controls. Significant differences were observed between the test group (TB patients) and their controls with respect to educational and occupational status ($P<0.001$). No significant difference ($P>0.05$) was found between TB patients and their controls with respect to age, gender and marital status. Some haemostatic parameters of tuberculosis patients and control subjects are presented in table 2. The platelet factor 4, prothrombin time, activated partial thromboplastin time and fibrinogen concentration were significantly higher ($P<0.001$) while the thrombin clotting time was significantly lower ($P<0.001$) than values obtained for control subjects. However, the mean platelet count of TB patients was not different from control.

Figure 1 shows three groups of TB patients based on treatment duration. The first group (23%) consists of newly diagnosed TB patients who were yet to commence treatment (untreated). The second group called the intensive phase (42%), refers to day one of treatment up to two months. The third group called the

TABLE 1 Demographic parameters of tuberculosis patients and control subjects

Parameters	TB Patients (N=120)	Control (N=120)	P-VALUE
Age (Years)	33.4±11.2	29.7±7.2	0.074
Gender			
Males	75 (62.5%)	38 (63.3%)	0.913
Females	45 (37.5%)	22 (36.7%)	
Marital Status			
Single	66 (55.0%)	27 (45.0%)	
Married	47 (39.2%)	32 (53.3%)	0.212
Divorced	3 (2.5%)	0 (0.0%)	
Widowed	4 (3.3%)	1 (1.7%)	
Educational Level			
Nil	14 (11.7%)	0 (0.0%)	
Primary	21 (17.5%)	2 (3.3%)	0.001
Secondary	59 (49.2%)	10 (16.7%)	
Tertiary	26 (21.6%)	48 (80.0%)	
Occupation			
Business/Trading	33 (27.5%)	5 (8.3%)	
Students	23 (19.2%)	8 (13.4%)	
Civil/Public Servant	11 (9.2%)	45 (75.0%)	0.001
Others	39 (32.5%)	2 (3.3%)	
Unemployed	14 (11.6%)	0 (0.0%)	

TABLE 2. Haemostatic indices of tuberculosis patients and control subjects

Parameters	TB PATIENTS (N=120)	CONTROL (N=120)	P-VALUE
Platelet Count ($X10^9$/L)	172.67±59.72	176.85±56.22	0.646
Platelet Factor 4 (pg/ml)	43.87±32.58	28.58±27.67	0.001
Prothrombin Time (s)	20.03±3.77	13.67±1.17	0.001
Activated Partial Thromboplastin Time (s)	46.07±8.81	34.63±4.49	0.001
Thrombin Clotting Time (s)	12.91±5.09	24.13±7.53	0.001
Fibrinogen (mg/dl)	349.96±137.75	150.42±73.18	0.001

Table 3. Haemostatic indices of tuberculosis patients based on duration of anti-tuberculosis therapy

PARAMETERS	New Patients (Untreated) (n=28)	Intensive Phase ≤ 2 Months (n=50)	Continuation Phase > 2-6 Months (n=42)	Significance Level
Platelet Count ($X10^9$/L)	178.75±56.83	178.08±57.37	162.17±64.16	NS
Platelet Factor 4 (pg/ml)	53.84±37.64	46.57±30.94	34.02±28.78	*b
Prothrombin Time (s)	19.75±3.56	19.76±3.91	20.55±3.77	NS
Activated Partial Thromboplastin Time (s)	46.71±7.65	46.00±9.44	45.74±8.93	NS
Thrombin Clotting Time (s)	9.11±1.52	10.78±1.82	17.98±5.30	***a,b,c
Fibrinogen (mg/dl)	484.39±92.81	390.28±90.99	212.33±76.03	***a,b,c

NS= No Significant difference; *= P<0.05; ***P<0.001, a= Difference between new and intensive phases, b= Difference between new and continuation phases c= Difference between intensive and continuation phases

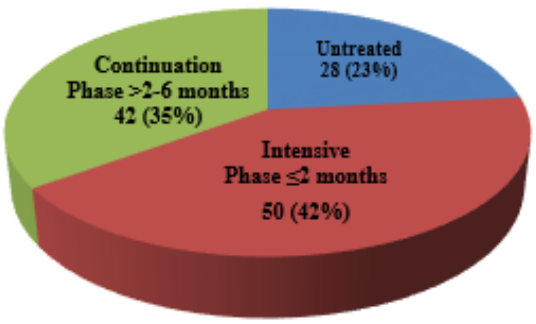

FIG. 1: Grouping of tuberculosis patients based on duration of anti-TB therapy

continuation phase (35%) refers to treatment period greater than two months up to the end of treatment at six months. The effect of duration of anti-tuberculosis therapy on some haematological parameters is presented in table 3. The platelet factor 4 level of the continuation group was significantly lower (P<0.05) than that of the untreated group. While the fibrinogen concentration decreased significantly (P<0.001) the thrombin clotting time increased significantly (P<0.001) from the value obtained for new patients to intensive and continuation phases as therapy progressed. However, no statistical difference (P>0.05) was observed for platelet count, prothrombin time and activated partial thromboplastin time among the three groups.

DISCUSSION

In this study, the TB patients were observed to belong to a lower socio-economic class with respect to educational level and occupation with 11.6% of them being unemployed (table 1). Poverty and poor nutrition are factors which predispose individuals to TB infection if exposed as supported by previous report (Cegielski and McMurray, 2004). The present finding also correlates with the WHO report that most TB cases occur in the economically active age group (15-50 years) with an adult losing about 3-4 months of work due to the disease (WHO, 2012). The platelet factor 4 was significantly higher for TB patients when compared to control (table 2). The increase observed could be attributed to abnormal platelet activation induced by the state of chronic inflammation. In normal physiology, platelets are inactive; protected by inhibitory factors like nitric oxide (NO) and prostacyclin (PGI_2) present in intact endothelium. However, in a state of chronic inflammatory response such as in TB, there is dysfunction of the endothelium resulting in raised synthesis of thromboxane A2 and von Willebrand factor with reduced levels of PGI_2 leading to platelet activation and increased reactivity. Following activation, platelets release bioactive peptides from their alpha and dense granules which include heparin neutralizing factor also called platelet factor 4 thus resulting in the elevation seen in TB patients. Previous reports support this finding (Gawaz *et al*., 2005; Lowenberg *et al*., 2010; Nurden, 2011; Speth *et al.*, 2013). The prothrombin and activated partial thromboplastin times of TB patients were significantly higher than values for control subjects. In addition, the PT and APTT of the TB patients were prolonged beyond the reference ranges of 12-16 seconds and 26-36 seconds respectively suggesting that the clotting factors assessed are depleted. When a state of chronic inflammation is developed; an integral part of the host defense geared towards eradication of the offending pathogen involves systemic activation of the haemostatic system as suggested by earlier reports (Schouten *et al*., 2008; Hoffbrand *et al*., 2011). However, an exaggerated systemic activation of the haemostatic system can result in disseminated intravascular coagulation (DIC) with consumption of clotting factors as a consequence. This explains the prolonged PT and APTT observed for TB patients and implies that both extrinsic and intrinsic pathways of blood coagulation are affected. The inability of blood to clot may pre-dispose these TB patients to bleeding problems in the event of injury. The thrombin clotting time of TB patients was lower than the control values. This is probably due to the fact that thrombin has been used up as a consequence of excessive activation of coagulation induced by the TB infection and inflammation. In inflammatory condition, thrombin suppresses clotting by activating protein C, a native anticoagulant that splits factors V and VIII, thus contrasting the procoagulant activities of thrombin; this is achieved via the binding of thrombin to thrombomodulin. The loss of procoagulant properties by thrombin further explains the reduction in thrombin levels as well as the prolonged PT and APTT observed for tuberculosis patients. The fibrinogen concentration of the TB patients was significantly higher ($p<0.05$) than values for control; this is in line with hyperfibrinogenaemia (increase in fibrinogen levels above the reference value) previously reported in tuberculosis disease (Awodu *et al*., 2007; Akpan *et al*., 2012). The increase in fibrinogen levels observed could be attributed to several factors. First, the interaction between *Mycobacteria* and the monocyte-macrophage system of the host results in the synthesis of large quantities of pro-inflammatory cytokines which in turn bring about hepatic acute-phase reactions that change coagulation proteins' levels; one of which is fibrinogen. Fibrinogen has indeed been known to be a key player in the regulation of immune response and inflammation in infection. Its role involves the increased formation of fibrin clots which serves to trap the offending pathogen (Degen *et al*., 2007; Esmon *et al*., 2011; Davalos, 2012). Again, since thrombin is responsible for clotting fibrinogen, a reduction in thrombin levels will lead to accumulation of fibrinogen as observed in this study.

Platelet factor 4 levels of TB patients decreased significantly while the thrombin clotting time increased significantly as therapy progressed (table 3). The decrease in platelet factor 4 as well as the increase in thrombin clotting time implies that the inflammation-induced activation of haemostasis is resolved with proper treatment. Thus, the platelet factor 4 and thrombin clotting time could be used to monitor response to anti-tuberculosis treatment. Conversely, the prothrombin time and activated partial thromboplastin time were similar for untreated TB patients as well as their counterparts who were on treatment. Although treatment of the underlying infection with appropriate antibiotics is expected to improve haemostatic interactions, it has been observed that abnormalities of the haemostatic system may proceed in some cases (Dellinger *et al*., 2008). This observation correlates with the findings in this study. A possible explanation is that the normal coagulation control systems are impaired by the infection-induced inflammation in tuberculosis. Previous report suggests that the protein C system is significantly affected through decreased synthesis, increased consumption and degradation as well as down-regulation of thrombomodulin and endothelial protein C receptor at the endothelial surface resulting in disturbed regulation of the haemostatic system (Marti-Carvajal *et al*., 2007). This may be responsible for the non-resolution of the prolonged PT and APTT as therapy progressed. It is also possible that it will take some

time for the clotting factors which have been depleted by excessive activation of coagulation, to be restored to normal levels in the plasma thus implying that TB patients may be prone to bleeding complications even after completion of treatment. Fibrinogen concentration of TB patients decreased significantly from the untreated through the intensive to the continuation phases of treatment (table 3). It is suggested that the anti-TB drugs inhibit the binding of thrombin to thrombomodulin thereby resulting in the availability of thrombin to bind fibrinogen. The acute phase response is also suppressed by the clearance of the offending pathogen hence fibrinogen which is an acute phase protein is no longer produced in large quantities by the liver leading to a decrease in its concentration with therapy. This indicates that fibrinogen concentration could be a useful marker in the monitoring of response to therapy in TB management.

In conclusion, this study has revealed abnormal activation of haemostasis in tuberculosis infection which may pre-dispose them to bleeding even after the completion of treatment. However, since platelet factor 4, thrombin clotting time and fibrinogen improved with therapy, these indices could be used as markers to monitor response to treatment in TB management.

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