CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb), a bacterium that most often affect the lungs (WHO, 2012). It is second only to Human immunodeficiency virus (HIV) infection as the greatest killer worldwide due to a single infectious agent. One third of the world's population is thought to have been infected with *M. tuberculosis.* In Nigeria, Tuberculosis is a major public health problem. According to CDC (2016), Nigeria is the country worst affected by tuberculosis in Africa and ranks third in the world (CDC, 2016). Tuberculosis burden in Nigeria is further compounded by the high prevalence of HIV/AIDS among the general population. The world TB situation is continually turning from bad to worse due to the emergence of multidrug-resistant (MDR) and virtually untreatable extensively drug-resistant (XDR) strains of *M. tuberculosis* (WHO, 2012).

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani *et al*., 2007), and is a protective response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. Inflammation plays a dual role in host immune response to mycobacteria. On the one part, it is a prerequisite for successful pathogen elimination. On the other part, it mediates tissue injury and disease progression. At the onset of the infection, inflammatory reactions are largely protective; during active disease, the deleterious effect of inflammation prevails, making inflammation a paramount pathogenic factor of TB progression. Inflammation in TB infection involves both immune cells such as Neutrophils, lymphocytes and monocytes/macrophages and cytokines such as IL-6, TNF-α, IL-10, TGF-β, IL-1β and IFN-Y (Tomioka *et al.*, 2011). Hence the measurement of inflammatory response in TB subjects involves the assessment of these cells and cytokines.

In TB patients, severe infection and inflammation are known to lead to haemostatic abnormalities, ranging from insignificant laboratory changes to severe disseminated intravascular coagulation (DIC) (Akpan *et al.*, 2012). Haemostasis is a balanced process that

halts bleeding after blood vessels have been traumatized and involves the entire process that maintains the flowing blood in a fluid state and confined to the circulatory system. It is the combination of cellular and biochemical events that function in harmony to keep blood within the veins and arteries, prevent blood loss from injuries by the formation of thrombi and re-establish blood flow during the healing process (Lewis *et al.*, 2006). Thus while inflammation aims at restoring the integrity of damaged or threatened tissues, most frequently because of injury or infectious pathogens, haemostasis is a physiological defence mechanism to stop bleeding due to vessel wall damage (Bonar *et al*., 2010).

Haemostasis and inflammation are closely linked, both in health and disease. They are tightly interrelated patho-physiologic processes that considerably affect each other. In this bidirectional relationship, inflammation leads to activation of the haemostatic system that in turn also considerably influences inflammatory activity (Verhamme and Hoylaerts, 2009). Local activation of the haemostatic system is an essential part of the host defence in both infectious and non-infectious inflammatory states. Such, that the haemostatic system acts in concert with the inflammatory cascade creating an inflammation-haemostasis cycle in which each activated process promotes the other and the two systems function in a positive feedback loop (Margetic, 2012). Failure of the complex balance between pro- and anti-coagulation, or between pro- and anti-inflammatory reactions because of genetic or acquired disturbances may result in disease. The many links between inflammation and coagulation help explain the pro-thrombotic tendency observed in patients with acute inflammatory or infectious diseases (Verhamme and Hoylaerts, 2009). The haemostatic system and the innate inflammatory response share a common ancestry and are coupled via common activation pathways and feedback regulation systems such that coagulation triggers inflammatory reactions and inflammation triggers the activation of the coagulation system. Thus extensive cross-talk exists between inflammation and coagulation. The vast crosstalk between immune (inflammatory) and haemostatic systems, involves all levels of haemostasis; the endothelium, platelets, clotting and anticoagulant pathways as well as the fibrinolytic system (Akpan *et al*., 2017).

The haemostatic process is divided into primary haemostasis and secondary haemostasis. Platelets which are blood cells mediate primary haemostasis leading to formation of a platelet plug, while the coagulation factors drive the secondary haemostasis leading to formation of a stable fibrin clot (Lewis *et al.*, 2006). The action of platelet in haemostasis involves adhesion, activation and change of shape from discoid to spiky sphere, release of granular content most of which have haemostatic effect and marks the haemostatic process and platelet aggregation.

Each of this action is mediated and marked by a number of measurable parameters such as Platelet factor -4, P-selectin, GP IIb/IIIa, and Thrombopoietin that regulates the formation of platelets in the bone marrow. It could be assumed that the general effect of inflammation on haemostasis which is already established due to the cross-talk between them would most likely affect these parameters that mediates and marks the activity of platelet in primary haemostasis. This assumption was put to test by this study as the changes that take place in these parameters with inflammation were monitored in TB infected subjects.

Malaria infection, gender and age are known confounders that affect inflammatory, haemostatic and other markers of human physiological processes. Platelets and its granular content (PF-4) has been shown to play a major role in Malaria parasite killing and according to Olaniyan *et al* (2018) *Plasmodium falciparum* co-infection with *Mycobacterium tuberculosis* could generate both innate and acquired immunity involving inflammatory responses which could affect the plasma levels of cytokines. Moreover, Pro- and anti-inflammatory cytokines are involved in the malarial pathogenesis and the outcome of malaria infection is determined by the balance in induction and counter-regulation of both pro- and anti-inflammatory cytokines (Harischandra *et al*., 2015). Similarly, hormonal differences between males and females are known to impact on the level of these markers. According to Popko *et al* (2010), gender is a major factor influencing the regulation of inflammatory factors; possibly due to a specific hormonal balance or adipose tissue distribution in women. Also, immune senescence and "inflamm-aging" which defines the deterioration in immunity and inflammatory changes that goes with aging cannot be overlooked in TB subjects because age is also known to affect some parameters in humans.

From the foregoing, it could be deduced that exploring the already established knowledge of a cross-talk between haemostasis and inflammation will strengthen our understanding of the patho-physiological relationship between haemostatic alterations and inflammatory changes in TB infected individuals. This study therefore explored inflammatory markers and haemostatic parameters of individuals infected with Tuberculosis and monitored the changes through the period of therapy in order to ascertain the changes in these parameters with treatment.

1.2. Statement of the problem

Despite various interventional measures and improved management schemes (including the Directly observed therapy) in recent times, tuberculosis still remains a major health problem with a global concern. Tuberculosis can cause a wide variety of complications. El-fekih *et al* (2009) reported a hypercoagulable state in active tuberculosis which predisposes tuberculosis patients to thrombotic complications with up to 4 % of pulmonary tuberculosis patients developing a deep vein thrombosis (DVT). In this light, an association between inflammation, haemostatic changes and a hypercoagulable state has been established (Naithani *et al.*, 2007). Thus, Akpan *et al* (2012) posits that the elevated risk of venous thromboembolism (VTE) in pulmonary tuberculosis patients may be related to presence of inflammation with associated haemostatic changes due to an acute phase response. Verhamme and Hoylaerts (2009) noted that there are many links between inflammation and coagulation that help explain the pro-thrombotic tendency observed in patients with acute inflammatory or infectious diseases. The implication of this is that inflammation in TB subjects which on the surface is of much benefit as the body's response against the pathogen may in the long run result in haemostatic alterations that may be harmful if not properly regulated. This may create a situation where aside the anti-tuberculosis therapy for the elimination of the mycobacteria, the clinician may in the long run have a haemostatic issue to manage as well in the same patient. The possibility of this was investigated with emphasis on some primary haemostatic parameters.

1.3. Justification of the study

According to Margetic (2012), local activation of the haemostatic system is an essential part of the host defence in both infectious and non-infectious inflammatory states. Such, that the haemostatic system acts in concert with the inflammatory cascade creating an inflammation-haemostasis cycle in which each activated process promotes the other and the two systems function in a positive feedback loop. Notably, the failure of the complex balance between pro- and anticoagulation, or between pro- and anti-inflammatory reactions because of genetic or acquired disturbances results in disease (Verhamme and Hoylaerts, 2009). Since there is an inseparable union between haemostasis and inflammation, this study leveraged on this existing knowledge to follow-up TB infected subjects in the course of therapy and monitor the changes in both the levels of inflammatory markers (cytokines and immune cells) and haemostatic parameters which have helped to define how the established cross-talk between

inflammation and haemostasis affects the levels of these parameters in TB infected subjects. A procoagulant shift in haemostatic parameters at the second month which is linked to inflammation was established and justifies the hypercoagulation tendencies in TB disease. These findings will be of great value in the laboratory monitoring and prognostic management of TB patients and have ruled out the necessity for the use of anti-inflammatory drugs as adjuncts in the treatment of TB infected patients.

1.4. Aim of the study

The study was aimed at assessing the changes in the levels of haemostatic and inflammatory markers in pulmonary *Mycobacterium tuberculosis* infected subjects before and after initiation of treatment.

1.5. Specific objectives of the study

1. To determine the levels of Tumor Necrosis Factor - alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-2 (IL-2), Interleukin – 10 (IL-10), Transforming growth factor – beta (TGF-β), Neutrophil count, Lymphocyte count, Monocyte count, Neutrophil-lymphocyte ratio (NLR) and Monocyte-lymphocyte ratio (MLR) in Tuberculosis infected subjects before treatment, at 2 month and 6 month treatment.

2. To determine the levels of P-selectin, Platelet Glycoprotein IIb/IIIa (GPIIb/IIIa) complex, Platelet activating factor (PAF), Platelet factor-4 (PF-4), Thrombopoietin hormone (TPO) and platelet count in Tuberculosis infected subjects before treatment, at 2 month and 6 month of treatment.

3. To correlate inflammatory markers (TNF-α, IL-6, IL-10, TGF-β, and IL-2) with primary haemostatic parameters (P-selectin, GPIIb/IIIa complex, PAF, PF-4, TPo and platelet count) of Tuberculosis infected subjects.

4. To compare the levels of the inflammatory and haemostatic parameters in TB infected subjects co-infected with malaria and those not infected with malaria.

5. To determine the influence of gender and age on the studied parameters based on period of treatment.

1.6. Research questions

1. Are there significant changes in the levels of the inflammatory markers in Tuberculosis infected subjects at 2 month and 6 month of treatment?

2. Are there significant changes in the levels of the haemostatic parameters in Tuberculosis infected subjects at 2 month and 6 month of treatment?

3. Is there any relationship between the inflammatory markers and haemostatic markers of TB infected subjects?

4. Does malaria co-infection alters the levels of inflammatory and haemostatic markers in TB subjects?

5. Does age and gender influence the studied parameters based on period of treatment?

1.7. Research hypotheses

1. H_O: There are no significant changes in the levels of inflammatory markers in Tuberculosis infected subjects at 2 month and 6 month of treatment.

   H_1: There are significant changes in the levels of inflammatory markers in Tuberculosis infected subjects at 2 month and 6 month of treatment.

2. H_O: There are no significant changes in the levels of haemostatic parameters in Tuberculosis infected subjects at 2 month and 6 month of treatment.

H_1: There are significant changes in the levels of haemostatic parameters in Tuberculosis infected subjects at 2 month and 6 month of treatment.

3. Ho: There is no correlation between the inflammatory and haemostatic markers of TB infected subjects.

   H_1: There is a correlation between the inflammatory and haemostatic markers of TB infected subjects.

4. Ho: Malaria co-infection alters the levels of haemostatic and inflammatory parameters in TB infected subjects.

   H_1: Malaria co-infection does not alter the levels of haemostatic and inflammatory parameters in TB infected subjects.

5. Ho: Age and gender does not influence the studied parameters based on period of treatment.

   H_1: Age and gender influences the studied parameters based on period of treatment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of tuberculosis

Mycobacterium tuberculosis (MTB) is the bacterium that is known to cause Tuberculosis (TB) which is an infectious disease that generally affects the lungs in cases of pulmonary tuberculosis, and other parts of the body in cases of extra-pulmonary tuberculosis (WHO, 2015). Tuberculosis infection may be asymptomatic in most cases in which it is said to be latent or with obvious symptoms in which case it is said to be active. About 10% of latent infections progress to active disease which, if left untreated, kills about half of those infected (WHO, 2015). The classic symptoms of active TB are a chronic cough with blood-containing sputum, fever, night sweats, and weight loss. However, infection of other organs can cause a wide range of symptoms (Dolin *et al*., 2010). Tuberculosis is spread through the air when active TB infected patients cough, spit, speak, or sneeze (CDC, 2012) It is thought that one-third of the world's population has been infected with TB with new infections occurring in about 1% of the population each year (WHO, 2013a). In 2016, there were more than 10 million cases of active TB which resulted in 1.3 million deaths (WHO, 2017). More than 95% of deaths occurred in developing countries such as Nigeria.

2.1.1 History of tuberculosis

Tuberculosis has been present in humans since antiquity (Lawn and Zumla, 2011). TB was not identified as a single disease until the 1820s and was not named "tuberculosis" until 1839, by J. L. Schönlein. The bacillus causing tuberculosis, *M. tuberculosis*, was identified and described on 24 March 1882 by Robert Koch. He received the Nobel Prize in physiology and medicine in 1905 for this discovery (Nobel Foundation, 2006). The World Tuberculosis Day is marked on 24 March each year, the anniversary of Koch’s original scientific announcement.

2.1.2 Epidemiology of tuberculosis infection

Roughly one-third of the world's population has been infected with *M. Tuberculosis* (WHO, 2010), with new infections occurring in about 1% of the population each year (WHO, 2013a). A report has shown that in Nigeria, pulmonary tuberculosis is a major public health problem with an estimated prevalence of 616 cases per 100,000 (Obeagu *et al*., 2019). However, most

infections with *M. tuberculosis* do not cause TB disease (CDC, 2011), and 90–95% of infections remain asymptomatic (Richard, 2011). Tuberculosis is the second-most common cause of death from infectious disease (after those due to HIV/AIDS) (Dolin *et al*., 2010). Tuberculosis is more common in developing countries; about 80% of the population in many Asian and African countries test positive when tuberculin screening tests is conducted. However, in developed countries, tuberculosis is less common and is found mainly in urban areas. Hopes of totally controlling the disease have been dramatically dampened because of a number of factors, including the difficulty of developing an effective vaccine, the expensive and time-consuming diagnostic process, the necessity of many months of treatment, the increase in HIV-associated tuberculosis, and the emergence of drug-resistant cases (Lawan and Zumla, 2011). The rate of TB varies with age. In Africa, it primarily affects adolescents and young adults, but in countries where incidence rates have declined dramatically TB is mainly a disease of older people and the immune-compromised (CDC, 2006).

2.1.3 Mechanism for transmission of tuberculosis

When subjects with active pulmonary TB infection cough, sneeze, speak, sing, or spit, they expel infectious aerosol droplets. It is worthy of note that a single sneeze can release up to 40,000 droplets. Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very small (the inhalation of fewer than 10 bacteria may cause an infection) (Nicas *et al*., 2005). People with prolonged, frequent, or close contact with people with TB are at particularly high risk of becoming infected (Ahmed and Hasnain, 2011). Transmission usually occurs from only people with active TB because those with latent infection are not thought to be contagious. The probability of transmission from one person to another depends upon several factors, including the number of infectious droplets expelled by the carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the *M. tuberculosis* strain, the level of immunity in the uninfected person, and others. The cascade of person-to-person spread can be circumvented by segregating those with active or overt TB and putting them on anti-TB drug regimens (Ahmed and Hasnain, 2011). If an infection occurs, it typically takes three to four weeks before the newly infected person becomes infectious enough to transmit the disease to others.

2.1.4 Pathogenesis of pulmonary tuberculosis

TB infection begins when the mycobacteria reach the alveolar air sacs of the lungs, where they invade and replicate within endosomes of alveolar macrophages (Queval *et al*., 2017). Macrophages identify the bacterium as foreign and attempt to eliminate it by phagocytosis. During this process, the bacterium is enveloped by the macrophage and stored temporarily in a membrane-bound vesicle called a phagosome. The phagosome then combines with a lysosome to create a phagolysosome. In the phagolysosome, the cell attempts to use reactive oxygen species and acid to kill the bacterium. However, *M. tuberculosis* has a thick, waxy mycolic acid capsule that protects it from these toxic substances. *M. tuberculosis* is able to reproduce inside the macrophage and will eventually kill the immune cell. The primary site of infection in the lungs, known as the "Ghon focus", is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (Kumar *et al.*, 2007). Tuberculosis is classified as one of the granulomatous inflammatory diseases. Macrophages, T lymphocytes, B lymphocytes, and fibroblasts aggregate to form granulomas, with lymphocytes surrounding the infected macrophages. When other macrophages attack the infected macrophage, they fuse together to form a giant multinucleated cell in the alveolar lumen. The granuloma may prevent dissemination of the mycobacteria and provide a local environment for interaction of cells of the immune system. However, more recent evidence suggests that the bacteria use the granulomas to avoid destruction by the host's immune system. Macrophages and dendritic cells in the granulomas are unable to present antigen to lymphocytes; thus the immune response is suppressed (Bozzano *et al*., 2014). Bacteria inside the granuloma can become dormant, resulting in latent infection. Progression from TB infection to overt TB disease occurs when the bacilli overcome the immune system defenses and begin to multiply. In primary TB disease (some 1–5% of cases), this occurs soon after the initial infection. However, in the majority of cases, a latent infection occurs with no obvious symptoms (Kumar *et al*., 2007). The risk of reactivation increases with immunosuppression, such as that caused by infection with HIV.

2.1.5. Risk factors for tuberculosis

People with compromised immune systems are most at risk of developing active tuberculosis. For instance, HIV suppresses the immune system, making it harder for the body to control TB bacteria. About 13% of all people with TB are infected by the virus (WHO, 2011a). People who are infected with both HIV and TB are around 20-30 percent more likely to

develop active TB than those who do not have HIV. Tobacco use has also been found to increase the risk of developing active TB. Those who smoke cigarettes have nearly twice the risk of TB compared to non-smokers (Smitt *et al*., 2010). And about 8 percent of TB cases worldwide are related to smoking. Tuberculosis is also closely linked to both overcrowding and malnutrition, making it one of the principal diseases of poverty (Lawn and Zumla, 2011). Those at high risk thus include: people who inject illicit drugs, inhabitants and employees of locales where vulnerable people gather (e.g. prisons and homeless shelters), medically underprivileged and resource-poor communities, high-risk ethnic minorities, children in close contact with high-risk category patients, and health-care providers serving these patients. Other conditions that can also increase the risk of developing tuberculosis include; alcoholism, diabetes mellitus certain cancers , malnutrition, kidney disease and people who are undergoing cancer therapy (Lawn and Zumla, 2011).

2.1.6. Management of tuberculosis

The majority of TB cases can be cured when the right medication is available and administered correctly. The precise type and length of antibiotic treatment depend on a person's age, overall health, potential resistance to drugs, whether the TB is latent or active, and the location of infection (i.e., the lungs, brain, kidneys). Treatment of TB involves the use of antibiotics to kill the bacteria. Effective TB treatment is difficult, due to the unusual structure and chemical composition of the mycobacterial cell wall, which hinders the entry of drugs and makes many antibiotics ineffective. Treatments can be prolonged, taking several months. Latent TB treatment usually employs a single antibiotic (Menzies *et al*., 2011), while active TB disease is best treated with combinations of several antibiotics to reduce the risk of the bacteria developing antibiotic resistance (Lawn and Zumla, 2011). People with latent infections are also treated to prevent them from progressing to active TB disease later in life (Menzies *et al.*, 2011). Directly observed therapy (which entails having a health care provider watch the person take their medications), is recommended by the WHO in an effort to reduce the number of people not appropriately taking antibiotics (Arch and Mainous, 2010). The recommended treatment of new-onset pulmonary tuberculosis is six months of a combination of antibiotics containing rifampicin, isoniazid, pyrazinamide, and ethambutol for the first two months, and only rifampicin and isoniazid for the last four months (Lawn and Zumla, 2011). Where resistance to isoniazid is high, ethambutol may be added for the last four months as an alternative (Lawn and Zumla, 2011). If multiple drug-resistant TB (MDR-TB) is detected,

treatment with at least four effective antibiotics for 18 to 24 months is usually applied (Lawn and Zumla, 2011). Primary resistance occurs when a person becomes infected with a resistant strain of TB. TB medication can be toxic to the liver, and although side effects are uncommon, when they do occur, they can be quite serious. Potential side effects include; Dark urine, Fever, Jaundice, Loss of appetite, Nausea and vomiting.

2.1.7. Diagnosis of tuberculosis

In clinical practice, rapid TB diagnosis can be difficult, and early pulmonary TB detection continues to be challenging for clinicians. Prompt diagnosis of active pulmonary TB is a priority for TB control, both for treating the individual and for public health intervention to reduce further spread in the community (WHO, 2011b). Therefore, an acid-fast bacilli (AFB) smear and bacteriological culture tests should be performed for patients with symptoms that are compatible with or suggestive of TB. However, mycobacterial culture, which has the highest sensitivity for diagnosing and confirming active TB, requires 2 to 6 weeks for interpretation3. Although sputum smear microscopy is a rapid, simple, and inexpensive tool for diagnosing pulmonary TB, it has low and variable sensitivity (WHO, 2011b). Recently, non-molecular and molecular assays have been developed for early detection of active TB with or without drug resistance detection. These Diagnostic Methods include;

1. Radiologic study: Chest X-ray is the primary radiologic evaluation of suspected or proven pulmonary TB. Radiological presentation of TB may be variable but in many cases is quite characteristic. Radiology also provides essential information for management and follow-up of these patients and is extremely valuable for monitoring complications. Chest X-ray is useful but is not specific for diagnosing pulmonary TB, and can be normal even when the disease is present (Yon, 2015). Therefore, it cannot provide a conclusive independent diagnosis and needs to be followed by sputum testing.

Although chest X-ray is the primary diagnostic tool for evaluating pulmonary TB, chest computed tomography (CT) is generally required to detect fine lesions that can be overlooked on chest X-ray, to define equivocal lesions, or to evaluate complications (Yon , 2015). Chest CT is an effective diagnostic method when plain films are normal or inconclusive, and it provides valuable information for managing the illness. Chest CT can add valuable information for detecting bacterial activity. Branching opacities, cavitation, or consolidation

are clear signs of active TB, but active disease must be confirmed by analyzing sputum for the presence of bacilli (Yon, 2015).

2. Acid fast bacilli (AFB) smear microscopy and culture: For pulmonary TB, sputum is the most critical sample for laboratory testing. Direct sputum smear microscopy is the most widely used method for diagnosing pulmonary TB (WHO, 2014). Smear microscopy may, however, be costly and inconvenient for patients, who must make multiple visits to health facilities and submit multiple sputum specimens over several days. Fortunately, good-quality microscopy of two consecutive sputum specimens has been shown to identify the vast majority (95%–98%) of smear-positive TB patients (WHO, 2011b; Davis *et al*., 2013). The WHO policy on case detection by microscopy was, therefore, revised to recommend a reduction in the number of specimens examined, from three to two in settings with appropriate external quality assurance and documented high-quality microscopy18. In addition, the WHO recommends that countries that have successfully implemented current WHO policies for a two-specimen case-finding strategy consider switching to same-day diagnosis, especially in settings where patients are likely to default from the diagnostic process (WHO, 2011b). Conventional light microscopy of Ziehl-Neelsen–stained smears prepared directly from sputum specimens is the most widely available test for diagnosing TB in resource-limited settings. Ziehl-Neelsen microscopy is highly specific, but its sensitivity is variable (20%–80%). Laboratory diagnosis of TB relies on direct microscopic examination of sputum specimens. However, the technique, although specific, has low and variable sensitivity and cannot identify drug-resistant strains. Clinicians have been advised to obtain culture confirmation of TB whenever possible. This not only confirms the diagnosis, but also obtains material for crucial drug susceptibility testing (DST) (WHO, 2011b). Mycobacterial culture is more sensitive, but growth of TB bacilli on traditional solid medium requires 4–8 weeks, which delays appropriate treatment in the absence of a confirmed diagnosis. Culturing mycobacteria is mainly done on solid media, the Lowenstein- Jensen slope, or in broth media. These methods are slow, with cultures from microscopy-positive material taking from 2–4 weeks and for microscopy-negative material from 4–8 weeks (Yon, 2015).

3. Molecular methods: These includes; (i) Nucleic acid amplification testing: Nucleic acid amplification (NAA) tests are a reliable way to increase the specificity of diagnosis, but the sensitivity is too poor to rule out disease, especially in smear-negative (paucibacillary) disease where clinical diagnosis is equivocal and where the clinical need is greatest (Ling *et*

al., 2008). (ii) Xpert MTB/RIF (Cepheid): The Xpert MTB/RIF assay is a novel, rapid, automated, and cartridge-based NAA test that can detect TB along with rifampicin resistance directly from sputum within 2 hours of collection (WHO, 2013b). The GeneXpert cartridges are pre-loaded with all of the necessary reagents for sample processing, DNA extraction, amplification, and laser detection of the amplified rpo B gene target. A major advantage of the Xpert MTB/RIF test is that it can be accurately administrated with minimal hands-on technical time. The sensitivity and specificity of this test has been reported to be acceptable for TB detection (Weyer *et al*., 2013; WHO, 2013b). The Xpert MTB/RIF test is a valuable, highly sensitive, and specific new tool for early TB detection and for determining rifampicin resistance.

2.2. Overview of Haemostasis

Haemostasis is a process that leads to stoppage of bleeding. It is the instinctive response for the body to stop bleeding and loss of blood. It occurs when blood is present outside of the body or blood vessels. The term comes from the Ancient Greek roots "heme" meaning blood, and "stasis" meaning halting which means the "halting of the blood (Marieb and Hoehn, 2010). Intact blood vessels are central to moderating blood's tendency to form clots. The endothelial cells of intact vessels prevent blood clotting with a heparin-like molecule and thrombomodulin and prevent platelet aggregation with nitric oxide and prostacyclin. Without the ability to stimulate haemostasis the risk of haemorrhaging is great. Haemostasis has three major steps that includes; vasoconstriction, temporary blockage of a break by a platelet plug, and blood coagulation, or formation of a fibrin clot. These processes seal the hole until tissues are repaired.

2.2.1. Mechanism of haemostasis

Haemostasis is maintained in the body via three mechanisms or steps that occur in a rapid sequence;

1. **Vascular spasm (Vasoconstriction) -** This is the first response to injury as the blood vessels constrict to allow less blood to be lost. Vasoconstriction is produced by vascular smooth muscle cells which are controlled by vascular endothelium, which releases intravascular signals to control the contracting properties. Collagen is exposed at the site of injury, the collagen promotes platelets to adhere to the injury site. Platelets release

cytoplasmic granules which contain serotonin, ADP and thromboxane A2, all of which increase the effect of vasoconstriction. The spasm response becomes more effective as the amount of damage is increased. Vascular spasm is much more effective in smaller blood vessels (Alturi, 2005).

2. **Platelet plug formation-** In this second step which is known as primary haemostasis, platelets adhere to damaged endothelium as well as stick together to form a temporary seal to cover the break in the vessel wall to form a platelet plug. It takes approximately sixty seconds until the first fibrin strands begin to intersperse among the wound. After several minutes the platelet plug is completely formed by fibrin. Plug formation is activated by a glycoprotein called von Willebrand factor (vWF), which is found in plasma. Platelets play one of major roles in the haemostatic process. When platelets come across the injured endothelium cells, they change shape, release granules and ultimately become 'sticky'. Platelets express certain receptors, some of which are used for the adhesion of platelets to collagen. When platelets are activated, they express glycoprotein receptors that interact with other platelets, producing aggregation and adhesion. Platelets release cytoplasmic granules such as adenosine diphosphate (ADP), serotonin and thromboxane A_2. Adenosine diphosphate (ADP) attracts more platelets to the affected area, serotonin is a vasoconstrictor and thromboxane A2 assists in platelet aggregation, vasoconstriction and degranulation. As more chemicals are released more platelets stick and release their chemicals; creating a platelet plug and continuing the process in a positive feedback loop. This is referred to as primary hemostasis (Li, 2010).

3. **Clot formation -** The third and last step is called coagulation or blood clotting. Coagulation reinforces the platelet plug with fibrin threads that act as a "molecular glue" (Marieb and Hoehn, 2010). Once the platelet plug has been formed by the platelets, the clotting factors (in an inactive state) are activated in a sequence of events known as 'coagulation cascade' which leads to the formation of Fibrin from inactive fibrinogen plasma protein. Thus, a Fibrin mesh is produced all around the platelet plug to hold it in place; this step is called "Secondary Hemostasis". During this process some red and white blood cells are trapped in the mesh which causes the primary hemostasis plug to become harder: the resultant plug is called as 'thrombus' or 'Clot'. Therefore 'blood clot' contains secondary hemostasis plug with blood cells trapped in it (Marieb and Hoehn, 2010).

2.2.2. Role of platelets in haemostasis

Platelets also called thrombocytes are fragments of cytoplasm that are derived from the megakaryocytes (Machlus *et al.*, 2014), of the bone marrow. Their function (along with the coagulation factors) is to react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot. Platelets have no cell nucleus and circulating unactivated platelets are biconvex discoid (lens-shaped) structures (Michelson, 2013). The main function of platelets is to contribute to haemostasis. They do this by gathering at the site of damaged endothelium and unless the interruption is physically too large, they plug the hole. First, platelets attach to substances outside the interrupted endothelium: adhesion. Second, they change shape, turn on receptors and secrete chemical messengers: activation. Third, they connect to each other through receptor bridges: aggregation (Yip *et al*., 2005). A low platelet concentration which is also known as thrombocytopenia could be due to either decreased production or increased destruction, while an Elevated platelet concentration which is known as thrombocytosis could be due to either congenital, reactive (to cytokines), or due to unregulated production as a result of one of the myeloproliferative neoplasms or certain other myeloid neoplasms. A disorder of platelet function is known as a thrombocytopathy. The normal range is 150–400 $\times 10^9$ per liter. Platelet production is regulated by thrombopoietin, a hormone produced in the kidneys and liver. Each megakaryocyte produces between 1,000 and 3,000 platelets during its lifetime and an average of 10^{11} platelets are produced daily in a healthy adult. The average life span of circulating platelets is 8 to 9 days which is controlled by the internal apoptotic regulating pathway (Mason *et al*., 2007).

2.2.3. Structure of platelets

Structurally the platelet can be divided into four zones, from peripheral to innermost as follows; (1) Peripheral zone which is rich in glycoproteins required for platelet adhesion, activation, and aggregation. For example, GPIb/IX/X, GPVI and GPIIb/IIIa. (2) Sol-gel zone which is rich in microtubules and microfilaments, allowing the platelets to maintain their discoid shape. (3) Organelle zone which is rich in platelet granules. Alpha granules contain clotting mediators such as factor V, factor VIII, fibrinogen, fibronectin, platelet-derived growth factor, and chemotactic agents. Delta granules, or dense bodies, contain ADP, calcium, serotonin, which are platelet-activating mediators. (4) Membranous zone – contains membranes derived from megakaryocytic smooth endoplasmic reticulum organized into a

dense tubular system which is responsible for thromboxane A_2 synthesis and is connected to the surface platelet membrane to aid thromboxane A_2 release.

2.2.4. Functions of platelet

i. Platelet adhesion

Endothelial cells are attached to the sub-endothelial collagen by von Willebrand factor (vWF) which these cells produce. vWF is also stored in the Weibel-Palade bodies of the endothelial cells and secreted constitutively into the blood. Platelets store vWF in their alpha granules. When the endothelial layer is disrupted, collagen and vWF anchor platelets to the subendothelium. Platelet GP1b-IX-V receptor binds with vWF; and GPVI receptor and integrin $alpha_{-2-}beta_1$ bind with collagen (Dubois *et al*., 2006).

ii. Platelet activation

Platelet activation begins seconds after adhesion occurs. It is triggered when collagen from the sub-endothelium binds with its receptors on the platelet. Intracellular calcium concentration determines platelet activation status, as it is the second messenger that drives platelet conformational change and de-granulation. Resting platelets maintain active calcium efflux via a cyclic AMP activated calcium pump. The intact endothelial lining inhibits platelet activation by producing nitric oxide, endothelial-ADPase (which degrades the platelet activator ADP), and PGI_2 (Prostacyclin). Tissue factor also binds to factor VII in the blood, which initiates the extrinsic coagulation cascade to increase thrombin production. Thrombin is a potent platelet activator. Thrombin also promotes secondary fibrin-reinforcement of the platelet plug. Platelet activation in turn de-granulates and releases factor V and fibrinogen, potentiating the coagulation cascade. So in reality the process of platelet plugging and coagulation are occurring simultaneously rather than sequentially, with each inducing the other to form the final clot.

GPIIb/IIIa activation: Platelets secrete thromboxane A2, which acts on the platelet's own thromboxane receptors on the platelet surface (hence the so-called "out-in" mechanism), and those of other platelets. These receptors trigger intra-platelet signaling, which converts GPIIb/IIIa receptors to their active form to initiate aggregation (Yip *et al*., 2005).

Granule secretion: Platelets contain majorly, dense granules and alpha granules. Activated platelets secrete the contents of these granules through their canalicular systems to the

exterior. Activated platelets degranulate to release platelet chemotactic agents to attract more platelets to the site of endothelial injury. The α-granules (alpha granules) contains P-selectin, platelet factor 4, transforming growth factor-β1, platelet-derived growth factor, fibronectin, B-thromboglobulin, vWF, fibrinogen, and coagulation factors V and XIII, while the δ-granules (delta or dense granules) contains ADP or ATP, calcium, and serotonin.

Morphology change: Mitochondria hyper-polarization is a key event in initiating changes in morphology (Matarrese *et al*., 2009). Intra-platelet calcium concentration increases, stimulating the interplay between microtubule/actin filament complex. These changes are all brought about by the interaction of the microtubule/actin complex with the platelet cell membrane and open canalicular system (OCS), which is an extension and invagination of that membrane.

Platelet-coagulation factor interactions (coagulation facilitation): In addition to interacting with vWF and fibrin, platelets interact with thrombin, Factors X, Va, VIIa, XI, IX, and prothrombin to complete clot formation via the coagulation cascade (Bouchard *et al*., 2010). Six studies suggested platelets express tissue factor, but the definitive study shows they do not (Bouchard *et al*., 2010).

iii. Platelet aggregation

Aggregation begins minutes after activation, and occurs as a result of turning on the GPIIb/IIIa receptor, allowing these receptors to bind with vWF or fibrinogen (Yip *et al*., 2005). When any one or more of at least nine different platelet surface receptors are turned on during activation, intra-platelet signaling pathways cause existing GpIIb/IIIa receptors to change shape – curled to straight – and thus become capable of binding (Yip *et al*., 2005). Since fibrinogen is a rod-like protein with nodules on either end capable of binding GPIIb/IIIa, activated platelets with exposed GPIIb/IIIa can bind fibrinogen to aggregate. GPIIb/IIIa may also further anchor the platelets to sub-endothelial vWF for additional clot structural stabilisation.

2.2.5. Role of platelets in Inflammation

In addition to being the cellular effector of haemostasis, platelets are rapidly deployed to sites of injury or infection, and potentially modulate inflammatory processes by interacting with leukocytes and by secreting cytokines, chemokines, and other inflammatory mediators

(Iannacone *et al*., 2005). Platelets also secrete platelet-derived growth factor (PDGF). Some drugs used to treat inflammation have the unwanted side effect of suppressing normal platelet function. Aspirin irreversibly disrupts platelet function by inhibiting cyclooxygenase-1 (COX1), and hence normal haemostasis. The resulting platelets are unable to produce new cyclooxygenase because they have no DNA. Normal platelet function will not return until the use of aspirin has ceased and enough of the affected platelets have been replaced by new ones, which can take over a week.

2.2.6. Role of platelet factor-4 (PF-4)

Platelet factor-4 (PF4) is a small cytokine belonging to the CXC chemokine family. It is usually released from alpha-granules of activated platelets during platelet aggregation, and promotes blood coagulation by moderating the effects of heparin-like molecules. Its major physiologic role appears to be neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation. Due to these roles, and being a strong chemoattractant for neutrophils, fibroblasts and monocytes it is predicted to play a role in wound repair and inflammation. The gene for human PF4 is located on human chromosome 4 (O'Donovan *et al.*, 1999). The human platelet factor 4 kills malaria parasites within erythrocytes by selectively lysing the parasite's digestive vacuole (Love *et al.*, 2012).

2.2.7. Role of p-selectin

P-selectin otherwise known as CD62P, Granule Membrane Protein 140 (GMP-140), and Platelet Activation-Dependent Granule to External Membrane Protein (PADGEM) is a protein encoded by the SELP gene in human beings (Ryan and Worthington, 1992). P-selectin is synthesized and found in endothelial cells and platelets where it is stored in Weibel-Palade bodies and α-granules, respectively. They functions as a cell adhesion molecule (CAM) on activated platelets and the surfaces of activated endothelial cells, which line the inner surface of blood vessels. Increased levels of P-selectin mRNA and protein are induced by inflammatory mediators such as tumor necrosis factor-a (TNF-a) and in response to inflammatory cytokines P-selectin is translocated to the plasma membrane in endothelial cells (Woltmann *et al*., 2000). P-selectin is very important in the recruitment and aggregation of platelets at areas of vascular injury. In quiescent platelet, P-selectin is located on the inner wall of α-granules. Platelet activation (through agonists such as thrombin, Type II collagen

and ADP) results in "membrane flipping" where the platelet releases α- and dense granules and the inner walls of the granules are exposed on the outside of the cell. The P-selectin then promotes platelet aggregation through platelet-fibrin and platelet-platelet binding. It also plays an essential role in the initial recruitment of leukocytes (white blood cells) to the site of injury during inflammation. P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on almost all leukocytes (neutrophils, eosinophils, lymphocytes, and monocytes) mediates tethering and adhesion of these cells. When endothelial cells are activated by molecules such as histamine or thrombin during inflammation, P-selectin moves from an internal cell location to the endothelial cell surface (Cleator *et al.*, 2006).

2.2.8. Role of thrombopoietin

Thrombopoietin (THPO) also known as Megakaryocytes Growth and Development Factor (MGDF) is a glycoprotein hormone that in humans is encoded by the THPO gene and regulates the production of platelets by stimulating the production and differentiation of megakaryocytes, the bone marrow cells that bud off large numbers of platelets (Kaushansky, 2006). Thrombopoietin is produced in the liver by both parenchymal cells and sinusoidal endothelial cells, as well as in the kidney by proximal convoluted tubule cells. Small amounts are also made by striated muscle and bone marrow stromal cells (Kaushansky, 2006). In the liver, its production is augmented by interleukin 6 (IL-6) (Kaushansky, 2006). However, the liver and the bone marrow stromal cells are the primary sites of thrombopoietin production. Its negative feedback is different from that of most hormones in endocrinology: The effector regulates the hormone directly. Thrombopoietin is bound to the surface of platelets by the mpl receptor (CD 110) and destroyed, thereby reducing megakaryocyte exposure to the hormone (Kaushansky, 2006). Therefore, the rising and dropping platelet concentrations regulate the thrombopoietin levels. Low platelets lead a higher degree of thrombopoietin exposure to the undifferentiated bone marrow cells, leading to differentiation into megakaryocytes and further maturation of these cells. On the other hand, high platelet concentrations lead to less availability of thrombopoietin to megakaryocytes.

2.2.9. Role of platelet glycoprotein IIb/IIIa

Glycoprotein IIb/IIIa (GPIIb/IIIa) is an integrin complex found on platelets. It aids platelet aggregation, is formed via calcium-dependent association of gpIIb and gpIIIa, a required step in normal platelet aggregation and endothelial adherence and is a receptor for von Willebrand

factor and fibrinogen (Shattil, 1999). The mechanism involves the binding of clotting mediators secreted with platelet activation to their respective receptors on platelet surfaces. This result in a cascade of events resulting in an increase in intracellular calcium. Hence, this calcium increase triggers the calcium-dependent association of gpIIb and gpIIIa to form the activated membrane receptor complex gpIIb/IIIa, which is capable of binding fibrinogen (factor I), resulting in many platelets "sticking together" as they may connect to the same strands of fibrinogen, resulting in a clot. The coagulation cascade then follows to stabilize the clot, as thrombin (factor IIa) converts the soluble fibrinogen into insoluble fibrin strands. These strands are then cross-linked by factor XIII to form a stabilized blood clot. Glanzmann's thrombasthenia is caused by defects in glycoprotein IIb/IIIa. Glycoprotein IIb/IIIa inhibitors can be used to prevent blood clots in an effort to decrease the risk of heart attack or stroke. Autoantibodies against IIb/IIIa can be produced in immune thrombocytopenic purpura (McMillan, 2007).

2.2.10. Role of platelet activating factor (PAF)

Platelet-activating factor (PAF) is a mediator of many leukocyte functions, platelet aggregation and degranulation, inflammation, and anaphylaxis. It is produced by a variety of cells, but especially those involved in host defense, such as platelets, endothelial cells, neutrophils, monocytes, and macrophages, it is continuously produced in low quantities under the control of PAF acetylhydrolases and produced in larger quantities by inflammatory cells in response to specific stimuli (Zimmerman *et al.*, 2002). It is important to the process of hemostasis because it causes platelets to aggregate and blood vessels to dilate. However, Unregulated PAF signaling can cause pathological inflammation. It initiates an inflammatory response in allergic reactions (McIntyre *et al.*, 2009). High PAF levels are associated with a variety of medical conditions. Some of these conditions include: Allergic reactions, Stroke, Sepsis, Myocardial infarction, Colitis, inflammation of the large intestine, Multiple sclerosis. There are two different pathways in which PAF can be synthesized: *de novo* pathway and remodeling. The remodeling pathway is activated by inflammatory agents and it is thought to be the primary source of PAF under pathological conditions. The *de novo* pathway is used to maintain PAF levels during normal cellular function. An increasing body of evidence supports the hypothesis that platelet-activating factor (PAF) plays a major role in the physiopathology of inflammatory reactions. The activity of PAF extends far beyond the

activation of platelets to include several events relevant for the development of inflammatory reactions.

2.3. Overview of Inflammation

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani *et al.*, 2007) and is a protective response involving immune cells, blood vessels, and molecular mediators. The inflammatory response is a defense mechanism that evolved in higher organisms to protect them from infection and injury. Its purpose is to localize and eliminate the injurious agent and to remove damaged tissue components so that the body can begin to heal. The response consists of changes in blood flow, an increase in permeability of blood vessels, and the migration of fluid, proteins, and white blood cells (leukocytes) from the circulation to the site of tissue damage. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Lichtman, 2009).

2.3.1. Causes of inflammation

The factors that can stimulate inflammation include microorganisms, physical agents, chemicals, inappropriate immunological responses, and tissue death. Infectious agents such as viruses and bacteria are some of the most common stimuli of inflammation. Viruses give rise to inflammation by entering and destroying cells of the body; bacteria release substances called endotoxins that can initiate inflammation. Physical trauma, burns, radiation, and frostbite can damage tissues and also bring about inflammation, as can corrosive chemicals such as acids, alkalis, and oxidizing agents. Malfunctioning immunological responses can incite an inappropriate and damaging inflammatory response. Inflammation can also result when tissues die from a lack of oxygen or nutrients, a situation that often is caused by loss of blood flow to the area. In summary, the causes of inflammation can be grouped as; Physical causes (Burns, Frostbite, Physical injury, Foreign bodies, including splinters, dirt and debris, Trauma, Ionizing radiation), Biological causes (Infection by pathogens, Immune reactions due to hypersensitivity, Stress), Chemical causes: (Chemical irritants, Toxins, Alcohol) and Psychological causes (Excitement) (Granger and Senchenkova, 2010; Hall, 2011;Piira *et al*., 2013).

2.3.2. Signs of inflammation

The four cardinal signs of inflammation are; redness, heat, swelling, and pain. Redness is caused by the dilation of small blood vessels in the area of injury. Heat results from increased blood flow through the area and are experienced only in peripheral parts of the body such as the skin. Fever is brought about by chemical mediators of inflammation and contributes to the rise in temperature at the injury. Swelling, called edema, is caused primarily by the accumulation of fluid outside the blood vessels. The pain associated with inflammation results in part from the distortion of tissues caused by edema, and it also is induced by certain chemical mediators of inflammation, such as bradykinin, serotonin, the prostaglandins and histamine that stimulate nerve endings. A fifth consequence of inflammation is the loss of function of the inflamed area, which may result from pain that inhibits mobility or from severe swelling that prevents movement in the area.

2.3.3. Classification of inflammation

Inflammation can be classified as either acute or chronic. An inflammatory response that lasts only a few days is called acute inflammation, while a response of longer duration is referred to as chronic inflammation.

2.3.4. The acute inflammatory response

Acute inflammation is a short-term process, usually appearing within a few minutes or hours and begins to cease upon the removal of the injurious stimulus. Acute inflammation may be regarded as the first line of defence against injury.It involves a coordinated and systemic mobilization response locally of various immune, endocrine and neurological mediators of acute inflammation. In a normal healthy response, it becomes activated, clears the pathogen and begins a repair process and then ceases (Kumar *et al*., 2004). The process of acute inflammation is initiated by resident immune cells already present in the involved tissue, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells and mast cells. Acute inflammatory response requires constant stimulation to be sustained. Inflammatory mediators are short-lived and are quickly degraded in the tissue. Hence, acute inflammation begins to cease once the stimulus has been removed. Since acute inflammation is an immune-vascular response to an inflammatory stimulus, this means that acute inflammation can be broadly divided into a vascular phase that occurs first and a cellular phase involving immune cells.

1. Vascular component of acute inflammation

The vascular component of acute inflammation involves the movement of plasma fluid, containing important proteins such as fibrin and immunoglobulins (antibodies), into inflamed tissue.

(a) Vasodilation and increased permeability

Acute inflammation is characterized by marked vascular changes, including vasodilation, increased permeability and increased blood flow, which are induced by the actions of various inflammatory mediators. When tissue is first injured, the small blood vessels in the damaged area constrict momentarily, a process called vasoconstriction. Following this transient event, which is believed to be of little importance to the inflammatory response, the blood vessels dilate (vasodilation), increasing blood flow into the area. Vasodilation occurs first at the arteriole level, progressing to the capillary level, and brings about a net increase in the amount of blood present, causing the redness and heat of inflammation. Next, the walls of the blood vessels, which normally allow only water and salts to pass through easily, become more permeable. Increased permeability of the vessels results in the movement of plasma into the tissues, with resultant *stasis* due to the increase in the concentration of the cells within blood – a condition characterized by enlarged vessels packed with cells. Stasis allows leukocytes to marginate (move) along the endothelium, a process critical to their recruitment into the tissues. Protein-rich fluid, called exudate, is now able to exit into the tissues. Substances in the exudate include clotting factors, which help prevent the spread of infectious agents throughout the body. Other proteins include antibodies that help destroy invading microorganisms. If the inflammatory stimulus is a lacerating wound, exuded platelets, coagulants, plasmin and kinins can clot the wounded area and provide haemostasis in the first instance.

(b) Plasma cascade systems

These includes the complement system which when activated, creates a cascade of chemical reactions that promotes opsonization, chemotaxis, and agglutination and the Kinin system which generates proteins capable of sustaining vasodilation and other physical inflammatory effects. Similarly the coagulation system or clotting cascade, which forms a protective protein mesh over sites of injury and the fibrinolysis system, which acts in opposition to the

coagulation system, to counterbalance clotting and generate several other inflammatory mediators.

(c) Plasma-derived mediators

The plasma derived mediators that play a role in acute inflammation includes; (i) Bradykinin which is a vasoactive protein produced by the Kinin system that is able to induce vasodilation, increase vascular permeability, cause smooth muscle contraction, and induce pain, (ii) C3 and C5a both of which stimulates histamine release by mast cells, thereby producing vasodilation. In addition, C3b is able to bind to bacterial cell walls and act as an opsonin, which marks the invader as a target for phagocytosis while C5a is able to act as a chemoattractant to direct cells via chemotaxis to the site of inflammation. (iii) Factor XII (Hageman factor) which is a protein that circulates inactively and when activated by collagen or platelets is able to activate three plasma systems involved in inflammation namely the Kinin system, fibrinolysis system and coagulation system. (iv) Plasmin which is able to break down fibrin clots, cleave complement protein C3 and activate factor XII while (v) Thrombin cleaves the soluble plasma protein fibrinogen to produce insoluble fibrin, which aggregates to form a blood clot.

2. Cellular component of acute inflammation

The cellular component involves leukocytes, which normally reside in blood and must move into the inflamed tissue via extravasation to aid in inflammation. Some act as phagocytes, ingesting bacteria, viruses, and cellular debris. Others release enzymatic granules that damage pathogenic invaders.

(a) Leukocyte extravasation

Various leukocytes, particularly neutrophils, are critically involved in the initiation and maintenance of inflammation. These cells must be able to move to the site of injury from their usual location in the blood, therefore mechanisms exist to recruit and direct leukocytes to the appropriate place. The process of leukocyte movement from the blood to the tissues through the blood vessels via chemotaxis, where they remove pathogens through phagocytosis and de-granulation is known as extravasation, and can be broadly divided up into a number of steps;

Leukocyte margination and endothelial adhesion: The white blood cells within the vessels which are generally centrally located move peripherally towards the walls of the vessels (Herrington, 2014). Activated macrophages in the tissue release cytokines such as IL-1 and TNF-α, which in turn leads to production of chemokines that bind to proteoglycans forming gradient in the inflamed tissue and along the endothelial wall. Inflammatory cytokines induce the immediate expression of P-selectin and E-selectin on endothelial cell surfaces.

Migration across the endothelium, known as transmigration, via the process of diapedesis: Chemokine gradients stimulate the adhered leukocytes to move between adjacent endothelial cells. The endothelial cells retract and the leukocytes pass through the basement membrane into the surrounding tissue using adhesion molecules such as ICAM-1 (Herrington, 2014).

(b) Phagocytosis

Extravasated neutrophils in the cellular phase come into contact with microbes at the inflamed tissue. Phagocytes express cell-surface endocytic pattern recognition receptors (PRRs) that have affinity and efficacy against non-specific microbe-associated molecular patterns (PAMPs). Upon endocytic PRR binding, actin-myosin cytoskeletal rearrangement adjacent to the plasma membrane occurs in a way that endocytoses the plasma membrane containing the PRR-PAMP complex, and the microbe. The endocytosed phagosome is transported to the intracellular lysosomes, where fusion of the phagosome and the lysosome produces a phagolysosome. The reactive oxygen species, superoxides and hypochlorite bleach within the phagolysosomes then kill microbes inside the phagocyte. Phagocytic efficacy can be enhanced by opsonization. Plasma derived complement C3b and antibodies that exude into the inflamed tissue during the vascular phase bind to and coat the microbial antigens. The co-stimulation of endocytic PRR and opsonin receptor increases the efficacy of the phagocytic process, enhancing the lysosomal elimination of the infective agent.

3. Events following acute inflammation

Once acute inflammation has begun, a number of outcomes may follow. These include healing and repair, suppuration, and chronic inflammation. The outcome depends on the type of tissue involved and the amount of tissue destruction that has occurred, which are in turn related to the cause of the injury.

(a) **Healing and repair**

During the healing process, damaged cells capable of proliferation regenerate. Different types of cells vary in their ability to regenerate. Some cells, such as epithelial cells, regenerate easily, whereas others, such as liver cells, do not normally proliferate but can be stimulated to do so after damage has occurred. Still other types of cells are incapable of regeneration.

(b) Suppuration

The process of pus formation, called suppuration, occurs when the agent that provoked the inflammation is difficult to eliminate. Pus is a viscous liquid that consists mostly of dead and dying neutrophils and bacteria, cellular debris, and fluid leaked from blood vessels. The most common cause of suppuration is infection with the pyogenic (pus-producing) bacteria, such as *Staphylococcus* and *Streptococcus*. Once pus begins to collect in a tissue, it becomes surrounded by a membrane, giving rise to a structure called an abscess. Because an abscess is virtually inaccessible to antibodies and antibiotics, it is very difficult to treat. Sometimes a surgical incision is necessary to drain and eliminate it. Some abscesses, such as boils, can burst of their own accord. The abscess cavity then collapses, and the tissue is replaced through the process of repair.

2.3.5. Chronic inflammation

If the agent causing an inflammation cannot be eliminated, or if there is some interference with the healing process, an acute inflammatory response may progress to the chronic stage. Repeated episodes of acute inflammation also can give rise to chronic inflammation. The physical extent, duration, and effects of chronic inflammation vary with the cause of the injury and the body's ability to ameliorate the damage. In some cases, chronic inflammation is not a sequel to acute inflammation but an independent response. Some of the most common and disabling human diseases, such as tuberculosis, rheumatoid arthritis, and chronic lung disease, are characterized by this type of inflammation. Chronic inflammation can be brought about by infectious organisms that are able to resist host defenses and persist in tissues for an extended period. These organisms include *Mycobacterium tuberculosis* (the causative agent of tuberculosis), fungi, protozoa, and metazoal parasites. Other inflammatory agents are materials foreign to the body that cannot be removed by phagocytosis or enzymatic breakdown. These include substances that can be inhaled, such as silica dust, and materials

that can gain entry to wounds, such as metal or wood splinters. The hallmark of chronic inflammation is the infiltration of the tissue site by macrophages, lymphocytes, and plasma cells (mature antibody-producing B lymphocytes). These cells are recruited from the circulation by the steady release of chemotactic factors. Macrophages are the principal cells involved in chronic inflammation and produce many effects that contribute to the progression of tissue damage and to consequent functional impairment. Granulomatous inflammation is a distinct type of chronic inflammation. It is marked by the formation of granulomas, which are small collections of modified macrophages called epithelioid cells and are usually surrounded by lymphocytes. A classic example of granulomatous inflammation is tuberculosis, and the granulomas formed are called tubercles (Kumar, 1998).

2.3.6. Resolution of inflammation

The inflammatory response must be actively terminated when no longer needed to prevent unnecessary "bystander" damage to tissues. Failure to do so results in chronic inflammation, and cellular destruction. Resolution of inflammation occurs by different mechanisms in different tissues. Mechanisms that serve to terminate inflammation include (Headland and Norling, 2015);

1. Short half-life of inflammatory mediators *in vivo*.

2. Production and release of transforming growth factor (TGF) beta from macrophages. Production and release of interleukin 10 (IL-10).

3. Production of anti-inflammatory specialized pro-resolving mediators, i.e. lipoxins, resolvins, maresins, and neuro-protectins.

4. Down-regulation of pro-inflammatory molecules, such as leukotrienes.

5. Up-regulation of anti-inflammatory molecules such as the interleukin 1 receptor antagonist or the soluble tumor necrosis factor receptor (TNFR).

6. Apoptosis of pro-inflammatory cells.

7. Increased survival of cells in regions of inflammation due to their interaction with the extracellular matrix (ECM).

8. Cleavage of chemokines by matrix metalloproteinases (MMPs) might lead to production of anti-inflammatory factors.

Table 2.1 Comparison between acute and chronic inflammation (Nordqvist, 2017)

	Acute inflammation	Chronic inflammation
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions
Major cells involved	neutrophils (primarily), basophils (inflammatory response), and eosinophils (response to helminth worms and parasites), mononuclear cells (monocytes, macrophages)	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months, or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis, necrosis

2.4. Inflammatory Response in Tuberculosis Infection

Infection with *Mycobacterium tuberculosis* (MTB) is usually accompanied by an intense local inflammatory response which obviously contributes to the pathogenesis of tuberculosis infection. Activation of components of the innate immune response, such as recruitment of polymorphonuclear (PMN) and mononuclear phagocytes and induction of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-alpha), by Mtb occurs early after Mtb infection, however, may persist as the organism establishes itself within granulomas. Mtb and its protein and non-protein components are potent in induction of cytokines and chemokines from PMN and monocytes.

2.4.1. Role of inflammatory cytokines in tuberculosis infection

Cytokines are proteins that can alter the behaviour or properties of the cell itself or of another cell (Romero-Adrian *et al*., 2015). These proteins are involved in the immunopathology of different diseases and the study of the cytokines in *Mycobacterium tuberculosis* infection is very important because they participate in the establishment, persistence and evolution of the infection (Romero-Adrian *et al.*, 2015). They are molecules that mediate mainly the intercellular communication in the immune system, being produced by different cell types. Cytokines have pleiotropic and regulatory effects and participate in the host defence and in inflammatory and tissue reparation processes (Gupta *et al*., 2012). In tuberculosis, an effective and coordinated participation of different cytokines was already identified (Tomioka *et al.*, 2011). Many events mediated by cytokines are important to the establishment of immunity against MTB and the expression of host resistance (Gupta *et al.*, 2012). Immunity and inflammation are intertwined processes. The interaction of Macrophages, one of the key elements involved in immunity to TB, with various mycobacterial strains is known to ensue in the differential induction and elaboration of several pro-inflammatory such as interleukin-1β (IL-1β), IL-6, IL-12, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF) and anti-inflammatory cytokines including IL-10. The intricate interplay of these cytokines is thought to orchestrate the induction and progression of an effective innate anti-mycobacterial immune response (Singh and Goyal, 2013).

2.4.2. Tumor necrosis factor-alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine which exerts multiple biological effects. Its expression is strictly controlled, since its super-production can mediate damaging effects found in the septic shock such as arterial hypotension, disseminated vascular coagulation, and lethal hypoglycaemia (Cavalcanti *et al*., 2012). TNF-α blocking has dramatic effects on the progression of tuberculosis in experimental models and stimulates the migration of immune cells to the infection site. However, Tumor necrosis factor (TNF-α) relative roles in Mtb have been a subject of controversy. It was described that mycobacteria decreases the production of TNF in human PBMCs, skill which probably contributes to its ability to establish chronic infections. Produced by macrophages, lymphocytes, neutrophils, and some endothelial cells, TNF-α coordinates the inflammatory response via induction of other cytokines (IL-1 and IL-6), and the recruitment of immune and inflammatory cells through the induction of chemokine and supra-regulation of adhesion molecules. TNF-α increases the capacity of macrophages to phagocytose and kill mycobacteria and stimulates apoptosis of macrophages, depriving bacilli of host cells and leading to death and presentation by dendritic cells of mycobacterial antigens. In vivo TNF-α is required for the formation and maintenance of granulomas. Research showed that neutralization of TNF-α produced by mice chronically infected with *M. tuberculosis* specific monoclonal antibodies disrupts the integrity of granulomas, exacerbates infection, and increases mortality (Robinson, *et al.*, 2012). TNF-α is also one of the most powerful controlling factors for the recruitment of monocytes and is a potent inducer of cell death by apoptosis. The important functions of TNF alpha in response to *Mtb* infection have been well documented in both mouse models and infected humans (Shi *et al*., 2016). Rapid *Mtb* growth and the death of the host have been clearly demonstrated following TNF-a blockade *in vitro*, *in vivo*, and in knockout mouse models, revealing the protective role of this cytokine in *Mtb* infection (Bourigault *et al*., 2013; Roh *et al*., 2013). In all, the overriding conclusion is that Tumor necrosis factor (TNF) plays a major role in the initial and long-term control of tuberculosis.

Mootoo *et al* (2009) observed that TNF-alpha is an essential component of the innate defence mechanism of the host against pathogenic challenge; it plays a major role in the pathology of certain diseases, such as tuberculosis. This disease is a striking example of the role of TNF-alpha as a 'double-edged sword', because apart from its role in controlling the *Mycobacterium tuberculosis* infection, it can also cause severe tissue damage (Mootoo *et al*., 2009). Host immune response against *Mycobacterium tuberculosis* is mediated by cellular immunity, in

which cytokines and Th1 cells play a critical role. In the process of control of the infection by mycobacteria, TNF-alpha seems to have a primordial function (Cavalcanti *et al*., 2012). According to Al-Attiyah *et al* (2012), increased levels of TNF-α are commonly detected in culture supernatants of peripheral blood mononucleated cells (PBMCs) from patients with pulmonary tuberculosis stimulated with mycobacterial antigens , also when the immune response of patients were evaluated prior to and after treatment it was noticed that patients with active pulmonary tuberculosis produced increased levels of TNF-α. One of the most overwhelming lines of evidence of the protective effects of TNF-α is, perhaps, provided by the observation that patients with rheumatoid arthritis under treatment with TNF-α antagonists (monoclonal antibodies against TNF-α or TNF-α soluble receptors) have a significant increased risk of reactivating latent TB (Solovic *et al*., 2010; Stallmach *et al*., 2010). On the other hand, there is also evidence showing that TNF-α may be associated with immunopathological responses in tuberculosis, aforementioned also as the head mediator of the destruction of the pulmonary tissue. Elevated levels of TNF-α are related to an excessive inflammation with necrosis and cachexy (Flynn *et al*., 2011). TNF-α is a cytokine with a long history in TB research and plays important roles in immune and pathological responses of TB patients (Mootoo *et al*., 2009). TNF was shown to be important in regulating chemokines, increasing adhesion molecule expression, mediating macrophage apoptosis (which can kill the infecting bacillus) and limiting excessive type 1 immune activation during intracellular infection (Zganiacz *et al*., 2004). Numerous laboratories have performed experiments with mouse models of tuberculosis that confirm that TNF plays a crucial role in the immune defence against Mtb. TNF is considered to be a pro-inflammatory cytokine, yet in the setting of tuberculosis disease it has also been shown to play an important disease-limiting effect (Zganiacz *et al*., 2004). Excess TNF appears to damage the host in advanced tuberculosis disease. Tuberculosis causes ill-health through weight loss and night sweats, which may be due to excessive TNF activity. This is consistent with the notion that TNF plays a crucial positive role in host defence during primary infection and in maintaining latency, whereas excessive TNF during tuberculosis disease is detrimental to the host. The importance of tumor necrosis factor-α (TNF-α) in the control of human TB was realized when rheumatoid arthritis patients latently infected with *Mtb* received anti-TNF therapy, which led to TB reactivation (Redford *et al*., 2011).

2.4.3. Interleukin-6 (IL-6)

IL-6 is a pleiotropic pro-inflammatory cytokine that is mainly secreted by monocytes. Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. In humans, it is encoded by the IL6 gene. Human IL-6 is made up of 212 amino acids, including a 28-amino-acid signal peptide, and its gene has been mapped to chromosome 7p21. Although the core protein is ~20 kDa, glycosylation accounts for the size of 21–26 kDa of natural IL-6 (Tanaka *et al.*, 2014). Interleukin 6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, leading to inflammation. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs). IL-6 is responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow. It supports the growth of B cells and is antagonistic to regulatory T cells. When psychologically stressed, the human body produces stress hormones like cortisol, which are able to trigger interleukin-6 release into the circulation. IL-6 has extensive anti-inflammatory functions in its role as a myokine. It was the first myokine that was found to be secreted into the blood stream in response to muscle contractions (Pedersen and Febbraio, 2008). In general, the cytokine response to exercise and sepsis differs with regard to TNF-α. Thus, the cytokine response to exercise is not preceded by an increase in plasma-TNF-α. Thus, when IL-6 is signalling in monocytes or macrophages, it creates a pro-inflammatory response, whereas IL-6 activation and signalling in muscle is totally independent of a preceding TNF-response or NFκB activation, and is anti-inflammatory (Brandt and Pedersen, 2010).

Interleukin 6 (IL-6), promptly and transiently produced in response to infections and tissue injuries, contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions (Tanaka *et al.*, 2014). Although its expression is strictly controlled by transcriptional and posttranscriptional mechanisms, dysregulated continual synthesis of IL-6 plays a pathological effect on chronic inflammation and autoimmunity (Tanaka *et al*., 2014). IL-6 is also involved in the regulation of serum iron and zinc levels via control of their transporters. As for serum iron, IL-6 induces hepcidin production, which blocks the action of iron transporter ferroportin 1 on gut and, thus, reduces serum iron levels (Nemeth *et al.* 2004). This means that the IL-6-hepcidin axis is responsible for hypoferremia and anaemia associated with chronic inflammation. When IL-6 reaches the bone marrow, it

promotes megakaryocyte maturation, thus leading to the release of platelets. These changes in acute phase protein levels and red blood cell and platelet counts are used for the evaluation of inflammatory severity in routine clinical laboratory examinations (Tanaka *et al*., 2014).

An immediate and transient expression of IL-6 is generated in response to environmental stress factors such as infections and tissue injuries. This expression triggers an alarm signal and activates host defense mechanisms against stress. Removal of the source of stress from the host is followed by cessation of IL-6-mediated activation of the signal-transduction cascade by negative regulatory systems (Tanaka *et al*., 2014). However, dys-regulated and persistent IL-6 production of mostly unknown etiology, leads to the development of various diseases (Tanaka *et al*., 2014). Conclusively, IL-6 is a pleiotropic pro-inflammatory cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis (Kishimoto, 2010). Hence, IL-6 is of critical importance for acquired immunity against *M. tuberculosis* infection (Martinez *et al*., 2013).

2.4.4. Interleukin-10 (IL-10)

Interleukin 10 (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. IL-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF made by cells such as macrophages and Th1 T cells.

IL-10 performs important functions in response to *Mtb* infection, which have been well documented in both mouse models and infected humans (Shi *et al*., 2016). IL-10 has been found at increased levels in tuberculosis patients, and elevated innate production of IL-10 is associated with greater susceptibility to this disease (Cooper *et al*., 2011; Sakamoto, 2012). IL-10 is a potent immune-modulatory cytokine that affects innate and acquired immune responses (Beamer *et al*., 2009). The immunological consequences of IL-10 production during pulmonary tuberculosis (TB) are currently unknown, although IL-10 has been implicated in reactivation TB in humans and with TB disease in mice (Beamer *et al*., 2009). According to Beamer *et al* (2009), IL-10 promotes TB disease progression which has important diagnostic and/or therapeutic implications for the prevention of reactivation TB in humans. The dominant function of IL-10 is to deactivate macrophages, resulting in

diminished Th1 cytokine production, decreased production of reactive nitrogen or oxygen species, and limited antigen presentation, which may have far-reaching consequences on both innate and acquired immunity in vivo (Beamer *et al*., 2009). This is supported by a previous findings that a mice engineered to over-express IL-10 had accelerated disease progression following *M.tb* infection confirming a clear relationship between IL-10 and poor disease outcome (Beamer *et al.*, 2009). These data demonstrate that increased IL-10 production exacerbates TB disease and provides supportive evidence that IL-10 can be a bio-marker of TB disease progression in humans, this suggests that IL-10 production during infection with *M.tb* leads to the poor T cell responses and impaired control of infection (Beamer *et al*., 2009). Due to its ability to inhibit the T lymphocyte production of cytokines, IL-10 was originally described as a cytokine synthesis inhibitory factor (CSIF).

IL-10 is considered primarily an inhibitory cytokine, important to an adequate balance between inflammatory and immunopathologic responses. The increase in IL-10 levels seems to support the survival of mycobacteria in the host (Cavalcanti *et al*., 2012). IL-10 acts by inhibiting the production of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-12) and the action of antigen presenting cells, blocking the activation of T lymphocytes through the inhibition of expression of MHC class II molecules. IL-10 is produced by macrophages and T lymphocytes during M. tuberculosis infection (Cavalcanti *et al*., 2012).

IL-10 is increased in samples obtained from patients with TB, and a higher capacity of IL-10 production is associated with an increase in the disease incidence. In human tuberculosis, IL-10 production is higher in anergic patients, suggesting that *M. tuberculosis* induces IL-10 production, suppressing an effective immune response. IL-10 may be required to modulate pro-inflammatory effects in patients and in healthy household tuberculosis individuals (Cavalcanti *et al*., 2012). IL-10 has also been thought to be responsible for the suppression of immune response against TB, and persistence of a long-term *M. tuberculosis* infection (Redford *et al*., 2011). Additionally, IL-10 is effective at decreasing levels of pro-inflammatory cytokines: IL-2, IL-6, IL-1β, IL-12, GM-CSF, TNF-α and IFN-γ, in both stimulatory and naïve situations related to the presence or absence of sufficient antigen (Shalev *et al*., 2011; Tang-Feldman *et al*., 2011). The reduction of these cytokines minimizes leukocyte maturation, recruitment and inflammation (Bijjiga and Martino, 2013).

Most leukocytes secrete IL-10 at some level. The majority of IL-10 secretion comes from monocytes and their common mature forms following differentiation: Macrophages and both

plasmacytoid and myeloid dendritic cells (Liu, 2011). While increased levels of IL-10 can result in severe immune-suppression, even normal levels of IL-10 can allow for chronic infection due to decreasing levels of pro-inflammatory cytokines and promoting effector T-cell anergy (Bijjiga and Martino, 2013). Anti-viral medication has been shown to decrease levels of IL-10 thereby allowing the immune system to mount a stronger attack against the persistent viral infection (Kobayashi *et al*., 2012; Langhans *et al*., 2012).

In the course of respiration the lungs are exposed to a vast variety of pathogens, allergens, and innocuous particles. A feature of the lung immune response to *Mycobacterium tuberculosis* (*Mtb*) is a balanced immune response that serves to restrict pathogen growth while not leading to host-mediated collateral damage of the delicate lung tissues. One immune-limiting mechanism is the inhibitory and anti-inflammatory cytokine interleukin (IL)-10 (Redford *et al*., 2011). IL-10 is made by many hematopoietic cells and it was proposed by Redford *et al*. (2011) that IL-10 is linked with the ability of *M.tb* to evade immune responses and mediate long-term infections in the lung. In some cases, too much IL-10 at one end of the scale may over-control otherwise protective T-cell responses, leading to chronic infection, and at the other end, too little or no IL-10 may tend toward fatal host-mediated pathology, thus, IL-10 manages a delicate balance between suppressing and activating host responses to virtually all pathogens (Redford *et al*., 2011). In human TB studies, IL-10 has been shown to be elevated in the lungs and serum of active PTB patients (Almeida, 2009).

2.4.5. Transforming growth factor-beta (TGF-β)

Transforming growth factor beta (TGF-β) is a multifunctional cytokine belonging to the transforming growth factor super-family that includes four different isoforms (TGF-β 1 to 4), and many other signaling proteins produced by all white blood cell lineages. TGF-β controls proliferation, differentiation, and other functions in many cell types (Massagué, 2012). TGF-β1 was first identified in human platelets as a protein with a molecular mass of 25 kilodaltons with a potential role in wound healing. TGF-β1 plays an important role in controlling the immune system, and shows different activities on different types of cell, or cells at different developmental stages. Some T cells (e.g. regulatory T cells) release TGF-β1 to inhibit the actions of other T cells. Similarly, TGF-β1 can inhibit the secretion and activity of many other cytokines including interferon-γ, tumor necrosis factor-alpha (TNF-α) and various interleukins (Wahl *et al.,* 2006). It can also decrease the expression levels of cytokine

receptors, such as the IL-2 receptor to down-regulate the activity of immune cells (Letterio and Roberts, 1998). The effects of TGF-β1 on macrophages and monocytes is predominantly suppressive; this cytokine can inhibit the proliferation of these cells and prevent their production of reactive oxygen (e.g. superoxide (O_2^-) and nitrogen (e.g. nitric oxide (NO) intermediates.

TGF-β1 produced by mononuclear phagocytes may play an important role in the pathogenesis of tuberculosis, in part by modulating the response to potentially protective cytokines such as TNF alpha and IFN-gamma. Tuberculosis (TB) is associated with excessive production and bio-activation of transforming growth factor beta (TGF-β) in situ (Mianda *et al.,* 2012). A prominent role for TGF-β in macrophage deactivation and suppression of T-cell responses to *M. tuberculosis* (MTB) is well-established. Excessive TGF-β activity is a feature of active pulmonary TB (Mianda *et al.*, 2012). Importantly, lung lavage from patients with active TB contain bioactive TGF-β (Bonecini-Almeida *et al*., 2004), implicating that conditions for TGF-β signaling are present in situ.

2.4.6. Interleukin-2 (IL-2)

Interleukin-2 (IL-2) is an interleukin, a type of cytokine signaling molecule in the immune system. It is a 15.5 - 16 kDa protein (Natalia *et al*., 2015) that regulates the activities of white blood cells (leukocytes, often lymphocytes) that are responsible for immunity. IL-2 is part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self" (Liao *et al*., 2011). IL-2 has essential roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells. In the thymus, where T cells mature, it prevents autoimmune diseases by promoting the differentiation of certain immature T cells into regulatory T cells, which suppress other T cells that are otherwise primed to attack normal healthy cells in the body (Natalia *et al*., 2015). IL-2 also promotes the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections (Liao *et al*., 2011). Together with other polarizing cytokines, IL-2 stimulates naive $CD4^+$ T cell differentiation into Th1 and Th2 lymphocytes while it impedes differentiation into Th17 and folicular Th lymphocytes (Liao *et al*., 2013). Its expression and secretion is tightly regulated and functions as part of both transient positive and negative feedback loops in mounting and dampening immune responses. Through its role in the development of T cell immunologic memory, which depends upon the expansion of the number and function of

antigen-selected T cell clones, it plays a key role in enduring cell-mediated immunity (Liao *et al,* 2011; Malek and Castro, 2010).

2.5. Haematological Changes in Tuberculosis Infection

Some studies have demonstrated that hematopoiesis may be perturbed by mycobacterial infection (Baldridge *et al.,* 2010) or the pathogen may infect bone marrow mesenchymal stem cells (Das *et al*., 2013). In tuberculosis various haematological derangements are seen including low haemoglobin, decreased lymphocyte count with its subsets, neutrophilia, monocytosis, monocytopenia, thrombocytopenia and thrombocytosis in few cases. Anaemia is one of the commonest findings seen in TB patients and considered to be responsible for poor prognosis (Alavi-Naini *et al*., 2013). Most commonly seen anaemia is iron deficiency anaemia that decreases hosts capacity in defending against foreign antigen resulting in impaired immune response (Isanaka *et al*., 2012). According to Iqbal *et al* (2015) more than three-fourths (¾) of TB patients recruited presented with anaemia (normocytic normochromic or iron deficiency anaemia). They explained further that Iron is a growth factor required by mycobacterium for growth and survival which prevents the release of iron from reticulo-endothelial system. Due to non-availability of iron to bone marrow, there is reduced erythropoiesis resulting in anaemia. They noted that with effective therapy anaemia improved in the studied patients after the completion of initiation phase of therapy. Amilo *et al* (2013) also found a significantly lower Haemoglobin and PCV values in pulmonary TB infected subjects compared to the uninfected control subjects.

Iqbal *et al* (2015) also found a total leukocyte count within normal range. Other similar studies showed that leukocyte count was significantly raised in slow responders whereas it was slightly raised in fast responder of anti-tuberculosis therapy (ATT) (Brahmbhatt *et al*., 2006), while Venestra *et al* (2006) found a significant increase in total leukocyte count that resulted from immune reaction taking place in response to foreign antigen *Mycobacterium tuberculosis*, that also resulted in increased cytokines levels which they claim causes further proliferation of white blood cells. Regarding lymphocyte count the results are found controversial; a group of scientists stated lymphopenia a marked finding of tuberculosis, whereas others found lymphocytosis (Okamura *et al.,* 2013). Iqbal *et al* (2015) also reported lymphopenia in half of the patients at the time of diagnosis which later improved with initial phase of treatment. They explained that the lymphopenia might be due to accumulation of lymphocytes at the site of infection leading to decrease number in peripheral blood (Djoba *et al*., 2009). Iqbal *et al* (2015) also reported a significant neutrophilia that improved with

successful treatment. This elevated neutrophil count they noted could be due to innate immune response of body against the antigen. However contrasting findings exists that found neutropenia that might be due to decreased production either because of bone marrow invasion or malnutrition in the patients leading to folate deficiency resulting in decreased haemopoiesis. The first line of defence against any foreign organism is innate immunity characterized by phagocytosis mediated by neutrophils and macrophages. Tuberculosis is characterized by granuloma formation hence neutrophils play a vital role in its formation.

Monocytosis is commonly seen in Tuberculosis because the microorganism after entering the body is engulfed by alveolar macrophages. However, some microorganisms escape the defence mechanisms resulting in infection with production of chemo-attractant substances which then invite other leukocytes and result in unopposed production of monocytes. But Iqbal *et al* (2015) found decreased monocyte count in TB group compared with the control healthy group.

Platelet count also has a significant role in immune functions and thrombocytosis is generally seen in chronic inflammation stated as 'reactive thrombocytosis' (Iqbal *et al*., 2015). Iqbal *et al* (2015) discovered that Platelet count was decreased at time of diagnosis till the completion of initiation phase when compared with controls. However, during continuation phase the normal platelet count was resumed. They noted that rifampicin is considered as a major drug that decreases platelet count in anti-tuberculosis therapy (ATT). Decreased platelet count in TB patient may also be due to release of IL-1 that act as a procoagulant on endothelial cells. Thrombocytopenia may also be caused by formation of antibodies that bind to platelets and undergo complement cascade resulting in lysis of these cells. These antibodies also suppress the production of platelets (Verma *et al*., 2010).

2.5.1. Role of white blood cells in tuberculosis and inflammation

According to the research by Ngahane *et al* (2017) the most common abnormalities found among tuberculosis patients were Lymphopenia (22.1%), Neutrophilia (14.2%), Monocytosis (23.5%), while MLR and NLR were significantly higher in patient group compared to control group and were fairly predictive of active TB. They discovered that a NLR<1.19 and MLR<0.29 were identified as cut-off values for discriminating healthy subjects from TB patients while the areas under the curves were 0.77 and 0.84 for the MLR and NLR respectively. Recent clinical analysis of peripheral blood mononuclear cells supports the hypothesis that a significantly high or low monocytes/lymphocytes count ratio is predictive of active TB and that neutrophils also play a role in early innate immunity against

Mycobacterium tuberculosis (Ngahane *et al*., 2017). Recent reports suggest that the lymphocyte-to-monocyte ratio (LMR) is a potential biomarker for predicting clinical outcomes (Zhang *et al*., 2018).

Once infection occurs, it may lead to Systemic Inflammatory Response Syndrome (SIRS), which can cause serious complications such as severe sepsis, renal dysfunction, encephalopathy, coagulopathy and multiple organ failure. SIRS is usually measured by peripheral blood count-based parameters, such as neutrophils, lymphocytes, monocytes, red blood cell distribution width (RDW), mean platelet volume or platelet count. These parameters have been reported to be independent predictive markers of clinical outcome in cancer and different states of HBV-related hepatic disorders (Lou *et al*., 2012; Karagoz *et al*., 2014; Liu *et al*., 2014). Among these inflammatory parameters, the neutrophil-lymphocyte ratio (NLR), RDW and monocyte ratio have been proposed as easily accessible and reliable markers (Karagoz *et al*., 2014; Liu *et al*., 2014; Lee *et al*., 2014). Monocytes are central mediators of the immune response. Inflammatory stimuli mainly affect the numbers of monocytes in the peripheral blood, which contributes to LMR changes (Zhang *et al*., 2018).

In tuberculous pleural effusion, neutrophilia is generally seen in its acute phase (Rekha *et al.*, 2012; Kashinkunti, 2014). Immune cells, both monocytes and lymphocytes have well defined role in innate as well as acquired immunity. The results with lymphocyte count are still controversial, increasing in some cases while decreasing in others and returning to normal with therapy (Al-Aska *et al*., 2011). Monocytosis is commonly seen in tuberculosis. The microorganism after entering the body is engulfed by alveolar macrophages. Some microorganisms escape the defense mechanisms and succeed to endure, resulting in infection with production of chemoattractant substances which then invites other leukocytes and results in unopposed production of monocytes (Iqbal *et al*., 2014). According to Iqbal *et al* (2014), Lymphopenia was seen in 50% of their tuberculosis infected subjects s at the time of diagnosis which later improved with treatment while none of the patient showed lymphocytosis. They posited that Lymphopenia is considered to be due to accumulation of lymphocytes at the site of infection leading to decreased number in peripheral blood. There are different studies available mentioning lymphocyte count in TB and the effect of TB on lymphocyte count is still uncertain, while some reported lymphocytosis, others reported Lymphopenia (Okamura *et al.*, 2013). Active tuberculosis patients had a significantly higher absolute count than the healthy subjects (Guadagnino *et al*., 2017).

According to Veenstra *et al* (2006), the total White cell count and absolute neutrophil counts were significantly elevated in patients at diagnosis relative to controls, but returned to normal levels by the end of treatment, while the absolute monocyte counts were also significantly elevated at diagnosis but then dropped dramatically to significantly depressed levels at week 26 (end of treatment). Though, Monocytes/macrophages are important components of the innate immune response to mycobacterial infections, they stated that the cause of the surprising finding that their numbers are significantly depressed in fully treated patients is unknown. But the absolute lymphocyte count of patients at diagnosis was significantly depressed at diagnosis, but counts were no longer significantly different from controls at the end of treatment. They concluded that peripheral blood white cell counts change rapidly during treatment and some counts at diagnosis hold promise as surrogate markers of treatment response. Previous published studies showed that the absolute cells number of monocytes or lymphocytes in peripheral blood or yet the ratio of monocytes to lymphocytes displayed the ability to predict the risk of active TB (Naranbhai *et al*., 2014; Agarwal *et al.*, 2016; Manna *et al*., 2017).

Monocytes play a pivotal role as cellular component of the innate immune response and represent a link to the activation and modulation of the adaptive immune response due to their role as antigen presenting cells (Manna *et al*., 2017). Therefore, all the factors that could perturb the functions of monocytes, may potentially affect an individual response in the course of infections or autoimmune diseases or tumors (Manna *et al*., 2017). CD4 T cells and monocytes/macrophages have previously been shown to be major effector cells in protecting the host against Mtb infection (Naranbhai *et al*., 2017). Additionally, alteration in monocyte functions may alter crosstalk with lymphocytes and adaptive immune responses (Yazdanbakhsh *et al*., 2013; Naranbhai *et al*., 2017); hence changes in monocyte functions alone may have a role in the detrimental immune response.

2.5.2. Neutrophil-lymphocyte ratio (NLR)

According to Yoon *et al* (2013) the neutrophil-lymphocyte count ratio (NLR), is a convenient marker of inflammation. It is a readily calculable laboratory marker used to evaluate systemic inflammation. L/N ratio is an expedient marker of inflammation for foreseeing bacterial infection (Iqbal *et al.*, 2014). Kashinkunti *et al* (2014) showed a significant increase in L/N ratio in patients who developed pleural effusion due to tuberculosis. However Iqbal *et al* (2015) reported that L/N ratio was similar in patients with and without TB pleural effusion, as

well as a significant change in L/N ratio in patients undergoing treatment with reduced inflammatory response because neutrophilia and lymphopenia improve with settlement of inflammation and appropriate treatment. De Jager *et al* (2010) demonstrated that lymphocytopenia and the NLR were superior to CRP level, and white blood cell (WBC) and neutrophil counts for predicting bacteraemia in an emergency care unit. The physiological immune responses of circulating leukocytes to various stressful events are characterized by an increased neutrophil count and decreased lymphocyte count and an increase in total WBC and neutrophils is an inflammatory reaction, particularly when caused by a bacterial infection. Lymphocytopenia has also been described as a diagnostic marker of bacterial infection. Therefore, the NLR is thought to have stronger discriminative power for predicting bacteremia compared to discrimination based on neutrophilia or lymphocytopenia alone (Yoon *et al*., 2013).

According to Ayed *et al* (2017) the peripheral blood neutrophil to lymphocyte ratio (NLR) has been reported to correlate with the prognosis of many acute or chronic infectious diseases. This was confirmed in their study in which Mean value of NLR was significantly higher in the Poor Prognosis group. They concluded that high NLR was an independent risk factor of predicting poor prognosis in Extra-Pulmonary TB patients. Chua *et al* (2011) reported that L/N ratio was a consistent prognostic marker to quantify the regression of tumor and survival chances by evaluating the chronic inflammatory response of disease.

2.5.3. Monocyte-lymphocyte ratio (MLR)

Based on a theory formulated by Cunningham and his associates in 1925 that the increase of the monocytes was an index of dissemination of the disease and the increase of the lymphocytes an index of resistance, they suggested that the ratio between the monocytes and the lymphocytes as expressed by the formula;

$$\frac{\text{Total number (or percentage) of monocytes}}{\text{Total number (or percentage) of lymphocytes}}$$

would give in mathematical form an index of the relationship of the processes of tuberculosis infection to those of resistance. They found that this monocyte-lymphocyte ratio was high when the disease was active and normal or low when the patient had good combative powers. In their opinion the changes of the blood were so consistent in relation to the clinical changes

that they felt it was equally possible to predict the clinical status from the blood findings or vice versa. The changes in the blood were therefore valuable from a prognostic and a diagnostic standpoint.

Recent studies focus on the prognostic value of lymphocytes, monocytes and calculated ratios in cancer and infected patients (Naranbhai *et al*., 2015; Werf *et al*., 2016; Manna *et al*., 2017; He *et al.*, 2017). Several studies suggest that the lymphocyte-to-monocyte ratio (LMR) is a cheap, readily available and reproducible test with potential for predicting clinical outcomes of patients with solid tumours and haematological malignancy (Lin *et al*., 2014; Paik *et al.*, 2014; Stotz *et al.*, 2015; Belotti *et al*., 2015). Recent human studies support historical animal studies that suggested an association between peripheral blood monocyte-lymphocyte (ML) ratio and tuberculosis (TB) disease (Naranbhai *et al*., 2014). Also, LMR has also been reported to predict survival and prognosis in various patient populations with malignant diseases (Lin *et al*., 2014; Paik *et al*., 2014; Stotz *et al*., 2015; Belotti *et al*., 2015) and a decreased LMR has been shown to be significantly associated with a high risk for critical limb ischaemia in patients with peripheral arterial occlusive disease (Gary *et al*., 2014). MLR is an easily available and low price biomarker (Zhang *et al*., 2018). According to Agarwal *et al* (2016), the L/M ratio may have potential as an effective biomarker response.

According to Iqbal *et al* (2014) the lymphocyte and monocyte count significantly differs in pre-treatment tuberculosis subjects compared to the control group, while M/L ratio was similar in both groups, the M/L ratio significantly decreased after initiation phase and two months of maintenance phase of treatment from the baseline value. They concluded that Tuberculosis is associated with increased M/L ratio, which declines and returns to normal with anti-tuberculous therapy and that M/L ratio can be considered as an independent prognostic marker and predictor of anti tuberculous treatment. Monocytes are the target cells for mycobacterial proliferation whereas lymphocytes provide resistance to the spread of infection causing mycobacterial clearance so it is reasonable to suggest that M/L ratio can also be used as a prognostic tool in TB (Iqbal *et al.*, 2014). M/L ratio has already been used as a prognostic marker in various malignances including colon cancer, non-Hodgkin lymphoma and multiple myeloma (Stotz *et al.*, 2014). M/L ratio increases with chronic inflammations including TB which then settles under the effect of antituberculous therapy (Iqbal *et al.*, 2014).

Another recent study by Naranbhai *et al* (2014) on 3 to 4 months old children suspecting TB showed that M/L ratio has a predictive role in TB, thus helps in early detection and prompt

treatment of the disease. Recent data confirmed the hypothesis suggested by historical studies that the ratio of peripheral blood monocytes to lymphocytes (M/L) is associated with the risk of tuberculosis (TB) disease (Guadagnino *et al*., 2017). According to Guadagnino *et al* (2017) the mean score of M/L ratio was higher in the active tuberculosis group compared to the healthy control group, Latent TB infected group, treated tuberculosis group and HIV/TB co-infected groups. In a recent study by Manna *et al* (2017) they discovered that patients with active TB disease had a very high ML ratio, as compared to both Healthy control subjects and Latent TB infected subjects, as well as cured TB patients also, additional analysis showed that the ML ratio value decreased close to the normal range after anti-mycobacterial therapy suggesting that the ML ratio is changed after anti-TB therapy and could be used a tool to evaluate treatment success. In active TB patients, the ML ratio was significantly correlated with increased monocyte counts and lower lymphocyte counts, indicating that both the monocyte count and the lymphocyte count contribute to the altered ML ratio. Moreover, ROC curves and cross-over plots showed that the ML ratio could contribute to distinguish patients with active TB from Healthy control subjects, but was of poor value to differentiate active TB patients from Latent TB infected subjects and cured TB patients.

2.6. Tuberculosis and Malaria Co-infection

Malaria remains one of world's major infectious diseases and an impediment to economic development. One third of the world's population are at risk of infection, around 250 million people develop clinical infections annually, and at least half a million die each year; most are children aged under five years (WHO, 2011c). The disease is caused by infection of circulating red blood cells by the protozoan *Plasmodium*, transmitted to humans by *Anopheles* mosquitoes. The most dangerous of the plasmodia infecting humans is *Plasmodium falciparum*. Malaria is the world's most prevalent parasitic disease and against which effective control measures are urgently needed.

Malaria and tuberculosis together account for more than 2 million deaths worldwide each year. Not only are malaria and tuberculosis important threats to public health in their own right, but malaria-tuberculosis co-infection appears to generate more severe pathology than either disease on its own, and malaria may exacerbate primary or re-activation tuberculosis. Considerable geographic overlap exists in the distribution of malaria and tuberculosis with concentration of both diseases in Africa, Asia, and South America (WHO, 2009). Individuals residing in zones of high transmission may become co-infected with both pathogens, and biological interactions may exist between *P. falciparum* and *M. tuberculosis*. While multiple

socio-economic factors may also in part explain the observed pattern of co-endemicity. Both diseases are endemic in tropical and impoverished areas of the world, and co-infection is likely to occur in individuals in these zones of intense transmission (Page and Jedlicka, 2005). In addition to the socio-economic health determinants that account in part for the significant geographic overlap of both pathogens, biological interactions within the host may play a role in malaria-tuberculosis co-infection. Host response to malaria and tuberculosis may result in clearance of the microbe without clinical manifestations, mild or uncomplicated disease without lasting sequellae, or severe complications with the development of pathology or death.

2.7. Crosstalk Between Haemostasis and Inflammation

Inflammation and haemostasis are tightly interrelated pathophysiologic processes that considerably affect each other. In this bidirectional relationship, inflammation leads to activation of the haemostatic system that in turn also considerably influences inflammatory activity (Verhamme and Hoylaerts, 2009). Coagulation and inflammation are closely linked, both in health and disease. Indeed, failure of the complex balance between pro- and anticoagulation, or between pro- and anti-inflammatory reactions because of genetic or acquired disturbances may result in disease. The many links between inflammation and coagulation help explain the prothrombotic tendency observed in patients with acute inflammatory or infectious diseases. In addition, chronic inflammatory diseases also predispose to venous thrombosis. Inflammation and coagulation cannot be considered as two separate processes because there are several interlocking points making them a unique defensive host reaction. The endothelium is one of the major links between the two since damaged endothelium during inflammation represents a surface where proteins involved in both coagulation and fibrinolysis and the development of inflammation are expressed.

The coagulation system and the innate inflammatory response share a common ancestry and are coupled via common activation pathways and feedback regulation systems (Verhamme and Hoylaerts, 2009). More evolved species have more complex and specialized systems, but a two-way relationship between both has persisted throughout evolution: coagulation triggers inflammatory reactions and inflammation triggers the activation of the coagulation system. Extensive cross-talk between inflammation and coagulation involves cell receptor-mediated signaling, cellular interactions and the production of cell derived microvesicles by endothelial cells, leukocytes and platelets (Simak and Gelderman, 2006). Immune cells are important in the initiation of coagulation pathways, while various inflammatory mediators are capable of

altering haemostasis. Vice versa, coagulation proteases have significant immune-modulatory effects. Inflammation and coagulation have reciprocal amplifying effects, potentially constituting an environment that is highly pro-inflammatory and procoagulant in severe disease states. Severe infections are characterised by an acute inflammatory response, and are almost invariably accompanied by alterations of the coagulation system. The two-way relationship between inflammation and coagulation is apparent in the procoagulant pathways, but also in the natural anticoagulant pathways that all have been attributed anti-inflammatory properties. Inflammation shifts endothelial cells towards a more prothrombotic state, downregulating natural anticoagulant defense mechanisms and leading to expression and *de novo* synthesis of prothrombogenic molecules such as P-selectin, von Willebrand factor and possibly also TF (Verhamme and Hoylaerts, 2009).

The role of platelets in (vascular) inflammation is illustrative for this two-way relationship. After adhering to an injured vessel wall, activated platelets release cytokines, growth factors, and numerous pro-inflammatory mediators (Blair and Flaumenhaft, 2009). Platelets also facilitate leukocyte recruitment to activated endothelium by forming P-selectin-PSGL-1 mediated conjugates with circulating leukocytes.

The extensive crosstalk between immune and haemostatic systems occurs at level of all components of the haemostatic system including vascular endothelial cells, platelets, plasma coagulation cascade, physiologic anticoagulants and fibrinolytic activity. During inflammatory response, inflammatory mediators, in particular pro-inflammatory cytokines, play a central role in the effects on haemostatic system by triggering its disturbance in a number of mechanisms including endothelial cell dysfunction, increased platelet reactivity, activation of the plasma coagulation cascade, impaired function of physiologic anticoagulants and suppressed fibrinolytic activity. Pro-inflammatory cytokines are important mediators of activation of coagulation. The main mediators of inflammation-induced activation of the haemostatic system are pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) (Levi and Poll, 2005). Hence, the pro-inflammatory cytokines IL-6 and TNF-α establish a procoagulant shift in the haemostatic balance, promoting fibrin generation in severe inflammatory states, both systemically and locally. To summarise the effects of severe inflammation on haemostasis, high levels of circulating pro-inflammatory cytokines cause massive systemic activation of coagulation while seriously inhibiting both fibrinolysis and natural anticoagulation. Inflammatory mediators trigger disturbance of the haemostatic system in a number of mechanisms including endothelial cell dysfunction, increased platelet activation, tissue factor (TF)

mediated activation of the plasma coagulation cascade, impaired function of physiologic anticoagulant pathways and suppressed fibrinolytic activity.

Platelet numbers increase following surgery, trauma and sepsis following a inflammatory response. Both IL-6 and thrombopoetin stimulate the production of platelets (Kaser *et al*., 2001). It appears that thrombopoietin is an acute phase reactant but not uniquely responsible for the rise in the platelet count during a reactive thrombocytosis but is probably aided and abetted by IL-6 (Klinger and Jelkmann, 2002). The inflammatory mediator, IL-6 (interleukin-6), increases platelet production and the newly formed platelets are more thrombogenic and Platelet activation contributes to the inflammatory response. Beside its important role in haemostasis, platelets also play a relevant function in inflammation acting as pro-inflammatory cells (Wagner and Burger, 2003). Additionally, in the setting of inflammation induced activation of haemostasis, platelets can be directly activated with inflammatory mediators such as pro-inflammatory cytokines or platelet-activating factor (PAF) in case of both non-infectious and infectious inflammatory states (Zimmerman *et al*., 2002).

2.8. Tuberculosis Treatment (Directly Observed Treatment Short-course)

Directly observed treatment, short-course (DOTS, also known as TB-DOTS) is the name given to the tuberculosis (TB) control strategy recommended by the World Health Organization in 2007. According to WHO, the most cost-effective way to stop the spread of TB in communities with a high incidence is by curing it and the best curative method for TB is known as DOTS (WHO, 1996). DOTS have five main components that includes; Government commitment (including political will at all levels, and establishment of a centralized and prioritized system of TB monitoring, recording and training), case detection by sputum smear microscopy, standardized treatment regimen directly of six to nine months observed by a healthcare worker or community health worker for at least the first two months, drug supply, a standardized recording and reporting system that allows assessment of treatment results. The initial emphasis was on "DOT, or directly observed therapy, using a specific combination of TB medicines known as short-course chemotherapy as one of the five essential elements for controlling TB (WHO, 1994). Current first line regimens consist of three or four agents that in concert can eradicate organisms within all compartments and prevent the development of drug resistance. Rifampicin (RIF) is bactericidal against all three populations. Isoniazid (INH) is bactericidal against extracellular and intracellular organisms. Pyrazinamide (PZA) is bactericidal against intracellular organisms. Ethambutol is also used (Dobbs and Webb, 2011). Standard treatment regimens are typically separated into two

phases. The initial phase includes the first 8 weeks of treatment. The continuation phase is an additional 4 to 7 months, depending on treatment response. While the initiation phase utilizes the potent sterilizing effects of RIF, PZA and INH to clear live bacilli from sputum in the majority of patient while the combination of INH and RIF in the continuation phase eradicates residual organisms, minimizing relapse of disease (Dobbs and Webb, 2011). Given the propensity for developing resistance and the prolonged treatment regimens required, adherence to therapy is very important. In line with this directly observed therapy (DOT) is the recommended approach to medication. Fluoroquinolones are currently utilized for drug-resistant strains and when first-line agents are not tolerated (Dobbs and Webb, 2011).

2.9. Side Effects of Anti-tuberculosis Therapy (DOTS)

The current internationally accepted therapy by Directly Observed Treatment, Short-Course (DOTS) for drug-susceptible TB consists of multiple expensive antibiotics and is lengthy (Espinal *et al.*, 2001). Adherence and compliance are critical for optimal efficacy of these drug regimens. The regimen chosen for treatment of TB is largely based on two indicators – first, whether the patient has previously been treated for tuberculosis and second, the drug-susceptibility status of infecting bacilli. Although current TB treatment eradicates *M.tb* from the host body it also causes severe hepatotoxicity and other adverse side effects, causing a large number of patients to withdraw early from therapy. Additionally, displaying a phenomenon called therapy-related immune impairment; TB-treated patients are vulnerable to reactivation or re-infection of the disease (Tousif *et al*., 2015).

According to a research by Sowmya and Anoop (2017) the most common symptoms of adverse drug reaction (ADR) observed in TB subjects on DOTS in their study were Gastrointestinal symptoms like nausea (56%), vomiting (30%), loss of taste (14%), dyspepsia (24%), abdominal pain (20%), diarrhoea (4%), jaundice (8%), others were malaise (16%) and skin rash (2%). The mean onset time of the adverse drug effects were observed within 7 days and mean duration time of the adverse drug effects were seen up to 2 months after start of anti-tuberculosis treatment.

Hepatotoxicity is the most serious complication arising from the first line of TB treatment. Isoniazid, rifampicin and pyrazinamide are potentially hepatotoxic drugs (Saukkonen *et al*., 2006). The probability to developing hepatotoxicity varies between 2% to 28% in all patients, and taking into consideration their comparative risk, associated with substantial morbidity and mortality. The liver metabolizes these drugs and hepatotoxicity is presumed by a 3- to 5-

fold increase in transaminase levels above the upper limit of the normal range (Au *et al.*, 2007). A published work has shown that treatment with Isoniazid (INH), one of the antibiotics used in DOTS, exhibits severe hepatotoxicity and also induces apoptosis in activated T cells (Tousif *et al.*, 2014). Considering T cells play a central role in host protective immune responses against *M.tb* and other organisms, it is likely that their elimination caused by treatment with INH is responsible for making the individual vulnerable to re-infection and or reactivation of the disease once the treatment is withdrawn (Cox *et al.*, 2008). Thus, while DOTS therapy for TB is very effective in controlling the disease, it is also associated with a significant number of adverse effects such as hepatotoxicity, thrombocytopenia, Eryptosis, immune impairment, neutropenia etc.

Rifampicin (RIF) enhances hepatotoxicity of Isoniazid (INH) by promoting the generation of the metabolites acetylhydrazine and hydrazine. It also competes with bilirubin for clearance at sinusoidal membranes in a dose-dependent manner, which leads to hepatic cell injury, mild asymptomatic unconjugated hyperbilirubinemia or jaundice without hepatocellular changes, or centrilobular necrosis, possibly associated with cholestasis (Tousif *et al*., 2015).. Histopathological findings from DOTS-treated patients show a range of pathologies from spotty to diffuse necrosis with more-or-less complete cholestasis (Tostmann *et al*., 2008). RIF, an integral component of DOTS therapy for normal *M.tb* as well as drug-resistant *M.tb* strains, is highly effective. However, many complications and adverse drug responses restrict its use. This adverse drug response is manifested as segmental necrotizing glomerulonephritis, acute renal failure and hemolytic anemia (Wiggins *et al*., 2007). It has also been reported that RIF stimulates suicidal eryptosis or erythrocyte death (Lang *et al*., 2010). Rifampicin stimulates Ca^{2+} permeable cation channels, which facilitates Ca^{2+} entry into RBC from extracellular spaces. Increases in cytosolic Ca^{2+} levels induce hyperpolarization of cells by release of K^+ via K^+ channels. Hyperpolarization of cells further induces Cl- release. Exodus of K^+ and Cl- ions promote water to be leached by osmosis, which ultimately leads to characteristic cell shrinkage and scrambling (Tousif *et al*., 2015). PZA is presumed as a serious contributor to hepatotoxicity and shows other complications such as rashes. Molecular mechanisms responsible for hepatotoxicity due to PZA are still not clear. It is well known that TB patients taking both PZA and Ethambutol (E) show a 4-fold increase in transaminase levels, whereas Ethambutol alone causes no significant increase in transaminase levels (Tousif *et al*., 2015).

Previous studies by Yakar *et al* (2013) have firmly established a role of drugs employed during DOTS in thrombocytopenia. INH and RIF are capable of causing thrombocytopenia either together or individually. RIF plays a prominent role in anti-TB-associated thrombocytopenia where it is known as drug-induced immune thrombocytopenia (DIIT). A characteristic feature of this clinical disorder is the presence of drug-dependent antibodies (DDAbs), which have the unique ability to bind antigen only in the presence of the respective drugs. The precise mechanism of INH-induced thrombocytopenia is not well defined, but may acutely cause leukocytosis, and may induce chronic anaemia (haemolytic, sideroblastic, aplastic, or megaloblastic), agranulocytosis, eosinophilia; disseminated intravascular coagulation and lymphadenopathy due to hypersensitivity reactions has also been reported. It exacerbates RIF-induced complications that might result in development of anti-tuberculosis drug induced hepatotoxicity (ADTH) (Tousif *et al*., 2015).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Area

This study was carried out at the TB Centre of Mile Four Hospital Abakaliki Ebonyi State. Mile 4 hospital is a renowned Catholic mission hospital known as a Special Tuberculosis and Leprosy Referral Centre. It is located in Abakaliki, the capital of Ebonyi State, South-Eastern Nigeria and serves patients of high, middle and lower socio-economic status and with Igbo as the dominant tribe.

3.2. Study Design

This is a longitudinal prospective study designed to measure the levels of haemostatic and inflammatory markers in Tuberculosis infected individuals before and following treatment.

3.3. Study Population

Individuals confirmed to be positive for pulmonary tuberculosis by Sputum-Smear Acid Fast Bacilli by Ziehl Neelsen's stain and GeneXpert MTB/RIF assay were recruited for the study. The baseline samples were collected before initiation of therapy (treatment naive) and participants followed up in the course of treatment. Samples collected after 2 months and 6 months therapy. Tuberculosis treatment (DOTS) regimen normally involves a six months treatment that is divided into two phases of treatment namely; two months Intensive phase in which the patients are given 4 fixed dose combination (Rifampicin, Isoniazid, Pyrazinamide and Ethambutol hydrochloride) and the Continuation phase in which the subjects are given Rifampicin and Isoniazid only for 4 months. The dosage of therapy is dependent on the body weight of the subjects.

3.4. Sampling Technique

The TB infected subjects were recruited before the initiation of therapy by Convenience sampling technique in which individuals that meets the inclusion criteria were recruited consecutively until the sample size was attained.

3.5. Sample Size Determination

Sample size was calculated using G*Power software (version 3.0.10). Power analysis for a repeated measures ANOVA with three measurements was conducted in G*Power to

determine a sufficient sample size using an alpha of 0.05, a power of 0.90 and a medium effect size (d=0.25) (Faul *et al*., 2013). Based on these assumptions, the calculated sample size of 58 has 90% power to detect a difference of 0.25 (medium effect size) at significance level of 0.05 (See Appendix 1).

3.6. Recruitment of Tuberculosis Infected subjects

A total of eighty (80) subjects aged 18 to 80 years were enlisted for this study to give room for attrition in the course of follow up at 2^{nd} month and 6^{th} month of treatment. Pulmonary tuberculosis subjects who presented at the TB DOTS centres were recruited as subjects after confirmation for *Mycobacterium tuberculosis* based on sputum-smear acid fast bacilli positive by Ziehl Neelsen's stain and GeneXpert MTB/RIF assay. A total of 80 individuals were recruited before initiation of therapy (pre-treatment), 71 of these subjects were followed up after 2 months of treatment and samples collected. After 6 months of treatment only 60 of these subjects were followed through and samples were collected.

3.7. Inclusion Criteria

Subjects of both gender positive for active pulmonary *Mycobacterium tuberculosis* and with or without malaria.

3.8. Exclusion Criteria

The following subjects were excluded from the study; Subjects with any known bleeding disorders or history of bleeding, pregnant women, subjects that had blood transfusion in the previous three months, those that withheld their consent before or in the course of the study, subjects on aspirin and anticoagulant therapy, females on oral contraceptives, smokers, those taking any local herbs or herbal concoctions, subjects that have other known clinical diseases such as cancer, HIV, diabetes, chronic infections, chronic kidney and liver disease.

3.9. Ethical Consideration

Ethical approval was obtained from the Ethics committee of Federal Teaching Hospital Abakaliki (FETHA) (See Appendix 2) and permission was sought and obtained from the management of Mile four hospital Abakaliki before sample collection (See Appendix 3).

3.10. Informed Consent

The reason for the research was explained to prospective participants and those who gave informed consent were recruited into the study and confidentiality was ensured (See Appendix 4).

3.11. Research Questionnaire

Socio-demographic information such as age, sex, educational status, marital status, occupation etc. And clinical details such as symptoms, history of infection, blood transfusion, smoking, alcohol use etc were obtained using a questionnaire (See Appendix 5).

3.12. Sample Collection

3.12.1. Blood sample collection

All the necessary precautions in collection, separation and processing of blood samples were observed. Eight millilitres (8ml) of blood was collected from each subject and processed ensuring the integrity of cellular elements and avoiding pre-analytical errors arising from sample collection and processing. Three millilitres (3mls) was dispensed into plain sample bottles. Serum was obtained after clotting by spinning at 3000rpm for 10 minutes and used for evaluation of Tumor Necrosis Factor - alpha (TNF-α), IL-10, IL-6, IL-2, Transforming growth factor-beta (TGF-β), Thrombopoietin and HIV screening. Also, two and half millilitres (2.5 ml) of blood were dispensed into 0.28ml (280µl) of 3.2% tri-sodium citrate to give a final blood: tri-sodium citrate ratio of 9:1. The sample was mixed properly by reverse uniform inversion and centrifuged at 3000rpm for 10 minutes at room temperature. The clear plasma was separated into a clean dry plastic container and used for the determination of P-selectin, Platelet activating factor, Platelet factor 4 and Gp IIb/IIIa complex. The remaining two and half millilitres (2.5ml) was dispensed into bottles containing di-potassium salt of Ethylenediamine tetra-acetic acid (K_2-EDTA) at a concentration of 1.5mg/ml of blood and used for total and differential white cell count, platelet count, malaria parasite screening and parasite density.

3.12.2. Sputum for TB diagnosis

Two sputum samples (consisting of one spot sample and one early morning sample) collected in a wide mouth container from the subjects was required for Acid fast bacilli (AFB)

test as well as for the automated GeneXpert MTB/RIF real-time nucleic acid amplification test for rapid and simultaneous detection of TB and Rifampicin resistance.

3.13. Methods of Sample Analysis

3.13.1. Ziehl-Neelsen technique for *Mycobacterium tuberculosis* diagnosis as described by WHO (2003).

Principle:

Ziehl-Neelsen (Zn) technique is used to stain *Mycobacterium species*, the Carbol fuchsin stain binds to the mycolic acid in the mycobacterial cell wall. After staining, an acid decolorizing solution is applied. This removes the red dye from the background cells, tissue fibres and any organisms in the smear except mycobacteria which retain (hold fast to) the dye. Following decolourization, the smear is counter-stained with malachite green or methylene blue which stains the background material, providing a contrast colour against which the red AFB can be seen.

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 labelled.

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

Ziehl –Neelsen staining: The slide containing the smear was placed on a slide rack and the smear covered with Carbol Fuchsin 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 the smear is sufficiently decolorized, i.e. pale pink. The slide was washed off with clean water. The smear was covered with methylene blue stain for 1-2 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.

Microscopic examination of Ziehl-Neelsen stained smear: The smear was examined microscopically using the 100X oil immersion objective. Scanning of the smear was done systematically and when any definite red bacillus is seen, it was reported as AFB positive.

Results: AFB - Red, straight or slightly curved rods, occurring singly or in small groups. Cells and background material appear blue.

3.13.2. Genexpert method for detection of *Mycobacterium tuberculosis* and rifampicin resistance (GeneXpert MTB/RIF) as described by Blakemore *et al* (2010).

Principle

The GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, California) is a nucleic acid amplification-based diagnostic system that performs sample processing and hemi-nested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens. The MTB/RIF assay detects *M. tuberculosis* and RIF resistance by PCR amplification of the 81-bp fragment of the *M. tuberculosis* rpoB gene and subsequent probing of this region for mutations that are associated with RIF resistance. The assay can generally be completed in less than 2 hours. The system consists of an instrument, personal computer, barcode scanner and preloaded software for running tests on collected samples and viewing the results. The system requires the use of single-use disposable GeneXpert catridges that hold the PCR reagents and host the PCR process. The primers in the GeneXpert MTB/RIF assay amplify a portion of the rpoB gene containing the 81 base pair "core" region. The probes (probes A to E) are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance. The internal control heminested *B. globigii* assay is multiplexed with the M. tuberculosis assay. Mutations in the rpoB gene target inhibit hybridization of one or more of the rpoB-specific molecular beacons, reducing or eliminating the signal from the corresponding probes. *Mycobacterium tuberculosis* is identified when at least two of the five rpoB-specific molecular beacons give a positive signal with cycle threshold (CT) values that are ≤ 38 and that differ by no more than two cycles. *B. globigii* DNA is detected when the single B. *globigii* molecular beacon produces a CT of <38 cycles. The difference in CT between the first (early CT) and last (later CT) *M. tuberculosis*-specific molecular beacon (Δ CT Max) is the basis of rpoB mutation and RIF resistance detection. RIF resistance is identified if the ΔCT Max is >3.5 cycles. Rifampicin susceptibility is identified if the ΔCT Max is ≤3.5 cycles. A sample is considered RIF indeterminate when the last probe returns a CT of >38 and the first probe has a CT value of >34.5 cycles because the assay terminates at cycle 38 and a ΔCT Max of >3.5 cannot be measured.

Procedure

The assay consists of a single-use multi-chambered plastic cartridge pre-loaded with the liquid buffers and lyophilized reagent beads necessary for sample processing, 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. Two (2) mls of the treated sample was transferred into the test cartridge, the cartridge was loaded into the GeneXpert instrument and an automatic step will complete the remaining assay steps. The assay cartridge also contained lyophilized *Bacillus globigii* spores which served as an internal sample processing and PCR control. The spores was automatically re-suspended and processed during the sample processing step and the resulting *B. globigii* DNA was amplified during PCR step. The standard user interface indicates the presence or absence of *M. tuberculosis*, the presence or absence or Rifampicin resistance and a semi quantitative estimate of *M. tuberculosis* concentration (high, medium, low and very low). Assays that are negative for *M. tuberculosis* and also negative for *B. globigii* internal control was reported as invalid.

3.13.3. Detection of antibodies to HIV-1 and HIV-2

Subjects were tested for HIV using Inverness Determine^{TM} 1 and 2 (Inverness Medicals Co. Ltd, Japan) and STAT-PAK (Chembio Diagnostic system, New York, USA). Uni-Gold (Trinity Biotech, Bray, Ireland) was used as the tie-breaker according to the national guidelines for HIV counselling and testing. Tests were carried out according manufacturer's instructions.

Principle of Determine

Determine HIV-1 and 2 is an in vitro, visually read, qualitative immunoassay for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma and whole blood. Determine is an immunochromatographic test which relies on the migration of liquid across the surface of a nitrocellulose membrane. Sample is added to the sample pad followed by the addition of running buffer. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patients window site. If antibodies to HIV-1 and/or HIV-2 are present in the samples, the antibodies bind to the antigen selenium colloid at the here patient window forming a red line at the patients window site. If antibodies to HIV-1 and/or HIV-2 are absent, the antigen-selenium colloid flows past the patients sample window and no red line is formed at the site.

There should always be a red line at the control window. This is the incorporated procedural control in the assay device to ensure assay validity.

Procedure

As described by the manufacturer, 50µl of patients samples were separated from the clotted whole blood samples and were delivered onto appropriately labelled specimen pads. After minutes of sample application, the result was read. This method has in built quality control which validates the result. Two visible red lines in the region labelled control and test represents an HIV seropositive reaction while a single red line in the region labelled control only represents an HIV seronegative reaction.

Principle of STAT-PAK

The Chembio HIV-1 and 2 STAT-PAK which is an immunochromatographic assay, relies on the migration of liquid across the surface of a nitrocellulose membrane. It employs a unique combination of a specific antibody binding protein which is conjugated colloidal gold dye. HIV-1 and 2 antigens are bound to the membrane solid phase. The sample is delivered onto the sample well followed by the addition of running buffer. The buffer facilitates the lateral flow of the released products and promotes the binding of antibodies to the antigen; if present, the antibodies bind to the gold conjugative antibody binding protein. In a reactive sample, the dye conjugate-immune complex migrates on the nitrocellulose membrane and is captured by the antigens immobilized in the test (T) area. The sample continues to migrate along the membrane and produces a pink/purple line in the control area containing immunoglobulins G antigens.

Procedure

The procedure followed was as described by the manufacturer. Five microlitres (5µl) of sample loop provided was used to collect the participants serum by touching it on the specimen and allowing the opening in the loop to fill. The samples were then dispensed on the sample wells in appropriately labelled sample pad. Three drops of buffer supplied with the test kit were added to the sample wells. The result was read after 5 minutes. This method also has inbuilt quality control. The presence of two pink/purple lines in the control and test regions indicates a HIV seropositive reaction while a single pink/purple line at the control region indicates a HIV sero-negative reaction.

3.13.4. Diagnosis of malaria (Cheesbrough, 2006).

Whole blood was used for the diagnosis of malaria using thick and thin blood smears for microscopic detection.

Preparation and staining of thick blood film

A drop of blood was placed on the centre of a clean grease free slide and spread in a circular manner on the slide with the corner of another slide, till it covered an area four times its original area. The film was allowed to dry properly before immersing in staining jar containing Giemsa stain for 30 minutes before washing off. The slides were stood upright to air dry. Slides were viewed using x100 objective (oil immersion) for detection of malaria parasite.

Preparation and staining of thin blood film

A small drop of blood was placed at the bottom of the slide and using a spreader, a thin film was made. This was air dried and stained using Leishman stain first for 2 minutes before double diluting in buffered water and staining further for 8 minutes. Subsequently, the slides were washed and air dried before viewing using x100 objective.

3.13.5. Procedure for malaria parasite count as described by WHO (2016).

The parasite counts were done using thick blood films. The films were stained using Giemsa stain as previously described. The density of malaria parasites was determined by counting the number of parasites against 200 white blood cells (WBCs) in the thick film. The parasite density (per µl) was obtained using the formula;

$$\frac{\text{No of parasite counted}}{\text{WBC counted (200)}} \times \frac{\text{WBC count}}{1}$$

3.13.6. Body Mass Index (BMI) as described by the WHO (2006).

Principle

The body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity. It is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). The current value settings are as follows; a BMI of 18.5 to 24.99 indicates optimal weight, <18.5 underweight, <16 severe thinness,

16.00 – 16.99 moderate thinness, 17.00 – 18.49 mild thinness. BMI ≥ 25.00 may indicate a person is overweight, while ≥ 30 suggests the person is obese and ≥ 40 suggests morbid obesity. A person may have a BMI below 18.5 due to disease.

Procedure

Weight measurement

A well calibrated weighing scale was used for weight measurements. Care was taken that the weighing scale was zeroed before weight measurement. The subjects were instructed to remove footwear and climb barefooted unto the scale. The weight was then read off and recorded in kilograms.

Height Measurement

A metre rule calibrated in meters was used to measure the heights of the subjects. The subjects were asked to remove foot wear before the measurement. Heights were recorded in metres.

The BMI for each subject was then calculated by dividing the weight by the square of the height in metres (kg/m^2).

3.13.7. Packed cell volume (PCV) as described by Lewis *et al* (2006).

Principle

The packed cell volume (PCV), also called haematocrit, is that volume of whole blood that is occupied by red cells, expressed as a ratio (litre/litre). Anticoagulated blood in capillary tube is centrifuged in a microhaematocrit centrifuge at 12 000g for 5 minutes to obtain constant packing of red cells. The PCV value is read from the scale of a microhaematocrit reader.

Procedure

Plain capillary blood was filled up to three quarters with well mixed EDTA anticoagulated blood. The unfilled end was heat-sealed with the flame of a Bunsen burner, while rotating the end of the capillary in the flame. The sealed capillary was placed in one of the numbered slots in the microhaematocrit rotor with the sealed end against the rim gasket. The inner lid was closed. It was centrifuged for 5 minutes at 12000g and the PCV was read using the PCV reader.

3.13.8. Total white cell count as described by Lewis *et al* (2006).

Principle

Whole blood is diluted 1 in 20 in an acid reagent which haemolyses the red cells, leaving the white cells to be counted. The white cells are counted microscopically using the Improved Neubauer counting chamber. The nuclei of the white blood cells stain deep violet black.

Test procedure

A 1 in 20 dilution of well mixed EDTA blood was made by adding 20µl of blood to 0.38ml of Turks solution in a small glass tube. The tube was corked and the diluted blood sample mixed. Using a capillary tube, one of the grids of the chamber was loaded with the sample. Precaution was taken not to overfill the chamber. The chamber was left undisturbed for 2 minutes to allow the white cells to settle. Using X40 objective, the cells in the four large corners were counted.

The number of white cell obtained was reported after using the calculation;

$$\frac{\text{Total number of cells counted x dilution factor x } 10^6}{\text{Area x depth (Volume) counted}}$$

3.13.9. Differential white cell count as described by Lewis *et al* (2006).

For the differential white cell count, thin blood films were made from EDTA anticoagulated blood. The air dried slides were covered with Leishman stain using a dropper and was left for three minutes. Twice the volume of pH 6.8 buffered water was added and allowed to further stain for 10 minutes. The stain was washed off; the back of the slide wiped clean and was placed on a draining rack to dry. Using oil immersion, the slide was viewed and white cells differentiated and counted.

3.13.10. Platelet count as described by Lewis *et al* (2006).

Principle

This was carried out by visual examination of diluted, lysed whole blood using an Improved Neubauer counting chamber. The diluent consists of 1% aqueous ammonium oxalate in which red cells is lysed and the platelets appear as small highly refractile particles.

Procedure

A 1 in 20 dilution of well mixed blood was made in diluent by adding 20µl of blood to 0.38ml of ammonium oxalate (10g/l). Before the dilution, the blood sample was examined to rule out the presence of any blood clot. The suspension was mixed properly and an Improved Neubauer counting chamber was filled with the suspension using a Pasteur pipette. The counting chamber was placed in a moist Petri dish and left untouched for 20 minutes to give time for the platelets to settle. The preparation was examined using X40 objective.

The platelet count was determined as follows;

$$\text{Platelet count per litre} = \frac{\text{No of cells counted x dilution x } 10^6}{\text{Volume counted (µl)}}$$

3.13.11. Determination of cytokines and haemostatic markers in serum and plasma

The protocol for each parameter was carried out according to the manufacturer's instructions. The samples for IL-10, IL-6, IL-2 and TNF-α were assayed using commercial ELISA test kits (U-CyTech Biosciences, Utrecht, Netherlands) while the samples for TGF-β, P-selectin, GP IIb/IIIa complex, Thrombopoietin, PF-4, and PAF were also assay using commercial ELISA test kits (Elabscience Biotechnology Inc., USA) according to the manufacturer's instructions. Readings were taken using Microplate reader at a wavelength of 450nm and the levels were quantified using reference standard curves. The sensitivity for the test kits were 2pg/ml for IL-10, 1pg/ml for IL-6, 2pg/ml for IL-2, 1pg/ml for TNF-α, 18.75pg/ml for TGF-β, 0.1ng/ml for P-selectin, 0.1ng/ml for Human GP IIb/IIIa complex, 18.75pg/ml for Thrombopoietin, 0.94ng/ml for PF-4, and 46.88pg/ml for PAF.

(a) Tumor Necrosis Factor Alpha (TNF-α) (Change *et al.,* 2004).

The human TNF-α ELISA test kit from U-CyTech Biosciences (Cat No CT209A; Lot No 23-32-12-29) is used for the in vitro quantitative determination of TNF-α in human fluids such as cell culture supernatant, plasma or serum.

Principle

The TNF-α ELISA kit is a sandwich immunoassay. The assay utilizes a coating antibody specific for TNF-α (analyte of interest) coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte

present binds to the immobilized coating antibody. After washing off the excess and unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody. The wells are washed again and a streptavidin-HRP (SPP) conjugate is added to the antibody-antigen-antibody complex. After another wash, a chromogenic substrate (TMB) is introduced, which produces a blue-coloured product of which the intensity is related to the amount of analyte in the sample. A sulphuric acid solution is added to stop the enzymatic reaction (changing the colour to yellow) and OD is read at 450nm.

Assay protocol

All the prepared reagents were thoroughly mixed before use. One hundred microlitre (100µl) of blank, diluted standard and samples were added to each well. The plates were sealed and incubated for 2 hours at 37^0C. After incubation the plates were washed six times with the Wash buffer using the automated microplate Washer. Then 100µl of diluted detection antibody solution was added to each well and the plate was sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer as done previously. Hundred microlitre (100µl) of diluted SPP conjugate was added to each well and the plates sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer. Afterwards, 100µl of TMB substrate solution was added into each well and left in the dark for 20 minutes at room temperature. After substrate incubation, the reaction was stopped by adding 100µl of Stop solution (resulting in a yellow solution) and it was read at 450nm within 30 minutes using the Microplate reader**.**

(b) Interleukin-10 (IL-10) (Chang *et al*., 2004).

The human IL-10 ELISA test kit from U-CyTech Biosciences (Cat No CT206A; Lot No 35-18-51-29) is used for the in vitro quantitative determination of IL-10 in human fluids such as cell culture supernatant, plasma or serum.

Principle

The IL-10 ELISA kit is a sandwich immunoassay. The assay utilizes a coating antibody specific for IL-10 (analyte of interest) coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte present binds to the immobilized coating antibody. After washing off the excess and unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody. The wells are washed again and a streptavidin-HRP (SPP) conjugate is added to the antibody-

antigen-antibody complex. After another wash, a chromogenic substrate (TMB) is introduced, which produces a blue-coloured product of which the intensity is related to the amount of analyte in the sample. A sulphuric acid solution is added to stop the enzymatic reaction (changing the colour to yellow) and OD is read at 450nm.

Assay protocol

All the prepared reagents were thoroughly mixed before use. After 5 minutes of reconstituting the IL-10 stock standard with the dilution buffer, serial dilutions were made (385, 192.5, 96.25, 48.13, 24.06, 12.03, 0 pg/ml) in seven tubes. One hundred microlitre (100µl) of blank, serially diluted standard and samples were added to each well. The plates were sealed and incubated for 2 hours at 37^0C. After incubation the plates were washed six times with the Wash buffer using the automated microplate Washer. Then 100µl of diluted detection antibody solution was added to each well and the plate was sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer as done previously. Hundred microlitre (100µl) of diluted SPP conjugate was added to each well and the plates sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer. Afterwards, 100µl of TMB substrate solution was added into each well and left in the dark for 20 minutes at room temperature. After substrate incubation, the reaction was stopped by adding 100µl of Stop solution (resulting in a yellow solution) and it was read at 450nm within 30 minutes using the Microplate reader**.**

(c) Interleukin-6 (IL-6) (Engela *et al.*, 2013).

The human IL-6 ELISA test kit from U-CyTech Biosciences (Cat No CT205A; Lot No 38-28-19-29.) is used for the in vitro quantitative determination of IL-6 in human fluids such as cell culture supernatant, plasma or serum.

Principle

The IL-6 ELISA kit is a sandwich immunoassay. The assay utilizes a coating antibody specific for IL-6 (analyte of interest) coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte present binds to the immobilized coating antibody. After washing off the excess and unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody. The wells are washed again and a streptavidin-HRP (SPP) conjugate is added to the antibody-antigen-antibody complex. After another wash, a chromogenic substrate (TMB) is

introduced, which produces a blue-coloured product of which the intensity is related to the amount of analyte in the sample. A sulphuric acid solution is added to stop the enzymatic reaction (changing the colour to yellow) and OD is read at 450nm.

Assay protocol

All the prepared reagents were thoroughly mixed before use. After 5 minutes of reconstituting the IL-6 stock standard with the dilution buffer, serial dilutions were made (405, 202.5, 101.3, 50.62, 25.3, 12.6, 0 pg/ml) in seven tubes. One hundred microlitre (100µl) of blank, serially diluted standard and samples were added to each well. The plates were sealed and incubated for 2 hours at 37^0C. After incubation the plates were washed six times with the Wash buffer using the automated microplate Washer. Then 100µl of diluted detection antibody solution was added to each well and the plate was sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer as done previously. Hundred microlitres (100µl) of diluted SPP conjugate was added to each well and the plates sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer. Afterwards, 100µl of TMB substrate solution was added into each well and left in the dark for 20 minutes at room temperature. After substrate incubation, the reaction was stopped by adding 100µl of Stop solution (resulting in a yellow solution) and it was read at 450nm within 30 minutes using the Microplate reader**.**

(d) Interleukin-2 (IL-2) (Baan *et al.*, 2007).

The human IL-2 ELISA test kit from U-CyTech Biosciences (Cat No CT202A; Lot No 56-23-15-29) is used for the in vitro quantitative determination of IL-2 in human fluids such as cell culture supernatant, plasma or serum.

Principle

The IL-2 ELISA kit is a sandwich immunoassay. The assay utilizes a coating antibody specific for IL-2 (analyte of interest) coated on the wells of a 96-well microtiter plate. The wells are washed and blocked. Standards and samples are added to the wells and any analyte present binds to the immobilized coating antibody. After washing off the excess and unbound materials, the bound analyte is allowed to associate with a biotinylated detection antibody. The wells are washed again and a streptavidin-HRP (SPP) conjugate is added to the antibody-antigen-antibody complex. After another wash, a chromogenic substrate (TMB) is introduced, which produces a blue-coloured product of which the intensity is related to the

amount of analyte in the sample. A sulphuric acid solution is added to stop the enzymatic reaction (changing the colour to yellow) and OD is read at 450nm.

Assay protocol

All the prepared reagents were thoroughly mixed before use. After 5 minutes of reconstituting the IL-2 stock standard with the dilution buffer, serial dilutions were made in seven tubes (540, 270, 135, 67.5, 33.75, 16.88, 0). One hundred microlitre (100µl) of blank, serially diluted standard and samples were added to each well. The plates were sealed and incubated for 2 hours at 37^0C. After incubation the plates were washed six times with the Wash buffer using the automated microplate Washer. Then 100µl of diluted detection antibody solution was added to each well and the plate was sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer as done previously. Hundred microlitres (100µl) of diluted SPP conjugate was added to each well and the plates sealed and incubated for 1 hour at 37^0C. After incubation, the plates were washed six times with Wash buffer. Afterwards, 100µl of TMB substrate solution was added into each well and left in the dark for 20 minutes at room temperature. After substrate incubation, the reaction was stopped by adding 100µl of Stop solution (resulting in a yellow solution) and it was read at 450nm within 30 minutes using the Microplate reader**.**

(e) Transforming Growth Factor-beta (TGF-β)

The human Transforming Growth Factor Beta (TGF-β) ELISA kit from Elabscience (Cat No: E-EL-H0110; Lot No 4EPGSN1RPZ) is used for the in vitro quantitative determination of human TGF-β concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human TGF-β. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human TGF-β and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human TGF-β, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured

spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human TGF-β.

Assay protocol

All the prepared reagents were mixed thoroughly before use without making any foam. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

(f) Human P-selectin

The human P-Selectin ELISA kit from Elabscience (Cat No E-EL-H0917; Lot No BQ3P4IS3ET) is used for the in vitro quantitative determination of human P-selectin concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human P-selectin. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human P-selectin and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human P-selectin, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the

addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human P-selectin.

Assay protocol

All the prepared reagents were mixed thoroughly before use without making any foam. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

(g) Human Platelet Glycoprotein IIb/IIIa (GP IIb/IIIa) complex

The human Platelet GlycoProtein IIb/IIIa complex (GP IIb/IIIa) ELISA kit from Elabscience (Cat No E-EL-H2202; Lot No GTXKBVIBPF) is used for the in vitro quantitative determination of human GP IIb/IIIa concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human GP IIb/IIIa. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human GP IIb/IIIa and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well.

Only those wells that contain human GP IIb/IIIa, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human GP IIa/IIIb.

Assay protocol

All the prepared reagents were mixed thoroughly before use without making any foam. After 10 minutes of reconstituting the human GP IIb/IIIa complex Reference standard producing a working solution of 10ng/ml, serial dilutions of the standard was made to produce dilution gradients of 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0 ng/ml. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

(h) Human Thrombopoietin (TPO)

The human Thrombopoietin ELISA kit from Elabscience (Cat No E-EL-H1588; Lot No VIXQCKUM75) is used for the in vitro quantitative determination of human TPO concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human TPO. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human TPO and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human TPO, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human TPO.

Assay protocol

All the prepared reagents were mixed thoroughly before use without making any foam. After 10 minutes of reconstituting the human Thrombopoietin Reference standard producing a working solution of 2000pg/ml, serial dilutions of the standard was made to produce dilution gradients of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/ml. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

(I) Platelet Factor-4 (PF-4)

The human Platelet Factor-4 (PF-4) ELISA kit from Elabscience (Cat No E-EL-H2216; Lot No EBSSJNW81H) is used for the in vitro quantitative determination of human PF-4 concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human PF-4. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human PF-4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human PF-4, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human PF-4.

Assay protocol

After 10 minutes of reconstituting the human PF-4 Reference standard producing a working solution of 100ng/ml, serial dilutions of the standard was made to produce dilution gradients of 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 ng/ml. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected

from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

(j) Platelet Activating Factor (PAF)

The human Platelet Activating Factor (PAF) ELISA kit from Elabscience (Cat No E-EL-H2199; Lot No ZLSLJXKVIS) is used for the in vitro quantitative determination of human PAF concentrations in serum, plasma and other biological fluids.

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to human PAF. When the standards or samples are added to the micro ELISA plate wells, they combine with the specific antibody. Then a biotinylated detection antibody specific for human PAF and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain human PAF, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of stop solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of human PAF.

Assay protocol

After 10 minutes of reconstituting the human PAF Reference standard producing a working solution of 5000pg/ml, serial dilutions of the standard was made to produce dilution gradients of 5000, 2500, 1250, 625, 312.5, 156.25, 78.13 and 0 pg/ml. One hundred microlitre (100µl) of Blank, Standard working solution and Samples were added to the bottom of the micro ELISA plate wells without touching the inside wall and avoiding foaming as much as possible. It was covered with the Sealer provided in the kit and incubated for 90 minutes at 37^0C. After incubation, the liquid was removed without washing. Then 100µl of Biotinylated Detection antibody working solution was immediately added to each well, covered with the Plate sealer, gently mixed and incubated for 1 hour at 37^0C. After incubation the plate was washed three times with 350µl of wash buffer and soak time of 2 minutes using a Microplate washer. Hundred microlitres (100µl) of HRP Conjugate working solution was added to each

well, covered with the Plate sealer and incubated for 30 minutes at 37^0C. The plate was washed five times as previously described. Afterwards 90µl of Substrate Reagent was added to each well, covered with a new plate sealer and incubated for 15 minutes at 37^0C well protected from light. Then 50µl of Stop Solution was added to each well in the same order as the substrate solution. The optical density (OD value) of each well was determined at once using a micro-plate reader set at 450nm.

3.14. Statistical methods

The Statistical Package for Social Sciences (IBM SPSS Inc, Illinois, USA) version 22 was used in the statistical analysis. In order to decide whether to use parametric tests (for normally distributed data) or non-parametric tests (for skewed or non-normally distributed data) to calculate significance and correlation, a normality test was conducted to assess the distribution of each variable using Kolmogorov-Smirnov statistic ($P>0.05$ indicates normality) as well as examining the histogram generated to check the shape of the distribution of the data. Data were expressed as Mean ± Standard deviation (Mean ± SD) for parametric tests, Median (Range) for non-parametric tests, frequencies and percentages in tables, Bar chart and Pie chart. For normally distributed variables, comparison of multiple repeated measurements (at Treatment naive, 2-months and 6-months) was carried out using One-way repeated-measures ANOVA with a Greenhouse-Geisser correction and a post-hoc test using the Bonferroni correction for significant comparisons. One-way between-groups ANOVA with Tukey's post-hoc test for significant comparison were used for comparison among age groups for each level of measurement (Treatment naive, 2-months and 6-months follow-up). Independent-samples t-test was used for comparison between groups (Gender and Malaria parasite infection) for each level of measurement and Pearson's product-moment correlation was used to test for relationship between variables. For comparisons involving skewed variables, Friedman ANOVA and post-hoc analysis with Wilcoxon Signed Rank test was applied for comparison of multiple repeated measurements (at Treatment naive, 2-months and 6-months follow-up). Kruskal-Wallis test with Dunns post-test was used for comparison among age groups for each level of measurement (Treatment naive, 2-months and 6-months follow-up). Mann-Whitney test was used for comparison between groups (Gender and Malaria parasite) for each level of measurement and Spearman Rank Order correlation was used to test relationship between variables. Generally, $P < 0.05$ were considered statistically significant, but for correlation analysis, * indicates significance at $P<0.05$ and ** indicates significance at $P<0.001$.

CHAPTER FOUR

RESULTS

4.1 Socio-demographic Variables of Participants

Majority of the participants were males (58.3%), aged 18-30 years (41.7%) and married (60%) (Table 4.1). The total mean age of the participants was 37.53 ± 15.65 years with an age-range of 18-80 years. The mean age for males was 38.23 ± 16.17 years while that of females was 35.76 ± 14.59 years. Also most of the participants were traders (42%), farmers (24%) and students (21%) (Figure 4.1). Moreover about 63% of the participants had secondary education while only a few (6%) had no educational qualification (Figure 4.2). The systolic and diastolic blood pressures were within the normal range at pre-treatment, 2-month and 6-month into treatment (Figure 4.3).

4.2. Pre-treatment (baseline) values of BMI and haematological parameters

Table 4.2 shows the baseline values of BMI and other haematological parameters in TB-infected individuals before the initiation of treatment.

4.3. White blood Cell Parameters of TB Subjects at pre-treatment, 2-month and 6-month into treatment

The Mean ± SD total white blood cell counts ($x10^9/l$) decreased significantly at 2-month and 6-month into treatment compared with pre-treatment value ($P<0.001$) and significantly increased at 6-month into treatment compared to 2-month into treatment value ($P=0.006$). Similarly, there was a significant decrease in Absolute Neutrophils count ($x10^9/l$) (NEUT) at 2-month into treatment and 6-month into treatment compared to pre-treatment value ($P<0.001$) and a slight but significance increase at 6-month compared to the value at 2-month into treatment ($P=0.007$). Also the mean absolute lymphocyte count (LYM) decreased significantly at 2-month (2.39 ± 0.85) and 6-month into treatment (2.68 ± 1.07) when compared to pre-treatment value (3.73 ± 1.90) ($P<0.001$). Moreover, there was a significant decrease in absolute Monocyte count ($x10^9/l$) at 2-month (0.17 ± 0.09) and 6-month into treatment (0.20 ± 0.14) compared to pre-treatment value (0.38 ± 0.28) ($P<0.001$). The Absolute eosinophil count ($x10^9/l$) also significantly decrease at 2-month into treatment (0.03 ± 0.01) compared with pre-treatment value (0.07 ± 0.03), as well as a significant increase at 6-month into treatment compared to 2-month into treatment ($P=0.011$) (See table 4.3).

Table 4.1: Some Socio-demographic variables of the subjects.

PARAMETER		Frequency	Percentage (%)
GENDER	Male	35	58.3
	Female	25	41.7
AGE GROUP	18 – 30 years	25	41.7
	31 – 50 years	21	35
	>50 years	14	23.3
MARITAL STATUS	Married	36	60
	Single	22	36.7
	Divorced	2	3.3

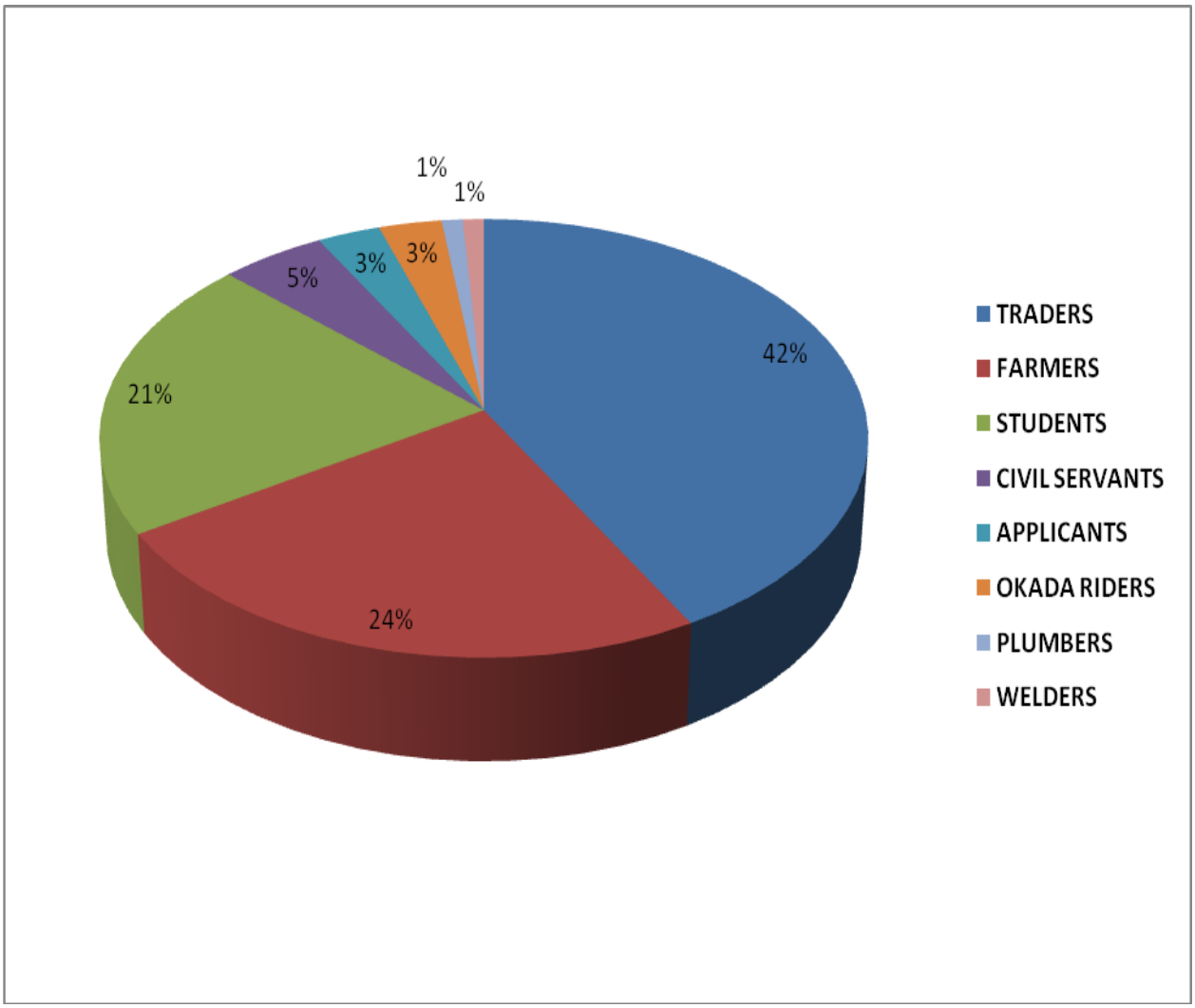

Figure 4.1: A pie chart showing the occupational distribution of the subjects.

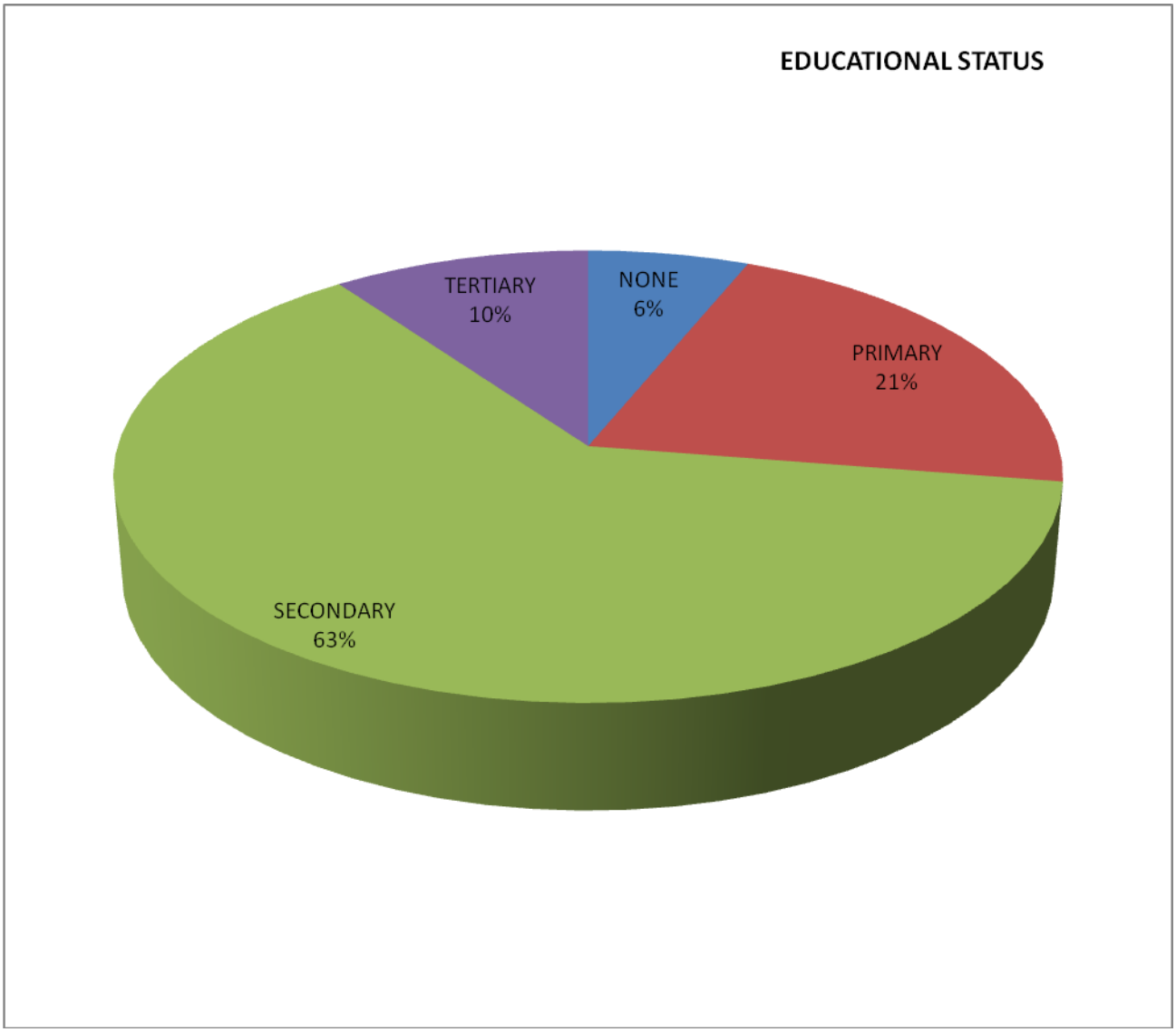

Figure 4.2: A pie chart showing the educational status of the subjects

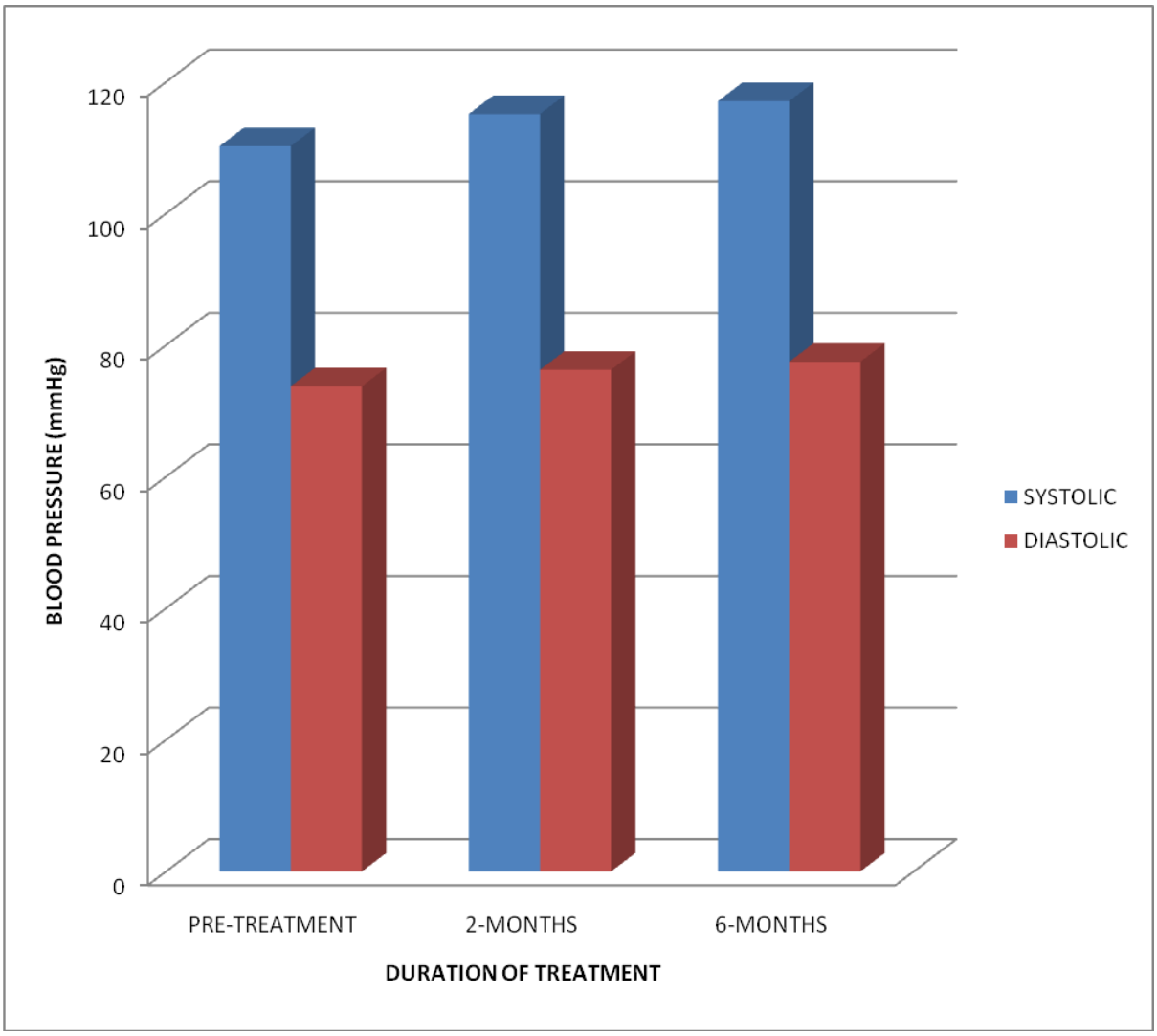

Figure 4.3: A bar chart showing the blood pressure values (mm/Hg) at pre-treatment, 2-month and 6-month into treatment.

Table 4.2: Pre-treatment (baseline) values (Mean ± SD) of BMI and haematological parameters in the TB-infected subjects.

Parameters	Mean ± SD
TWBC ($x10^9$/l)	10.72 ± 6.13
NEUT ($x10^9$/l)	6.24 ± 3.94
LYM ($x10^9$/l)	4.02 ± 2.81
MONO ($x10^9$/l)	0.43 ± 0.32
EOS ($x10^9$/l)	0.08 ± 0.03
PCV (l/l)	0.33 ± 0.06
BMI (kg/m^2)	19.81 ± 2.63
NLR	2.00 ± 1.12
MLR	0.18 ± 0.10
PLT ($x10^9$/l)	212.96 ± 105.20

TWBC = Total white blood cell count

NEUT = Absolute neutrophils count

LYM = Absolute Lymphocytes count

MONO = Absolute monocytes count

EOS = Absolute eosinophils count

PCV = Packed cell volume

BMI = Body mass index

NLR = Neutrophil lymphocyte ratio

MLR = Monocyte lymphocyte ratio

PLT = Total platelet count

Table 4.3: Values of white cell parameters at pre-treatment, 2-month and 6-month into treatment (Mean ± SD).

DURATIONS	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
(1) Pre-treatment (n=60)	10.27 ± 4.94	6.11 ± 3.62	3.73 ± 1.90	0.38 ± 0.28	0.07 ± 0.03
(2) 2-month (n=60)	5.41 ± 1.61	2.87 ± 0.97	2.39 ± 0.85	0.17 ± 0.09	0.03 ± 0.01
(3) 6-month (n=60)	6.59 ± 2.61	3.54 ± 1.54	2.68 ± 1.07	0.20 ± 0.14	0.06 ± 0.02
F value	41.46	39.63	18.513	25.74	6.861
P-value	<0.001*	<0.001*	<0.001*	<0.001*	0.002*
1 vs 2: *P*-value	<0.001*	<0.001*	<0.001*	<0.001*	0.001*
1 vs 3: *P*-value	<0.001*	<0.001*	<0.001*	<0.001*	1000
2 vs 3: *P*-value	0.006*	0.007*	0.255	0.643	0.011*

*$P<0.05$ = Significant

TWBC = Total white blood cell count

NEUT = Absolute neutrophils count

LYM = Absolute Lymphocytes count

MONO = Absolute monocytes count

EOS = Absolute eosinophils count

1 vs 2 = Comparison of parameters at pre-treatment and 2-month into treatment.

1 vs 3 = Comparison of parameters at pre-treatment and 6-month into treatment.

2 vs 3 = Comparison of parameters at 2-months and 6-month into treatment.

4.4. BMI and Other Haematological Parameters of TB subjects at pre-treatment, 2-month and 6-month into treatment

There was a significant increase in Packed Cell Volume (PCV) (l/l) at 2-month into treatment (0.36 ± 0.05) compared to pre-treatment value (0.33 ± 0.05) (P=0.043) and a significant decrease at 6-month into treatment (0.31 ± 0.06) compared to 2-month into treatment (P<0.001). Similarly, there was a significant increase in BMI at 2-month into treatment compared to pre-treatment value (P<0.001), as well as a significant increase at 6-month into treatment compared to 2-month and pre-treatment (P<0.001). The Mean ± SD of Neutrophil-Lymphocyte ratio (NLR) decreased significantly after 2-month (1.26 ± 0.33) and 6-month into treatment (1.32 ± 0.41) compared to pre-treatment value (1.67 ± 0.79) (P=0.001 and 0.007). Moreover, there was a significant decrease in Monocyte Lymphocyte ratio (MLR) at 2-month into treatment and 6-month into treatment compared to pre-treatment (P=0.011 and 0.045), but no significant change at 6-month compared to 2-month into treatment value (P>0.05). Conversely, the Mean ± SD of total platelet count ($x10^9$/l) increased significantly at 2-month into treatment (281.92 ± 93.22) compared to pre-treatment (209.02 ± 103.55) (P<0.001) and there was a further significant increase at 6-month (340.69 ± 71.00) compared to values at 2-month into treatment and pre-treatment (P<0.001) (See table 4.4)

4.5. Pre-treatment (baseline) values of inflammatory and haemostatic parameters

Table 4.5 shows the baseline values of inflammatory and haemostatic parameters in TB-infected individuals before the initiation of treatment.

Table 4.4: Values of BMI and other haematological parameters at pre-treatment, 2-month and 6-month into treatment (Mean ± SD).

DURATIONS	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT ($x10^9$/l)
(1) Pre-treatment (n=60)	0.33 ± 0.05	19.81 ± 2.63	1.67 ± 0.79	0.11 ± 0.08	209.02 ± 103.55
(2) 2-month (n=60)	0.36 ± 0.05	20.72 ± 2.82	1.26 ± 0.33	0.08 ± 0.04	281.92 ± 93.22
(3) 6-month (n=60)	0.31 ± 0.06	21.72 ± 2.85	1.32 ± 0.41	0.08 ± 0.06	340.69 ± 71.00
F value	8.128	151.09	11.27	4.78	34.50
P-value	<0.001*	<0.001*	<0.001*	0.013*	<0.001*
1 vs 2: *P*-value	0.043*	<0.001*	0.001*	0.011*	<0.001*
1 vs 3: *P*-value	0.146	<0.001*	0.007*	0.045*	<0.001*
2 vs 3: *P*-value	<0.001*	<0.001*	0.802	1000	<0.001*

*$P<0.05$ = Significant

PCV = Packed cell volume

BMI = Body mass index

NLR = Neutrophil lymphocyte ratio

MLR = Monocyte lymphocyte ratio

PLT = Total platelet count

1 vs 2 = Comparison of parameters at pre-treatment and 2-month into treatment.

1 vs 3 = Comparison of parameters at pre-treatment and 6-month into treatment.

2 vs 3 = Comparison of parameters at 2-month and 6-month into treatment.

Table 4.5: Pre-treatment (baseline) values of inflammatory and haemostatic parameters in the TB-infected individuals (Median (range)

Parameters	Median	Range
TNF-A (pg/ml)	1.55	1.55 – 38.99
IL-6 (pg/ml)	66.12	4.08 - 267.65
IL-10 (pg/ml)	1.46	1.46 – 10.39
TGF-B (pg/ml)	23.83	23.83 – 148.83
IL-2 (pg/ml)	11.36	11.36 – 15.36
PF4 (ng/ml)	0.37	0.35 – 3.32
PSEL (ng/ml)	22.12	20.13– 23.46
GP IIb/IIIa (ng/ml)	4.87	0.36 – 14.20
PAF (pg/ml)	3156.98	654.42 – 8963.97
TPO (pg/ml)	3603.03	2898.14 – 5112.56

TNF-α = Tumor Necrosis Factor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

TGF-β = Transforming growth factor beta

IL-2 = Interleukin-2

PF-4 = Platelet factor 4

P-SEL = P-Selectin

GP IIb/IIIa = Human platelet glycoprotein IIb/IIIa

PAF = Platelet Activating Factor

TPO = Thrombopoietin hormone

4.6. Inflammatory Cytokines of TB Subjects at pre-treatment, 2-month and 6 month into treatment.

The median value of TNF-α (pg/ml) was significantly increased at 2-month (86.54) compared to the pre-treatment value (1.55) (P<0.001). However, there was a significant decline at 6-month into treatment compared to the value at 2-month into treatment (P=0.025). Similarly, the level of IL-6 (pg/ml) significantly increased at 2-month into treatment (172.90) compared to the level at pre-treatment (66.12) (P<0.001), while the level declined at 6-month into treatment (74.06) compared to the value at 2-month (P=0.006). Furthermore, there was no significant change in the median values for IL-10 (pg/ml) and TGF-β (pg/ml) at 2-month into treatment compared to the level at pre-treatment (P>0.05). However, there was a significant increase at 6-month into treatment compared to the level at pre-treatment and 2-month into treatment (P=0.020 and 0.001). Also, there was a significant increase in IL-2 (pg/ml) at 2-month into treatment compared to the pre-treatment level (P<0.001) and a significant decline at 6-month into treatment compared to the the level at 2-month into treatment (P<0.001) (See table 4.6).

4.7. Haemostatic Parameters of TB Subjects at pre-treatment, 2-month and 6-month into treatment.

There was no significant change in the median values of PF-4 (ng/ml) at 2-month into treatment (0.38) compared to pre-treatment (0.37) (P>0.05), but there was a significant decline at 6-month into treatment (0.35) compared to 2-month into treatment and pre-treatment (P=0.030). Also, the median values of PSEL (ng/ml) increased significantly at 2-month into treatment compared to pre-treatment (P=0.004) and a significant decline below the pre-treatment value at 6-month into treatment compared to 2-month into treatment (P<0.001). Moreover, there was a significant increase in GP IIb/IIIa (ng/ml) at 2-month into treatment (6.63) compared with pre-treatment (4.87) (P=0.037) and a significant decline at 6-month into treatment (3.47) compared to the value at 2-month into treatment (P=0.007). There was no significant change in the median values of PAF (pg/ml) at 2-month into treatment and 6-month into treatment compared to the pre-treatment values (P>0.05). Furthermore, there was a significant increase in the median values of TPO (pg/ml) at 2-month into treatment compared to pre-treatment (P=0.017). But no significant change at 6-month into treatment compared to 2-month into treatment (P>0.05) (See table 4.7).

Table 4.6: Comparison of median (range) values of inflammatory markers at pre-treatment, 2-months and 6-months follow up treatment.

DURATION	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-B (pg/ml)	IL-2 (pg/ml)
(1) Pre-treatment (n=60)	1.55 (1.55 –38.99)	66.12 (4.08-267.65)	1.46 (1.46-10.39)	23.83 (23.83-148.83)	11.36 (11.36-15.36)
(2) 2-months (n=60)	86.54 (1.23-934.95)	172.90 (4.08-259.47)	2.77 (1.46-9.20)	23.83 (23.83-273.83)	16.98 (11.36-467.48)
(3) 6-months (n=60)	18.51 (1.55-478.03)	74.06 (4.08-247.53)	4.46 (1.46-20.24)	173.83 (23.83-5198.83)	12.55 (11.36-36.81)
X^2 value	35.854	30.79	8.158	25.34	28.99
P-value	0.000*	0.000*	0.017*	0.000*	0.000*
1 vs 2 *P*-value	0.000*	0.000*	0.817(ns)	0.182(ns)	0.000*
1 vs 3 *P*-value	0.000*	0.020*	0.002*	0.000*	0.030*
2 vs 3 *P*-value	0.025*	0.006*	0.020*	0.001*	0.000*

*$P<0.05$ is significant

ns = not significant (P>0.05)

TNF-α = Tumor Necrosis Factor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

TGF-β = Transforming growth factor beta

IL-2 = Interleukin-2

1 vs 2 = Comparison of parameters at pre-treatment and 2-months follow up treatment.

1 vs 3 = Comparison of parameters at pre-treatment and 6-months follow up treatment.

2 vs 3 = Comparison of parameters at 2-months and 6-months follow up treatment.

Table 4.7: Comparison of median (range) values of haemostatic parameters at pre-treatment, 2-months and 6-months follow up treatment.

DURATION	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
(1) Pre-treatment (n=60)	0.37 (0.35-3.32)	22.12 (20.13-23.46)	4.87 (0.36-14.20)	3156.98 (654.4-8963.97)	3603.03(2898-5112.56)
(2) 2-months (n=60)	0.38 (0.35-6.29)	23.22 (16.17-24.62)	6.63 (0.22-16.04)	2895.91 (251.6-7557.91)	3966.04(2957-4754.31)
(3) 6-months (n=60)	0.35 (0.35-2.41)	21.94 (16.86-25.06)	3.47 (0.97-11.55)	2351.39 (665.6-5499.17)	3899 (2661-5640.82)
X^2 value	6.379	9.138	8.138	0.470	9.270
P-value	0.044*	0.015*	0.035*	0.791(ns)	0.010*
1 vs 2: *P*-value	0.062(ns)	0.004*	0.037*	0.581(ns)	0.017*
1 vs 3: *P*-value	0.041*	0.114(ns)	0.094(ns)	0.098(ns)	0.020*
2 vs 3: *P*-value	0.030*	0.001*	0.007*	0.245(ns)	0.478(ns)

*$P<0.05$ = Significant

ns = not significant (P>0.05)

PF-4 = Platelet factor 4

P-SEL = P-Selectin

GP IIb/IIIa = Human platelet glycoprotein IIb/IIIa

PAF = Platelet Activating Factor

TPO = Thrombopoietin hormone

1 vs 2 = Comparison of parameters at Pre-treatment and 2-months follow up treatment.

1 vs 3 = Comparison of parameters at Pre-treatment and 6-months follow up treatment.

2 vs 3 = Comparison of parameters at 2-months and 6-months follow up treatment.

4.8. Correlation of Haemostatic and Inflammatory Parameters.

There was a weak significant positive correlation between TNF-α and PF4 (r=0.295; P=0.045) and a moderate significant positive correlation between TGF-β and PF4 (r=0.463; P=0.006). Also, there was a weak significant positive correlation between IL-6 and P-selectin (r=0.413; P=0.027) as well as between IL-6 and TPO (r=0.335; P=0.046). Conversely, there was a weak significant negative correlation between TGF-β and PAF (r = -0.368; P=0.032) (See table 4.8).

4.9. Pre-treatment (baseline) values of inflammatory and haemostatic parameters in the TB-infected individuals with and without malaria

Table 4.9 shows the baseline values of inflammatory and haemostatic parameters in TB-infected individuals with and without malaria before the initiation of treatment.

4.10. Inflammatory Cytokines in TB Subjects with (TB/MP$^+$) and without Malaria (TB/MP-) at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment the median values of IL-6 (pg/ml) was significantly lower in TB/MP+ subjects (61.02) compared to TB/MP- (90.47) (P=0.045). Similarly, the median IL-10 (pg/ml) at pre-treatment was significantly lower in TB/MP+ (1.87) compared to TB/MP- (4.71) (P=0.039). At 2-month into treatment, the median value of TNF-α (pg/ml), IL-2 (pg/ml) and IL-6 (pg/ml) was significantly higher in TB/MP+ compared to TB/MP- (P=0.044, 0.045 and 0.027). Conversely, the median IL-10 (pg/ml) was significantly lower in TB/MP+ (3.96) compared with TB/MP- (5.37) (P<0.044). However, there was no significant difference in the median TGF-β (pg/ml) between TB/MP+ and TB/MP- (P>0.05). At 6-month into treatment, the median TNF-α (pg/ml), IL-6 (pg/ml) and TGF-β (pg/ml) was significantly lower in TB/MP+ compared with TB/MP- (P = 0.047, 0.046 and 0.023). However, no significant difference was observed in the median values of IL-10 (pg/ml) and IL-2 (pg/ml) between TB/MP+ and TB/MP- (P>0.05) (See table 4.10).

4.11. Haemostatic Parameters in TB Subjects with (TB/MP+) and without Malaria (TB/MP-) at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment, the median P-Selectin (ng/ml), GP IIb/IIIa (ng/ml), PAF (pg/ml), was significantly higher in TB/MP+ compared with TB/MP- (P=0.045, 0.025 and 0.044). However, no significant difference was observed in the median PF-4 (ng/ml) and TPO (pg/ml) when compared between TB/MP+ and TB/MP- (P>0.05). At 2-month into treatment,

the median PAF (pg/ml) was significantly lower in TB/MP+ (2784.02) compared with TB/MP- (4163.98) (P=0.030). At 6-month into treatment, the median PF-4 (ng/ml), GP IIb/IIIa (ng/ml) and TPO (pg/ml) was significantly lower in TB/MP+ compared with TB/MP- (P=0.024, 0.047, 0.044). Conversely, the median P-Selectin (ng/ml) was significantly higher in TB/MP+ (22.01) compared with TB/MP- (20.24) (P=0.048) (See table 4.11).

4.12. Pre-treatment (baseline) values of BMI and haematological parameters in the TB-subjects with and without malaria (Mean ± SD)

Table 4.12 shows the baseline values of BMI and haematological parameters in TB-infected individuals with and without malaria before the initiation of treatment.

4.13. White Blood Cell Parameters in TB Subjects with (TB/MP+) and without Malaria (TB/MP-) at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment, the mean total white cell count ($x10^9$/l), mean absolute neutrophils count ($x10^9$/l) and absolute lymphocyte count ($x10^9$/l) was significantly lower in TB/MP+ compared with TB/MP- (P<0.05). Additionally, the mean eosinophil count ($x10^9$/l) was significantly higher in TB/MP+ (0.09 ± 0.10) compared with TB/MP- (0.06 ± 0.11) (P=0.043). At 2-month into treatment, there was no significant difference in the mean values of white cell parameters between TB/MP+ and TB/MP- (P>0.05). At 6-month into treatment, the mean eosinophil count ($x10^9$/l) was significantly higher in TB/MP+ (0.07 ± 0.07) compared with TB/MP- (0.04 ± 0.06) (P=0.045) (See table 4.13).

4.14. BMI and Other Haematological Parameters in TB Subjects with (TB/MP+) and without Malaria (TB/MP-) at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment, the mean value of Neutrophil-lymphocyte ratio and mean platelet count ($x10^9$/l) was significantly lower in TB/MP+ compared to TB/MP- (P=0.036 and 0.026). At 2-month into treatment, there was no significant difference in the mean values of BMI and other haematological parameters when compared between TB/MP+ and TB/MP- (P>0.05). At 6-month into treatment, the mean PCV (l/l) was significantly lower in TB/MP+ (0.30 ± 0.06) compared with TB/MP- (0.33 ± 0.05) (P<0.046) (See table 4.14).

Table 4.8: Correlation of haemostatic and inflammatory parameters in TB subjects.

Parameters	Correlation (rho) value	*P*-value
TNFA vs PF4	0.295	0.045*
TNFA vs P-SEL	0.169	0.380
TNFA vs GP IIb/IIIa	0.372	0.031*
TNFA vs PAF	0.219	0.172
TNFA vs TPO	0.101	0.604
TNFA vs PLT	0.116	0.390
IL6 vs PF4	0.025	0.897
IL6 vs P-SEL	0.413	0.027*
IL6 vs GP IIa/IIIa	0.016	0.936
IL6 vs PAF	0.092	0.635
IL6 vs TPO	0.335	0.046*
IL6 vs PLT	0.189	0.159
IL10 vs PF4	-0.223	0.244
IL10 vs P-SEL	-0.256	0.181
IL10 vs GP IIa/IIIb	0.033	0.864
IL10 vs PAF	0.138	0.475
IL10 vs TPO	-0.168	0.384
IL10 vs PLT	0.098	0.470
PF4 vs TGFB	0.463	0.006*
TGFB vs P-SEL	0.025	0.888
TGFB vs GP IIa/IIIb	-0.302	0.083
TGFB vs PAF	-0.368	0.032*
TGFB vs TPO	0.043	0.809
TGFB vs PLT	-0.140	0.485
IL2 vs PF4	-0.202	0.293
IL2 vs P-SEL	-0.086	0.659
IL2 vs GP IIa/IIIb	0.089	0.645
IL2 vs PAF	0.216	0.261
IL2 vs TPO	0.073	0.706
IL2 vs PLT	0.074	0.586

P<0.05 = Significant.

Table 4.9: Pre-treatment (baseline) values of inflammatory and haemostatic parameters in the TB-infected individuals with and without malaria (Median (range)

Parameters	TB/MP$^+$ (n=25)	TB/MP$^-$ (n=35)
TNF-A (pg/ml)	1.55 (1.55 – 38.03)	3.63 (1.55 – 38.99)
IL-6 (pg/ml)	61.02 (5.60 – 228.37)	90.47 (16.02 – 267.65)
IL-10 (pg/ml)	1.87 (1.46 – 10.39)	4.71 (1.46 – 10.39)
TGF-B (pg/ml)	23.83 (23.83 – 148.83)	23.83 (23.83 – 148.83)
IL-2 (pg/ml)	11.36 (11.36 – 15.36)	11.36 (11.36 – 14.36)
PF4 (ng/ml)	0.35 (0.35 – 2.41)	0.35 (0.35 – 2.41)
PSEL (ng/ml)	23.83 (20.13 – 23.46)	21.90 (20.13 – 23.46)
GP IIb/IIIa (ng/ml)	6.66 (1.66 – 14.20)	3.06 (0.36 – 11.02)
PAF (pg/ml)	4637.64 (1821.79 – 8963.97)	3350.92 (654.42 – 5965.37)
TPO (pg/ml)	3906.26 (2661.50 – 5191.50)	3716.00 (2661.50 – 5640.82)

TNF-α = Tumor Necrosis Factor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

TGF-β = Transforming growth factor beta

IL-2 = Interleukin-2

PF-4 = Platelet factor 4

P-SEL = P-Selectin

GP IIb/IIIa = Human platelet glycoprotein IIb/IIIa

PAF = Platelet Activating Factor

TPO = Thrombopoietin hormone

Table 4.10: Comparison of inflammatory markers between TB subjects with and without malaria infection at pre-treatment, 2-months and 6-months follow up treatment.

DURATION	STATUS	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-B (pg/ml)	IL-2 (pg/ml)
Pre-treatment	TB/MP+ (n=25)	1.55 (1.55-38.03)	61.02 (5.60-228.37)	1.87 (1.46-10.39)	23.83 (23.83-148.83)	11.36 (11.36-15.36)
	TB/MP- (n=35)	3.63 (1.55-38.99)	90.47 (16.02-267.65)	4.71 (1.46-10.39)	23.83 (23.8-148.8)	11.36 (11.36-14.36)
	z-value	-0.687	-1.929	-2.015	-0.089	-0.345
	p-value	0.492(ns)	0.045*	0.039*	0.929 (ns)	0.730 (ns)
2-Months	TB/MP+ (n=24)	208.91 (1.55-934.95)	180.51 (8.89-248.25)	3.96 (1.46-20.24)	23.83 (23.83-273.83)	34.19 (11.36-467.48)
	TB/MP- (n=36)	49.55 (1.23-934.95)	109.69 (4.08-259.47)	5.37 (1.46-20.24)	23.83 (23.83-273.83)	11.36 (11.36-467.48)
	z-value	-1.989	-2.211	-1.992	-1.293	-1.923
	p-value	0.044*	0.027*	0.044*	0.196(ns)	0.045*
6-Months	TB/MP+ (n=33)	20.59 (1.55-478.03)	83.10 (4.08-247.53)	2.99 (1.46-9.20)	98.83 (23.83-5198.83)	11.36 (11.36-15.42)
	TB/MP- (n=27)	60.91 (1.55-478.03)	135.28 (4.08-237.27)	1.65 (1.46-9.20)	986.33 (23.83-5198.83)	11.36 (11.36-15.42)
	z-value	-1.902	-1.919	-0.500	-2.352	-0.752
	p-value	0.047*	0.046*	0.617(ns)	0.023*	0.452(ns)

*$P<0.05$ = Significant

ns = not significant ($P>0.05$)

TB/MP+ = Tuberculosis and Malaria parasite positive subjects

TB/MP- = Tuberculosis and Malaria parasite negative subjects

Table 4.11: Comparison of haemostatic parameters between TB subjects with and without malaria infection at pre-treatment, 2-months and 6-months follow up treatment.

DURATION	STATUS	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
Pre-treatment	TB/MP+ (n=25)	0.35 (0.35-2.41)	23.83 (20.13-23.46)	6.66 (1.66-14.20)	4637.64 (1821.79-8963.97)	3906.26 (2661.50-5191.50)
	TB/MP- (n=35)	0.35 (0.35-2.41)	21.90 (20.13-23.46)	3.06 (0.36-11.02)	3350.92 (654.42-5965.37)	3716.00 (2661.50-5640.82)
	z-value	-0.134	-1.922	-2.244	-1.991	-0.696
	p-value	0.893 (ns)	0.045*	0.025*	0.044*	0.486 (ns)
2-Months	TB/MP+ (n=24)	0.35 (0.35-6.29)	21.86 (16.17-24.62)	5.71 (0.22-15.36)	2784.02 (251.63-7557.91)	3815.18 (2957.00-4626.80)
	TB/MP- (n=36)	0.35 (0.35-6.29)	22.74 (17.25-24.62)	5.99 (0.73-16.04)	4163.98 (654.42-7557.91)	3888.04 (2971.17-4754.31)
	z-value	-0.060	-1.216	-0.121	-2.159	-0.712
	p-value	0.952 (ns)	0.224(ns)	0.903 (ns)	0.030*	0.477(ns)
6-Months	TB/MP+ (n=33)	0.30 (0.35-2.41)	22.01 (16.86-25.06)	3.08 (1.20-9.63)	2613.40 (1139.27-5461.88)	3649.21 (3222.14-4881.82)
	TB/MP- (n=27)	0.35 (0.35-2.41)	20.24 (16.86-25.06)	5.84 (1.25-11.55)	3302.44 (665.61-5499.17)	4008.47 (2898.30-5112.56)
	z-value	-2.239	-1.902	-1.909	-1.195	-1.970
	p-value	0.024*	0.048*	0.047*	0.232(ns)	0.044*

*$P<0.05$ = Significant

ns = not significant (P>0.05)

MP+ = Tuberculosis and Malaria parasite positive subjects

MP- = Tuberculosis and Malaria parasite negative subjects

Table 4.12: Pre-treatment (baseline) values of BMI and haematological parameters in the TB-individuals with and without malaria (Mean ± SD)

Parameters	TB/MP$^+$	TB/MP$^-$
TWBC (x10^9/l)	10.89 ± 5.10	12.52 ± 5.16
NEUT (x10^9/l)	6.29 ± 3.42	7.75 ± 4.28
LYM (x10^9/l)	3.53 ± 1.87	4.46 ± 2.04
MONO (x10^9/l)	0.40 ± 0.31	0.37 ± 0.24
EOS (x10^9/l)	0.09 ± 0.10	0.06 ± 0.11
PCV (l/l)	0.33 ± 0.06	0.33 ± 0.05
BMI (kg/m^2)	20.05 ± 2.83	18.82 ± 1.67
NLR	1.45 ± 0.58	1.85 ± 1.00
MLR	0.10 ± 0.08	0.09 ± 0.06
PLT (x10^9/l)	228.17 ± 116.59	275.14 ± 99.09

TWBC = Total white blood cell count

NEUT = Absolute neutrophils count

LYM = Absolute Lymphocytes count

MONO = Absolute monocytes count

EOS = Absolute eosinophils count

PCV = Packed cell volume

BMI = Body mass index

NLR = Neutrophil lymphocyte ratio

MLR = Monocyte lymphocyte ratio

PLT = Total platelet count

Table 4.13: Comparison of White blood cell parameters between TB subjects with and without malaria infection at pre-treatment, 2-months and 6-months follow up treatment

GROUP	STATUS	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
Pre-treatment	TB/MP+ (n=25)	10.89 ± 5.10	6.29 ± 3.42	3.53 ± 1.87	0.40 ± 0.31	0.09 ± 0.10
	TB/MP- (n=35)	12.52 ± 5.16	7.75 ± 4.28	4.46 ± 2.04	0.37 ± 0.24	0.06 ± 0.11
	t-value	-2.091	-2.350	-1.964	0.486	2.045
	p-value	0.041*	0.022*	0.046*	0.629 (ns)	0.043*
2-Months	TB/MP+ (n=24)	5.43 ± 1.65	2.89 ± 0.97	2.35 ± 0.82	0.16 ± 0.08	0.03 ±0.04
	TB/MP- (n=36)	5.37 ± 1.59	2.85 ± 0.97	2.42 ± 0.87	0.18 ± 0.11	0.03 ± 0.04
	t-value	0.154	0.189	-0.348	-0.623	-0.012
	p-value	0.878 (ns)	0.851 (ns)	0.729 (ns)	0.538 (ns)	0.990 (ns)
6-Months	TB/MP+ (n=33)	6.53 ± 2.52	3.60 ± 1.60	2.56 ± 1.02	0.21 ± 0.14	0.07 ± 0.07
	TB/MP- (n=27)	6.67 ± 2.78	3.46 ± 1.48	2.83 ± 1.12	0.19 ± 0.14	0.04 ± 0.06
	t-value	-0.220	0.383	-1.044	0.556	1.976
	p-value	0.827 (ns)	0.703 (ns)	0.300 (ns)	0.580 (ns)	0.045*

*$P<0.05$ = Significant

ns = not significant (P>0.05)

TB/MP+ = Tuberculosis and Malaria parasite positive subjects

TB/.MP- = Tuberculosis and Malaria parasite negative subjects

Table 4.14: Comparison of BMI and other haematological parameters between TB subjects with and without malaria at pre-treatment, 2-months and 6-months follow up

GROUP	STATUS	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT (x10^9/l)
Pre-treatment	TB/MP+ (n=25)	0.33 ± 0.06	20.05 ± 2.83	1.45 ± 0.58	0.10 ± 0.08	228.17 ± 116.59
	TB/MP- (n=35)	0.33 ± 0.05	18.82 ± 1.67	1.85 ± 1.00	0.09 ± 0.06	275.14 ± 99.09
	t-value	0.078	1.939	-2.133	0.554	-2.230
	p-value	0.938 (ns)	0.059 (ns)	0.036*	0.582 (ns)	0.026*
2-Months	TB/MP+ (n=24)	0.35 ± 0.06	20.61 ± 2.31	1.28 ± 0.36	0.07 ± 0.04	275.71 ± 100.69
	TB/MP- (n=36)	0.34 ± 0.05	20.72 ± 3.25	1.24 ± 0.30	0.08 ± 0.05	278.33 ± 91.83
	t-value	0.708	-0.162	0.442	-0.341	-0.112
	p-value	0.481 (ns)	0.872 (ns)	0.660 (ns)	0.734 (ns)	0.911 (ns)
6-Months	TB/MP+ (n=33)	0.30 ± 0.06	21.73 ± 2.84	1.38 ± 0.46	0.09 ± 0.06	343.61 ± 64.92
	TB/MP- (n=27)	0.33 ± 0.05	21.72 ± 3.13	1.24 ± 0.33	0.08 ± 0.06	336.65 ± 79.82
	t-value	-1.958	0.022	1.433	0.640	0.378
	p-value	0.046*	0.983 (ns)	0.157 (ns)	0.524 (ns)	0.707 (ns)

*$P<0.05$ = Significant

ns = not significant (P>0.05)

TB/MP+ = Tuberculosis and Malaria parasite positive subjects

MP- = Tuberculosis and Malaria parasite negative subjects

4.15. Correlation of Median Parasite Count (MPC) with Haemostatic, Inflammatory and other Parameters

There was a moderate significant positive correlation between MPC and MONO (r=0.629; P<0.001), NEUT (r=0.693; P<0.001) and LYM (r=0.609; P<0.001) respectively and a strong significant positive correlation between MPC and TWBC (r=0.775; P<0.001) as well as a weak significant positive correlation between MPC and EOS (r=0.440; P<0.017). There was a weak positive correlation between MPC and PF4 (r=0.480; P=0.046) and a moderate positive correlation with PSEL (r=0.589; P=0.008) (See table 4.15).

The median (range) parasite count in malaria parasite-infected subjects were 1512 (312-2712) at pre-treatment, 678 (288-1032) at 2-months into treatment and 912 (252-4575) at 6-months into treatment respectively.

4.16. Pre-treatment (baseline) values of inflammatory and haemostatic parameters of male and female TB subjects

Table 4.16 shows the baseline values of inflammatory and haemostatic parameters of male and female TB-infected subjects.

4.17. Inflammatory Cytokines in Male and Female TB Subjects at pre-treatment, 2-month and 6-month into Treatment.

At pre-treatment, the median IL-6 (pg/ml) was significantly higher in male TB subjects (81.59) compared to female TB subjects (55.59) (P=0.046). While at 2-month into treatment, the median TNF-α (pg/ml) was significantly higher in males (100.11) compared with females (62.11) (P=0.008). Similarly, the median IL-10 (pg/ml) was significantly higher in males (5.27) compared with females (3.71) (P=0.045). Conversely, the median IL-6 (pg/ml) was significantly higher in female TB subjects (176.71) compared to the males (136.10) (P=0.042). However, at 6-month into treatment, there was no significant difference in the median values of the inflammatory cytokines when compared between males and female TB subjects (P>0.05) (See table 4.17).

Table 4.15: Correlation of median parasite count (MPC) and other parameters in TB subjects.

PARAMETERS	Correlation (rho) value	p-value
MPC vs EOS	0.440	0.017*
MPC vs MONO	0.629	<0.001**
MPC vs NEUT	0.693	<0.001**
MPC vs LYM	0.609	<0.001**
MPC vs TWBC	0.775	<0.001**
MPC vs PAF	-0.128	0.678
MPC vs PF4	0.480	0.046*
MPC vs GP	-0.159	0.604
MPC vs PSEL	0.589	0.008*
MPC vs TPO	-0.379	0.202
MPC vs IL10	0.252	0.271
MPC vs IL6	-0.187	0.381
MPC vs IL2	0.085	0.693
MPC vs TNFA	-0.336	0.109
MPC vs TGFB	0.235	0.440
MPC vs PLT	-0.599	0.022*

*P<0.05 = Significant

**P<0.001 = Significant

Table 4.16: Pre-treatment (baseline) values of inflammatory and haemostatic parameters of male and female TB subjects (Median (range)

Parameters	Males	Females
TNF-A (pg/ml)	1.55 (1.55 – 38.99)	1.55 (1.55 – 38.03)
IL-6 (pg/ml)	81.59 (4.08 – 267.65)	55.59 (4.08 – 267.65)
IL-10 (pg/ml)	2.09 (1.46 – 10.39)	1.46 (1.46 – 10.39)
TGF-B (pg/ml)	23.83 (23.83 – 148.83)	23.83 (23.83 – 123.83)
IL-2 (pg/ml)	11.36 (11.36 – 15.36)	11.36 (11.36 – 14.36)
PF4 (ng/ml)	0.35 (0.35 – 3.32)	0.35 (0.35 – 3.32)
PSEL (ng/ml)	22.58 (21.07 – 23.46)	21.36 (20.13 – 23.46)
GP IIb/IIIa (ng/ml)	4.23 (0.36 – 14.20)	6.69 (0.54 – 14.20)
PAF (pg/ml)	2914.56 (645.42 – 7464.67)	3697.78 (710.37 – 8963.97)
TPO (pg/ml)	3898.16 (261.50 – 5642)	3904.23 (2661.50 – 528.29)

TNF-α = Tumor Necrosis Factor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

TGF-β = Transforming growth factor beta

IL-2 = Interleukin-2

PF-4 = Platelet factor 4

P-SEL = P-Selectin

GP IIb/IIIa = Human platelet glycoprotein IIb/IIIa

PAF = Platelet Activating Factor

TPO = Thrombopoietin hormone

Table 4.17: Comparison of median values of inflammatory cytokines between male and female TB subjects at pre-treatment, 2-months and 6-months follow up treatment.

GROUP	GENDER	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-B (pg/ml)	IL-2 (pg/ml)
Pre-treatment	MALE (n=35)	1.55 (1.55-38.99)	81.59 (4.08-267.65)	2.09 (1.46-10.39)	23.83(23.83-148.83)	11.36 (11.36-15.36)
	FEMALE (n=25)	1.55 (1.55-38.03)	55.59 (4.08-267.65)	1.46 (1.46-10.39)	23.83(23.83-123.83)	11.36 (11.36-14.36)
	z-value	-1.336	-1.908	-0.970	-1.194	-0.570
	p-value	0.181 (ns)	0.046*	0.332 (ns)	0.232 (ns)	0.569 (ns)
2-Months	MALE (n=35)	100.11 (1.55-928.54)	136.10 (4.08-259.47)	5.27 (1.46-20.24)	23.83(23.83-273.83)	16.98 (11.36-464.27)
	FEMALE (n=25)	62.11 (1.23-934.95)	176.71 (4.08-241.19)	3.71 (1.46-20.24)	23.8323.83-273.83)	12.09 (11.36-467.48)
	z-value	-2.645	-1.072	-1.910	-1.300	-0.399
	p-value	0.008*	0.042*	0.045*	0.194 (ns)	0.690
6-Months	MALE (n=35)	15.31 (1.55-478.03)	74.06 (4.08-247.53)	2.21 (1.46-9.20)	198.83(23.83-5198.83)	11.36 (11.36-36.81)
	FEMALE (n=25)	28.91 (1.55-478.03)	74.30 (4.08-240.39)	2.90 (1.46-9.20)	148.83(23.83-5198.83)	11.36 (11.36-15.42)
	z-value	-0.131	-0.035	-0.673	-0.925	-0.466
	p-value	0.896 (ns)	0.972 (ns)	0.501 (ns)	0.355	0.641 (ns)

*Significant (P<0.05)

ns = not significant (P>0.05)

4.18. Haemostatic Parameters in Male and Female TB Subjects at pre-treatment, 2-month and 6-month into Treatment.

At pre-treatment, the median P-SEL (ng/ml) was significantly higher in male TB subject (22.58) compared to females (21.36) (P=0.044). However, no significant difference exists in the median values of PF-4 (ng/ml) GP IIb/IIIa (ng/ml), PAF (pg/ml) and TPO (pg/ml) when compared between male and female TB subjects (P>0.05). At 2-month into treatment, the median P-SEL (ng/ml) and TPO (pg/ml) was significantly higher in male TB subjects compared to females (P=0.046 and 0.030). But there was no significant difference in the median values of PF-4 (ng/ml) GP IIb/IIIa (ng/ml) and PAF (pg/ml) when compared between male and female TB subjects (P>0.05). At 6-month into treatment, the median values of PSEL (ng/ml) was significantly higher in male TB subjects (22.01) compared with females (18.27) (P=0.028). But there was no significant difference in the median values of PF-4 (ng/ml), GP IIb/IIIa (ng/ml), PAF (pg/ml) and TPO (pg/ml) when compared between male and female TB subjects (P>0.05) (See table 4.18).

4.19. Pre-treatment (baseline) values of BMI and haematological parameters of male and female TB subjects

Table 4.19 shows the baseline values of BMI and haematological parameters of male and female TB-infected subjects.

4.20. White Blood Cell Parameters in Male and Female TB Subjects at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment, there was no significant difference in the mean values of white blood cell parameters ($x10^9$/l) when compared between male and female TB subjects (P>0.05). But at 2-month into treatment, the mean value of total white blood cell count ($x10^9$/l), mean neutrophil count ($x10^9$/l), mean lymphocyte count ($x10^9$/l), mean monocyte count ($x10^9$/l) and mean eosinophil count ($x10^9$/l) were significantly higher in male TB subjects compared with females (P=0.022, 0.044, 0.029, 0.036 and 0.042). At 6-month into treatment, there was also no significant sex differences in the mean values of white blood cell parameters in TB subjects (P>0.05) (See table 4.20).

Table 4.18: Comparison of haemostatic parameters between male and female TB subjects at pre-treatment, 2-months and 6-months follow up

GROUP	GENDER	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
Pre-treatment	MALE (n=35)	0.35 (0.35-3.32)	22.58 (21.07-23.46)	4.23(0.36-14.20)	2914.56 (645.42-7464.67)	3898.16 (2661.50-5640.82)
	FEMALE (n=25)	0.35 (0.35-3.32)	21.36 (20.13-23.46)	6.69 (0.54-14.20)	3697.78 (710.37-8963.97)	3904.23 (2661.50-5258.29)
	z-value	-1.188	-2.012	-0.442	-0.387	-0.443
	p-value	0.235 (ns)	0.044*	0.659	0.699*	0.658 (ns)
2-Months	MALE (n=35)	0.35 (0.35-6.29)	22.42 (16.17-24.62)	6.10 (0.73-15.36)	2854.89 (654.42-7557.91)	3981.14 (2971.17-4754.31)
	FEMALE (n=25)	0.35 (0.35-6.29)	21.55 (16.17-24.62)	6.12 (0.22-16.04)	3300.58 (251.63-5495.44)	3696.77(2957.00-4549.89)
	z-value	-1.711	-1.911	-0.117	-0.293	-2.168
	p-value	0.087(ns)	0.046*	0.907 (ns)	0.770 (ns)	0.030*
6-Months	MALE (n=35)	0.35 (0.35-2.41)	22.01 (16.86-25.06)	3.53 (0.97-11.55)	2567.71(665.61-5461.88)	3803.03 (2898.30-5112.56)
	FEMALE (n=25)	0.35 (0.35-2.41)	18.27 (16.86-24.74)	3.02 (1.20-7.91)	2260.02 (1139.27-5499.17)	3765.59 (3019.74-5005.29)
	z-value	-0.408	-2.204	-0.933	-1.091	-0.695
	p-value	0.683 (ns)	0.028*	0.351 (ns)	0.275 (ns)	0.487 (ns)

*$P<0.05$ = Significant

ns=not significant (P>0.05)

Table 4.19: Pre-treatment (baseline) values of BMI and haematological parameters of male and female TB subjects (Mean ± SD)

Parameters	Males	Females
TWBC ($x10^9$/l)	10.26 ± 5.24	10.39 ± 4.24
NEUT ($x10^9$/l)	6.32 ± 3.80	5.74 ± 3.19
LYM ($x10^9$/l)	3.53 ± 1.94	4.17 ± 1.74
MONO ($x10^9$/l)	0.37 ± 0.28	0.41 ± 0.29
EOS ($x10^9$/l)	0.08 ± 0.10	0.07 ± 0.09
PCV (l/l)	0.34 ± 0.05	0.31 ± 0.05
BMI (kg/m^2)	19.92 ± 2.49	19.55 ± 2.95
NLR	1.78 ± 0.82	1.35 ± 0.67
MLR	0.11 ± 0.08	0.10 ± 0.08
PLT ($x10^9$/l)	236.75 ± 110.17	202.82 ± 89.41

TWBC = Total white blood cell count

NEUT = Absolute neutrophils count

LYM = Absolute Lymphocytes count

MONO = Absolute monocytes count

EOS = Absolute eosinophils count

PCV = Packed cell volume

BMI = Body mass index

NLR = Neutrophil lymphocyte ratio

MLR = Monocyte lymphocyte ratio

PLT = Total platelet count

Table 4.20: Comparison of White blood cell parameters between male and female TB subjects at pre-treatment, 2-months and 6-months follow up treatment.

GROUP	GENDER	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
Pre-treatment	MALES (n=35)	10.26 ± 5.24	6.32 ± 3.80	3.53 ± 1.94	0.37 ± 0.28	0.08 ± 0.10
	FEMALES (n=25)	10.39 ± 4.24	5.74 ± 3.19	4.17 ± 1.74	0.41 ± 0.29	0.07 ± 0.09
	t-value	-0.103	0.666	-1.364	-0.475	0.233
	p-value	0.918 (ns)	0.508 (ns)	0.179 (ns)	0.637 (ns)	0.817 (ns)
2-Months	MALES (n=35)	5.64 ± 1.53	3.01 ± 0.97	2.51 ± 0.84	0.19 ± 0.09	0.03 ± 0.04
	FEMALES (n=25)	4.86 ± 1.66	2.56 ± 0.90	2.13 ± 0.83	0.14 ± 0.10	0.02 ± 0.03
	t-value	2.294	2.012	2.183	2.136	2.082
	p-value	0.022*	0.044*	0.029*	0.036*	0.042*
6-Months	MALES (n=35)	6.61 ± 2.61	3.59 ± 1.56	2.67 ± 1.03	0.19 ± 0.13	0.05 ± 0.07
	FEMALES (n=25)	6.56 ± 2.68	3.44 ± 1.52	2.70 ± 1.16	0.22 ± 0.16	0.07 ± 0.08
	t-value	0.066	0.371	-0.098	-0.801	-1.291
	p-value	0.947 (ns)	0.712 (ns)	0.922 (ns)	0.426 (ns)	0.201 (ns)

*$p<0.05$ = Significant

ns = not significant

4.21. BMI and Other Haematological Parameters in Male and Female TB Subjects at pre-treatment, 2-month and 6-month into treatment.

At pre-treatment, the mean PCV (l/l), NLR and the mean platelet count ($x10^9$/l), was significantly higher in male TB subject compared to females (P=0.022, 0.040 and 0.039). At 2-month into treatment, the mean MLR was significantly higher in males (0.10 ± 0.04) compared with females (0.06 ± 0.04) (P=0.036). Conversely, the mean platelet count ($x10^9$/l) was significantly higher in females (315.18 ± 104.43) than males (259.92 ± 85.62) (P=0.022). At 6-month into treatment, there was no significant difference in the mean values of BMI and other haematological parameters when compared between male and female TB subjects (P>0.05). (See table 4.21)

4.22. Pre-treatment (baseline) values of inflammatory and haemostatic parameters among TB subjects of different age groups (Median (range)

Table 4.22 shows the baseline values of inflammatory and haemostatic parameters among TB-infected subjects of different age groups.

4.23. Inflammatory Cytokines of Different Age-groups at pre-treatment.

The median IL-6 and IL-10 (pg/ml) was significantly higher in TB subjects that were >50 years compared to those that were 18-30 years and 31-50 years age groups respectively (P=0.028 and 0.003). Moreover, the median value of IL-2 was significantly lower in TB subjects aged >50 years (1.55) compared to those 18-30 years (11.36) and 31-50 years (11.36) respectively (P=0.041 and 0.040). (Table 4.23)

4.24. Haemostatic Parameters of Different Age-group at pre-treatment.

The median value of GP IIb/IIIa (ng/ml) was significantly higher in TB subjects aged 18-30 years (6.28) compared to those aged 31-50 years (2.82) and >50 years (4.23) respectively (P=0.002 and 0.020) (Table 4.24).

Table 4.21: Comparison of BMI and other haematological parameters between male and female TB subjects at pre-treatment, 2-months and 6-months follow up treatment.

GROUP	GENDER	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT ($x10^9$/l)
Pre-treatment	MALES (n=35)	0.34 ± 0.05	19.92 ± 2.49	1.78 ± 0.82	0.11 ± 0.08	236.75 ± 110.17
	FEMALES (n=25)	0.31 ± 0.05	19.55 ± 2.95	1.35 ± 0.67	0.10 ± 0.08	202.82 ± 89.41
	t-value	2.376	0.543	2.118	0.362	2.065
	p-value	0.022*	0.590 (ns)	0.040*	0.720 (ns)	0.039*
2-Months	MALES (n=35)	0.34 ± 0.06	20.81 ± 2.81	1.26 ±0.34	0.10 ± 0.04	259.92 ± 85.62
	FEMALES (n=25)	0.36 ± 0.04	20.54 ± 2.90	1.25 ± 0.31	0.06 ±0.04	315.18 ±104.43
	t-value	-1.695	0.387	0.043	-2.142	-2.337
	p-value	0.096 (ns)	0.700 (ns)	0.966 (ns)	0.036*	0.022*
6-Months	MALES (n=35)	0.31 ± 0.05	21.79 ± 2.89	1.35 ± 0.41	0.08 ± 0.05	337.33 ± 65.79
	FEMALES (n=25)	0.31 ± 0.06	21.56 ± 2.80	1.26 ± 0.41	0.09 ± 0.07	348.32 ± 83.04
	t-value	-0.179	0.331	0.814	-1.011	-0.559
	p-value	0.859 (ns)	0.742 (ns)	0.419 (ns)	0.315 (ns)	0.578 (ns)

*$P<0.05$ = Significant

ns = not significant ($P>0.05$)

Table 4.22: Pre-treatment (baseline) values of inflammatory and haemostatic parameters among TB subjects of different age groups (Median (range)

Parameters	18-30 years (n=25)	31-50 Years (n=21)	>50 years (n=14)
TNF-A (pg/ml)	1.55 (1.55 – 38.99)	1.55 (1.55 – 38.99)	4.43 (1.55 – 38.03)
IL-6 (pg/ml)	61.80 (4.08 – 231.98)	64.00 (5.60 – 267.65)	208.49 (4.08 – 267.65)
IL-10 (pg/ml)	1.46 (1.46 – 10.39)	1.46 (1.46 – 10.39)	3.15 (1.46 – 8.14)
TGF-B (pg/ml)	23.83 (23.83 – 123.83)	23.83 (23.83 – 148.83)	23.83 (23.83 – 148.83)
IL-2 (pg/ml)	11.36 (11.36 – 14.36)	11.36 (11.36 – 14.36)	1.55 (1.55 – 38.99)
PF4 (ng/ml)	0.35 (0.35 – 3.32)	0.35 (0.35 – 2.50)	0.35 (0.35 – 2.41)
PSEL (ng/ml)	22.68 (20.13 – 23.46)	22.19 (20.13 – 23.46)	21.83 (20.13 – 23.46)
GP IIb/IIIa (ng/ml)	6.28 (1.58 – 11.28)	2.82 (0.36 – 14.20)	4.23 (0.54 – 11.02)
PAF (pg/ml)	32.39 (2168.64 – 6834.4)	3350.9 (654.4 – 8964)	2493.1 (710.37 – 5965.37)
TPO (pg/ml)	4337.4 (2732.3 – 5258.3)	3786.8 (2661.5 – 5258)	4027.70 (2661.5 – 5640.8)

TNF-α = Tumor Necrosis Factor alpha

IL-6 = Interleukin-6

IL-10 = Interleukin-10

TGF-β = Transforming growth factor beta

IL-2 = Interleukin-2

PF-4 = Platelet factor 4

P-SEL = P-Selectin

GP IIb/IIIa = Human platelet glycoprotein IIb/IIIa

PAF = Platelet Activating Factor

TPO = Thrombopoietin hormone

Table 4.23: Levels of inflammatory cytokines among age groups of TB subjects at pre-treatment (Median (Range).

AGE GROUP	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-β (pg/ml)	IL-2 (pg/ml)
(1) 18-30 years (n=25)	1.55 (1.55 - 38.99)	61.80 (4.08 - 231.98)	1.46 (1.46 - 10.39)	23.83 (23.83 -123.83)	11.36 (11.36 -14.36)
(2) 31-50 years (n=21)	1.55 (1.55 - 38.99)	64.00 (5.60 - 267.65)	1.46 (1.46 - 10.39)	23.83 (23.83 -148.83)	11.36 (11.36 -14.36)
(3) >50 years (n=14)	4.43 (1.55 - 38.03)	208.49 (4.08 - 267.65)	3.15 (1.46 - 8.14)	23.83 (23.83 -148.83)	1.55 (1.55 -38.99)
X^2-value	0.641	10.849	8.861	2.148	9.998
P-value	0.726	0.001*	0.006*	0.342	0.004*
1 vs 2 *P*-value	0.928	0.243	0.827	0.210	0.968
1 vs 3 *P*-value	0.482	0.028*	0.042*	0.157	0.041*
2 vs 3 *P*-value	0.474	0.003*	0.034*	0.712	0.040*

*$P<0.05$ = Significant

1 vs 2 = Comparison of parameters between TB subjects aged 18-30 years and 31-50 years at pre-treatment.

1 vs 3 = Comparison of parameters between TB subjects aged 18-30 years and > 50 years at pre-treatment.

2 vs 3 = Comparison of parameters between TB subjects aged 31-50 years and >50 years at pre-treatment.

Table 4.24: Comparison of median (range) values of haemostatic parameters among age groups of TB subjects at pre-treatment.

AGE GROUP	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
(1) 18-30 years (n=25)	0.35 (0.35-3.32)	22.68 (20.13-23.46)	6.28 (1.58-11.28)	3239.04 (2168.64-6834.37	4337.37 (2732.34-5258.29)
(2) 31-50 years (n=21)	0.35 (0.35-2.50)	22.19 (20.13-23.46)	2.82 (0.36-14.20)	3350.92 (654.42-8963.97)	3786.84 (2661.50-5258.29)
(3) >50 years (n=14)	0.35 (0.35-2.41)	21.83 (20.13-23.46)	4.23 (0.54-11.02)	2493.12 (710.37-5965.37)	4027.70 (2661.50-5640.82)
X^2-value	0.221	1.221	7.628	1.550	1.075
P-value	0.895 (ns)	0.543 (ns)	0.013*	0.461 (ns)	0.584 (ns)
1 vs 2 *P*-value	0.677 (ns)	0.366 (ns)	0.002*	0.366 (ns)	0.336 (ns)
1 vs 3 *P*-value	1000 (ns)	0.297 (ns)	0.020*	0.203 (ns)	0.562 (ns)
2 vs 3 *P*-value	0.720 (ns)	0.799 (ns)	0.775 (ns)	0.679 (ns)	0.610 (ns)

P<0.05 = Significant

ns = not significant (P>0.05)

1 vs 2 = Comparison of parameters between 18-30 year and 31-50 years at pre-treatment.

1 vs 3 = Comparison of parameters between 18-30 years and > 50 years pre-treatment.

2 vs 3 = Comparison of parameters between 31-50 and >50 years at pre-treatment.

4.25. Pre-treatment (baseline) values of haematological parameters among TB subjects of different age groups

Table 4.25 shows the baseline values of haematological parameters among TB-infected subjects of different age groups.

4.26. White Blood Cell Parameters of Different Age-groups at pre-treatment.

The mean total white cell count (TWBC) and neutrophil count (NEUT) was significantly higher in TB subjects aged >50 years compared to those aged 18-30 years and 31-50 years respectively (P=0.001 and 0.002). Also, the mean eosinophil count (EOS) was significantly higher in TB subjects aged >50 years (0.11 ± 0.11) compared to 31-50 years (0.05 ± 0.08) (P=0.004). On the other hand, there was no significant difference in the mean values of lymphocyte count ($x10^9$/l) and monocyte count ($x10^9$/l) among the different age groups (P>0.05) (Table 4.26).

4.27. BMI and Other Haematological Parameters of Different Age-groups at pre-treatment.

The mean PCV was significantly lower in TB subjects aged >50 years (0.30 ± 0.04) compared to 18-30 years (0.33 ± 0.06) and 31-50 years (0.33 ± 0.05) respectively (P=0.041 and 0.040). Also, the mean NLR was significantly higher in TB subjects aged >50 years (1.95 ± 0.82) compared to 18-30 years (1.53 ± 0.81) age group (P=0.021). Moreover, no significant difference was observed in the mean values of BMI (kg/m2), MLR and platelet count ($x10^9$/l) among the different age groups (P>0.05) (Table 4.27).

4.28. Inflammatory Cytokines among TB Subjects of Different Age-groups at 2-month into treatment

The median value of IL-10 (pg/ml) was significantly higher in TB subjects aged 18-30 years (5.71) compared to 31-50 years (3.06) and >50 years (3.40) age group respectively (P=0.029 and 0.018). Also, the median value of IL-2 (pg/ml) was significantly higher in TB subjects aged >50 years (41.35) compared to 31-50 years (11.36) and 18-30 years (12.82) respectively (P=0.018 and 0.013) (Table 4.28).

Table 4.25: Pre-treatment (baseline) values of haematological parameters among TB subjects of different age groups (Median (range)

Parameters	18 – 30 years	31 – 50 years	>50 years
TWBC ($x10^9$/l)	9.81 ± 5.21	9.33 ± 4.15	12.04 ± 5.00
NEUT ($x10^9$/l)	5.76 ± 4.01	5.53 ± 2.94	7.30 ± 3.21
LYM ($x10^9$/l)	3.90 ± 2.11	3.27 ± 1.57	3.77 ± 1.76
MONO ($x10^9$/l)	0.36 ± 0.28	0.35 ± 0.27	0.50 ± 0.31
EOS ($x10^9$/l)	0.08 ± 0.10	0.05 ± 0.08	0.11 ± 0.11
PCV (l/l)	0.33 ± 0.06	0.33 ± 0.05	0.30 ± 0.04
BMI (kg/m^2)	20.01 ± 2.36	19.69 ± 2.38	19.79 ± 3.60
NLR	1.53 ± 0.81	1.70 ± 0.73	1.95 ± 0.82
MLR	0.10 ± 0.08	0.10 ± 0.07	0.13 ± 0.09
PLT ($x10^9$/l)	216.83 ± 102.55	207.70 ± 112.30	212.00 ± 103.08

TWBC = Total white blood cell count

NEUT = Absolute neutrophils count

LYM = Absolute Lymphocytes count

MONO = Absolute monocytes count

EOS = Absolute eosinophils count

PCV = Packed cell volume

BMI = Body mass index

NLR = Neutrophil lymphocyte ratio

MLR = Monocyte lymphocyte ratio

PLT = Total platelet count

Table 4.26: Values of White blood cell parameters among age groups of TB subjects at pre-treatment (Mean ± SD).

AGE GROUP	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
(1) 18-30 years (n=25)	9.81 ± 5.21	5.76 ± 4.01	3.90 ± 2.11	0.36 ± 0.28	0.08 ± 0.10
(2) 31-50 years (n=21)	9.33 ± 4.15	5.53 ± 2.94	3.27 ± 1.57	0.35 ± 0.27	0.05 ± 0.08
(3) >50 years (n=14)	12.04 ± 5.00	7.30 ± 3.21	3.77 ± 1.76	0.50 ± 0.31	0.11 ± 0.11
F-value	6.788	5.188	0.772	1.507	7.332
P-value	0.003*	0.007*	0.466	0.229	0.026*
1 vs 2 *P*-value	0.933	0.972	0.449	0.982	0.625
1 vs 3 *P*-value	0.001*	0.045*	0.973	0.285	0.649
2 vs 3 *P*-value	0.002*	0.034*	0.702	0.250	0.004*

*$P<0.05$ = Significant

1 vs 2 = Comparison of parameters in TB subjects aged between 18-30 year and 31-50 years at pre-treatment.

1 vs 3 = Comparison of parameters in TB subjects aged between 18-30 years and > 50 years at pre-treatment.

2 vs 3 = Comparison of parameters in TB subjects aged between 31-50 and >50 years at pre-treatment.

Table 4.27: Values of BMI and other haematological parameters among age groups of TB subjects at pre-treatment (Mean ± SD).

AGE GROUP	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT ($x10^9$/l)
(1) 18-30 years (n=25)	0.33 ± 0.06	20.01 ± 2.36	1.53 ± 0.81	0.10 ± 0.08	216.83 ± 102.55
(2) 31-50 years (n=21)	0.33 ± 0.05	19.69 ± 2.38	1.70 ± 0.73	0.10 ± 0.07	207.70 ± 112.30
(3) >50 years (n=14)	0.30 ± 0.04	19.79 ± 3.60	1.95 ± 0.82	0.13 ± 0.09	212.00 ± 103.08
F-value	4.887	0.114	5.464	0.739	0.049
P-value	0.048*	0.893	0.046*	0.481	0.952
1 vs 2 *P*-value	0.946	0.889	0.726	0.999	0.948
1 vs 3 *P*-value	0.041*	0.956	0.021*	0.494	0.989
2 vs 3 *P*-value	0.040*	0.993	0.588	0.547	0.992

*$P<0.05$ = Significant

1 vs 2 = Comparison of parameters in TB subjects aged between 18-30 year and 31-50 years at pre-treatment.

1 vs 3 = Comparison of parameters in TB subjects aged between 18-30 years and > 50 years at pre-treatment.

2 vs 3 = Comparison of parameters in TB subjects aged between 31-50 years and >50 years at pre-treatment.

Table 4.28: Comparison of median values of inflammatory cytokines among age groups of TB subjects at 2-months follow-up treatment.

AGE GROUP	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-B (pg/ml)	IL-2 (pg/ml)
(1) 18-30 years (n=25)	71.47 (7.31-925.34)	176.02 (8.49-248.25)	5.71 (1.46-20.24)	23.83 (23.83-273.83)	12.82 (11.36-462.67)
(2) 31-50 years (n=21)	72.44 (1.23-934.95)	113.34 (4.08-259.47)	3.06 (1.46-20.24)	23.83 (23.83-273.83)	11.36 (11.36-467.48)
(3) >50 years (n=14)	89.97 (8.27-934.95)	192.66 (12.10-249.21)	3.40 (1.46-20.24)	23.83 (23.83-273.83)	41.35 (11.36-467.48)
X^2-value	0.785	0.596	6.812	1.523	7.256
P-value	0.675 (ns)	0.742 (ns)	0.045*	0.467 (ns)	0.024*
1 vs 2 *P*-value	0.569 (ns)	0.465 (ns)	0.029*	0.278 (ns)	0.723 (ns)
1 vs 3 *P*-value	0.608 (ns)	0.918 (ns)	0.018*	0.962 (ns)	0.013*
2 vs 3 *P*-value	0.431 (ns)	0.586 (ns)	0.703 (ns)	0.307 (ns)	0.018*

P<0.05 = Significant

ns = not significant (P>0.05)

1 vs 2 = Comparison of parameters between 18-30 year and 31-50 years at 2-months.

1 vs 3 = Comparison of parameters between 18-30 years and > 50 years at 2-months.

2 vs 3 = Comparison of parameters between 31-50 and >50 years at 2-months.

4.29. Haemostatic Parameters among TB Subjects of Different Age-groups at 2-month into treatment.

The median value of PF-4 (ng/ml) was significantly higher in TB subjects aged 31-50 years (1.18) compared to 18-30 years (0.35) and >50 years (0.35) respectively (P=0.028 and 0.032). However, there was no significant difference in the median values of P-SEL (ng/ml), GP IIb/IIIa (ng/ml), PAF (pg/ml) and TPO (pg/ml) among the different age groups at 2-month into treatment (P>0.05) (Table 4.29).

4.30. White Blood Cell Parameters among TB Subjects of Different Age-groups at 2-month into treatment.

At 2-month into treatment, the mean EOS was significantly higher in TB subjects aged 31-50 years (0.05 ± 0.50) compared to >50 years (0.03 ± 0.04) (P=0.003). Also no significant difference was observed in the mean values of TWBC ($x10^9$/l), NEUT ($x10^9$/l), LYM ($x10^9$/l) and MONO ($x10^9$/l) among the different age groups at 2-month into treatment (P>0.05) (See table 4.30).

4.31. BMI and Other Haematological Parameters among Different Age-groups of TB Subjects at 2-month into treatment.

The mean PCV was significantly higher in TB subjects aged 18-30 years (0.36 ± 0.06) compared to >50 years (0.32 ± 0.05) at 2-months into treatment (P=0.012). Similarly, the mean MLR was significantly higher in TB subjects aged 31-50 years (0.09 ± 0.05) compared to >50 years (0.06 ± 0.05) (P=0.031). Also, the mean platelet count (PLT) was significantly higher in TB subjects aged >50 years (309.67 ± 91.88) compared to 31-50 years (258.91 ± 107.06) (P=0.030) (See table 4.31)

4.32. Inflammatory Cytokines among Different Age-groups of TB Subjects at 6-month into treatment.

The median value of TNF-α (pg/ml) was significantly higher in TB subjects aged 18-30 years (54.83) compared to 31-50 years (13.87) and >50 years (5.71) respectively (P=0.048 and 0.043). Moreover, the median value of IL-6 (pg/ml) was significantly lower in TB subjects aged >50 years (9.77) compared to 31-50 years (65.28) and 18-30 years (114.14) respectively (P=0.046 and 0.005). Also, the median value of TGF-β (pg/ml) was significantly higher in TB subjects aged 18-30 years (1961.33) compared to 31-50 years (161.33) and >50 years (61.33) age group respectively (P=0.042 and 0.046) (Table 4.32).

Table 4.29: Comparison of median values of haemostatic parameters among age groups of TB subjects at 2-months follow-up treatment.

AGE GROUP	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
18-30 years (n=25)	0.35 (0.35-6.29)	22.12 (16.17-24.62)	5.99 (1.23-15.36)	2854.89 (1075.87-7557.91)	3888.04 (2971.17-4626.80)
31-50 years (n=21)	1.18 (0.35-6.29)	22.30 (16.17-24.62)	5.98 (0.73-16.04)	2618.06 (654.42-7557.91)	3966.98 (2957.00-4754.31)
>50 years (n=14)	0.35 (0.35-6.29)	22.26 (16.17-24.62)	4.22 (0.22-12.41)	3308.04 (251.63-4492.18)	3934.59 (3029.86-4537.74)
X^2-value	8.944	0.439	0.192	0.379	0.008
P-value	0.008*	0.803 (ns)	0.908 (ns)	0.828 (ns)	0.996 (ns)
1 vs 2 *P*-value	0.028*	0.636 (ns)	0.719 (ns)	0.559 (ns)	0.893 (ns)
1 vs 3 *P*-value	1000 (ns)	0.590 (ns)	0.772 (ns)	0.649 (ns)	0.967 (ns)
2 vs 3 *P*-value	0.032*	0.676 (ns)	0.760 (ns)	0.978 (ns)	0.978 (ns)

P<0.05 = Significant

ns = not significant (P>0.05)

1 vs 2 = Comparison of parameters between 18-30 year and 31-50 years at 2-months.

1 vs 3 = Comparison of parameters between 18-30 years and > 50 years at 2-months.

2 vs 3 = Comparison of parameters between 31-50 and >50 years at 2-months.

Table 4.30: Values of White blood cell parameters among age groups of TB subjects at 2-month into treatment (Mean ± SD).

AGE GROUP	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
(1) 18-30 years (n=25)	5.01 ± 1.20	2.60 ± 0.70	2.25 ± 0.64	0.15 ± 0.09	0.01 ± 0.02
(2) 31-50 years (n=21)	5.56 ± 1.73	2.94 ± 0.99	2.44 ± 0.94	0.19 ± 0.10	0.05 ± 0.05
(3) >50 years (n=14)	5.61 ± 1.93	3.12 ± 1.23	2.49 ± 1.07	0.16 ± 0.11	0.03 ± 0.04
F-value	1.108	1.819	0.516	1.378	5.788
P-value	0.336	0.170	0.559	0.259	0.005*
1 vs 2 *P*-value	0.425	0.388	0.705	0.247	0.003*
1 vs 3 *P*-value	0.448	0.186	0.652	0.965	0.489
2 vs 3 *P*-value	0.993	0.828	0.983	0.513	0.196

*$P<0.05$ = Significant

1 vs 2 = Comparison of parameters in TB subjects aged between 18-30 years and 31-50 years at 2-month into treatment.

1 vs 3 = Comparison of parameters in TB subjects aged between 18-30 years and > 50 years at 2-month into treatment.

2 vs 3 = Comparison of parameters in TB subjects aged between 31-50 years and >50 years at 2-month into treatment.

Table 4.31: Values of BMI and other haematological parameters among age groups of TB subjects at 2-month into treatment (Mean ± SD).

AGE GROUP	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT ($x10^9$/l)
(1) 18-30 years (n=25)	0.36 ± 0.06	21.07 ± 2.54	1.20 ± 0.34	0.07 ± 0.04	276.23 ± 79.55
(2) 31-50 years (n=21)	0.34 ± 0.05	20.56 ± 2.75	1.30 ± 0.34	0.09 ± 0.05	258.91 ± 107.06
(3) >50 years (n=14)	0.32 ± 0.05	20.58 ± 3.61	1.29 ± 0.31	0.06 ± 0.05	309.67 ± 91.88
F-value	6.779	0.286	0.739	5.991	4.980
P-value	0.017*	0.752	0.482	0.045*	0.048*
1 vs 2 *P*-value	0.681	0.776	0.520	0.255	0.778
1 vs 3 *P*-value	0.012*	0.836	0.640	0.939	0.490
2 vs 3 *P*-value	0.522	1.000	0.998	0.031*	0.030*

*$P<0.05$ = Significant

1 vs 2 = Comparison of parameters in TB subjects aged between 18-30 years and 31-50 years at 2-month into treatment.

1 vs 3 = Comparison of parameters in TB subjects aged between 18-30 years and > 50 years at 2-month into treatment.

2 vs 3 = Comparison of parameters in TB subjects aged between 31-50 years and >50 years at 2-month into treatment.

Table 4.32: Comparison of median (range) values of Inflammatory cytokines among age groups of TB subjects at 6-months follow-up treatment.

AGE GROUP	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-B (pg/ml)	IL-2 (pg/ml)
(1) 18-30 years (n=25)	54.83 (1.55-478.03)	114.14 (4.08-247.53)	2.93 (1.46-9.20)	1961.33 (23.83-5198.83)	11.36 (11.36-15.42)
(2) 31-50 years (n=21)	13.87 (1.55-478.03)	65.28 (4.08-186.68)	2.90 (1.46-9.20)	161.33 (23.83-3423.83)	11.36 (11.36-15.42)
(3) >50 years (n=14)	5.71 (1.55-350.35)	9.77 (4.08-186.68)	2.52 (1.46-9.20)	61.33 (23.83-5198.83)	11.36 (11.36-14.28)
X^2-value	5.707	6.188	0.422	8.273	4.847
P-value	0.009*	0.023*	0.810 (ns)	0.004*	0.089 (ns)
1 vs 2 *P*-value	0.048*	0.472 (ns)	0.959 (ns)	0.042*	0.125 (ns)
1 vs 3 *P*-value	0.043*	0.005*	0.572 (ns)	0.046*	0.394 (ns)
2 vs 3 *P*-value	0.304 (ns)	0.046*	0.542 (ns)	0.423 (ns)	0.052 (ns)

P<0.05 = Significant

ns = not significant (P>0.05)

1 vs 2 = Comparison of parameters between 18-30 year and 31-50 years at 6-months follow-up.

1 vs 3 = Comparison of parameters between 18-30 years and > 50 years at 6-months follow-up.

2 vs 3 = Comparison of parameters between 31-50 and >50 years at 6-months follow-up.

4.33. Haemostatic Parameters among Different Age-groups of TB Subjects at 6-month into treatment.

The median value of GP IIb/IIIa was significantly higher in TB subjects aged 18-30 years (5.42) compared to 31-50 years (2.66) and >50 years (2.86) respectively (P=0.041 and 0.025). Moreover, there was no significant difference in the median values of PF-4 (ng/ml), P-SEL (ng/ml), PAF (pg/ml) TPO (pg/ml) and among the different age groups at 6-months (P>0.05) (Table 4.33).

4.34. White Blood Cell Parameters among Different Age-groups of TB Subjects at 6-month into treatment.

There was no significant difference in the mean values of white blood cell parameters among the different age groups at 6-months (P>0.05) (See table 4. 34).

4.35. BMI and Other Haematological Parameters among Different Age-groups of TB Subjects at 6-month into treatment.

There was no significant difference in the mean values of BMI and other haematological parameters among the different age groups at 6-months (P>0.05) (See table 4. 35).

Table 4.33: Comparison of median values of haemostatic parameters among age groups of TB subjects at 6-months follow-up treatment.

AGE GROUP	PF-4 (ng/ml)	P-SEL (ng/ml)	GP IIb/IIIa (ng/ml)	PAF (pg/ml)	TPO (pg/ml)
(1) 18-30 years (n=25)	0.35 (0.35-2.41)	21.49 (16.86-25.06)	5.42 (1.20-11.55)	3046.96 (1445.10-5461.88)	3603.67 (3019.74-4452.74)
(2) 31-50 years (n=21)	0.35 (0.35-0.68)	22.02 (18.24-25.06)	2.66 (0.97-9.63)	2191.02 (665.61-4656.28)	3846.55 (2898.30-5112.56)
(3) >50 years (n=14)	0.35 (0.35-2.41)	22.03 (16.86-23.65)	2.86 (1.73-8.86)	2420.39 (1139.27-5461.88)	3652.25 (3323.34-4881.82)
X^2-value	2.361	0.845	10.377	2.071	0.778
P-value	0.307 (ns)	0.655 (ns)	0.002*	0.355 (ns)	0.678 (ns)
1 vs 2 *P*-value	0.308 (ns)	0.319 (ns)	0.041*	0.160 (ns)	0.412 (ns)
1 vs 3 *P*-value	0.667 (ns)	0.703 (ns)	0.025*	0.957 (ns)	0.704 (ns)
2 vs 3 *P*-value	0.111 (ns)	0.967 (ns)	0.650 (ns)	0.386 (ns)	0.620 (ns)

P<0.05 = Significant

ns = not significant (P>0.05)

1 vs 2 = Comparison of parameters between 18-30 year and 31-50 years at 6-months follow-up.

1 vs 3 = Comparison of parameters between 18-30 years and > 50 years at 6-months follow-up.

2 vs 3 = Comparison of parameters between 31-50 and >50 years at 6-months follow-up.

Table 4.34: Values of White blood cell parameters among age groups of TB subjects at 6-month into treatment (Mean ± SD).

AGE GROUP	TWBC ($x10^9$/l)	NEUT ($x10^9$/l)	LYM ($x10^9$/l)	MONO ($x10^9$/l)	EOS ($x10^9$/l)
(1) 18-30 years (n=25)	6.77 ± 2.44	3.56 ± 1.35	2.81 ± 1.11	0.19 ± 0.13	0.05 ± 0.07
(2) 31-50 years (n=21)	6.82 ± 2.64	3.80 ± 1.67	2.66 ± 1.05	0.21 ± 0.15	0.06 ± 0.06
(3) >50 years (n=14)	6.05 ± 3.09	3.15 ± 1.79	2.52 ± 1.09	0.21 ± 0.15	0.07 ± 0.09
F-value	0.438	0.742	0.345	0.091	0.201
P-value	0.648	0.480	0.710	0.914	0.819

*$P<0.05$ = Significant

Table 4.35: Values of BMI and other haematological parameters among age groups of TB subjects at 6-month into treatment (Mean ± SD).

AGE GROUP	PCV (l/l)	BMI (kg/m^2)	NLR	MLR	PLT ($x10^9/l$)
(1) 18-30 years (n=25)	0.32 ± 0.06	21.96 ± 2.46	1.28 ± 0.39	0.08 ± 0.06	349.00 ± 75.47
(2) 31-50 years (n=21)	0.31 ± 0.04	21.61 ± 2.85	1.43 ± 0.44	0.08 ± 0.06	334.05 ± 67.16
(3) >50 years (n=14)	0.30 ± 0.06	21.50 ± 3.55	1.20 ± 0.42	0.09 ± 0.07	340.14 ± 76.50
F-value	0.380	0.187	1.516	0.018	0.244
P-value	0.685	0.830	0.227	0.982	0.784

*$P<0.05$ = Significant

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1. Discussion

Inflammation and haemostasis are two biological processes that considerably affect each other. Tuberculosis infection is marked by changes in inflmammatory markers (immune cells and cytokines) as well as alterations in haemostatic parameters. According to Yoon *et al* (2013), the physiological immune responses of circulating leukocytes to various stressful events and inflammation are characterized by an increased neutrophil count and decreased lymphocyte count and an increase in total WBC particularly when caused by a bacterial infection. This generally agrees with the findings of this study at pre-treatment except in the lymphocyte count where a significant increase was found as well.

A statistically significant higher TWBC was observed at pre-treatment compared to 2-month and 6-month into treatment. This agrees with that of Venestra *et al* (2006) that posited that this increase in total leukocyte count resulted from immune reaction taking place in response to foreign antigen (TB) resulting in an increased cytokine levels which they suggested caused further proliferation of white blood cells. Veenstra *et al* (2006) also found a decreased TWBC with treatment which aligns with the finding of a significant decrease at 2-month and 6-month into treatment compared to the pre-treatment value in this study. This response of TWBC with treatment could point to its possible utility as surrogate marker of treatment response in TB subjects. The reason for the significant increase at 6-months into treatment compared to 2-month into treatment could be due to the anti-tuberculosis therapy. However, Brahmbhatt *et al* (2006) showed that the level of leukocyte count during therapy could be dependent on whether TB patients are slow responders or fast responders of anti-tuberculosis therapy.

This study found that neutrophil count was higher at pre-treatment compared to 2-month and 6-month treatment. The first line of defence against any foreign organism is innate immunity characterised by phagocytosis mediated by neutrophils and macrophages and the neutrophils play a vital role in granuloma formation that characterizes TB disease (Ngahane *et al*., 2017). Iqbal *et al* (2015) corroborated this by stating that innate immune response of body against the antigen (TB) could result in elevated neutrophil count. Rekha *et al* (2012) and Kashinkunti (2014) also stated that Neutrophilia is generally seen in the acute phase of TB infection. These may explain the significantly higher neutrophil count (NEUT) observed at

pre-treatment. Similarly, this finding agrees with Iqbal *et al* (2015) that reported a significant neutrophilia (increase in neutrophils) that improved (decreased) with treatment which aligns with the significant decrease observed with treatment at 2-month and 6-month into treatment. The significant decrease at 2-month into treatment could be as a result of decreased production due to therapy or bone marrow invasion by the bacterium.

This study showed that the value of absolute lymphocyte count (LYM) at pre-treatment significantly decreased at 2-month and 6-month into treatment. Previous studies have reported conflicting findings on lymphocyte count in TB subjects, some research findings reported an increase in lymphocyte count and others a decrease in lymphocyte count with return to normal values with therapy (Al-Aska *et al*., 2011; Okamura *et al.*, 2013). Iqbal *et al* (2015) reported lymphopenia in half (50%) of the patients at the time of diagnosis which later improved with initial treatment and Veenstra *et al* (2006) found that absolute lymphocyte count of patients at diagnosis was significantly depressed at diagnosis, but counts were no longer significantly decreased at the end of treatment. Both of these disagree with the findings of this study in which the value at pre-treatment decreased at 2-month and 6-month into treatment. This decrease observed in this study may be due to accumulation of lymphocytes at the site of infection leading to decreased number in peripheral blood as posited by Djoba *et al* (2009). It may also have a link to the therapy administered at 2-month (initiation phase) and 6-month (continuation phase) into treatment.

According to Naranbhai *et al* (2017), monocytes/macrophages are one of the major effector cells in protecting the host against Mtb infection. They are central mediators of the immune response and inflammatory stimuli mainly affect the numbers of monocytes in the peripheral blood (Zhang *et al*., 2018). The finding of a significantly higher monocyte count (MONO) in this study at pre-treatment in comparison to 2-month and 6-month treatment is in line with the findings of Veenstra *et al* (2006) that tuberculosis infection causes monocytosis which then settles as the infection resolves. This increase in monocytes commonly seen in TB infection is because the micro-organism is engulfed by alveolar macrophages after entering the body, but some evade the defence mechanism resulting in infection that leads to production of chemo-attractant substances that mobilize other leukocytes and unopposed production of monocytes. The decrease in monocyte count at 2-month and 6-month into treatment is also in line with the significantly decreased level observed in the course of treatment by Veenstra *et al* (2006) after a significantly elevated level at diagnosis. We posit

that the cause of the decrease from 2-month into treatment could be the anti-tuberculosis therapy. However, according to Iqbal *et al* (2015) it may be due to malnutrition because TB is a disease of low socio-economic society that prevails more in poor and malnourished individuals.

Though it is a common knowledge that eosinophils play a major role in parasitic infections of which TB is not one, but according to Tocheny *et al* (2017) eosinophils have comparable phagocytic function to neutrophils and an overlapping repertoire of granular contents capable of limiting bacterial growth. This may explain the similar pattern of response we found in neutrophils and eosinophils in our study. The significant higher eosinophils at pre-treatment in comparison to 2-month and 6-month treatment may also be relative to the findings of Tocheny *et al* (2017) that *Mtb* directly affects eosinophil degranulation, and eosinophils are able to contribute to *Mtb*-driven inflammation. They also reported that eosinophils are being actively recruited to the lung in response to *Mtb in vivo.* This may suggest that the decrease observed at 2-month into treatment may be due to this mobilization of eosinophils to the lungs away from the peripheral circulation. The decrease may also be due to the chemotherapeutic effects of the initiation phase drugs. However, the significant increase at 6-month into treatment implies that whatever could be the reason for the decrease at 2-month into treatment did not continue till 6-month into treatment.

A significant increase in platelet count (PLT) was found at 2-month into treatment compared to pre-treatment and a further significant increase at 6-month into treatment. According to Iqbal *et al* (2015), platelet count has a significant role in immune functions and "reactive thrombocytosis" is generally seen in chronic inflammation. Platelets are principal regulators of haemostasis and integrate host immune responses through production of immune-modulatory molecules and via cell-to-cell interactions with white blood cells (WBC) and may have host-protective roles in infectious disease (Semple *et al*., 2011). This seems to explain the findings of this study. However, the findings of this study did not agree with that of Koju *et al* (2015). They discovered a decreased platelet count during the initiation phase. A decreased platelet count could be due to the release of IL-1 that act as a procoagulant on endothelial cells and formation of antibodies that binds to platelet and suppress the production of platelets (Verma *et al.,* 2010). This study also conflicts with that of Iqbal *et al* (2015) that discovered a decreased platelet count from diagnosis till completion of initiation phase (at 2-month) with resumption of normal platelet count during continuation phase of treatment. They attributed this observed decrease to Rifampicin which is considered as a

major drug that decreases platelet count in anti-tuberculosis therapy. However the reason for the exception in this study is not clear. However, clinically low platelet counts are often associated with poor prognosis and increased risk for infection. In the light of this, the increased platelet count in our study is a sign of enhanced prognosis with treatment since the platelet count values were within the normal range.

This study showed a significantly lower packed cell volume (PCV) at pre-treatment compared to 2-month into treatment. This is supported by the report that reduction in PCV is one of the commonest findings seen in TB patients and considered to be responsible for poor prognosis (Alavi-Naini *et al.*, 2013). According to Iqbal *et al* (2015) more than ¾ of TB patients present with normocytic normochromic or iron deficiency anaemia. Iron deficiency anaemia that decreases host capacity in defending against foreign antigen resulting in impaired immune response is the most seen in TB subjects (Isanaka *et al*., 2012). Iron is a growth factor required by *Mycobacterium tuberculosis* for growth and survival which prevents the release of iron from reticulo-endothelial system and due to reduced/non-availability of iron to bone marrow, there is reduced erythropoiesis resulting in anaemia.

The significant increase in PCV observed at 2-month into treatment may be explained by the findings of Iqbal *et al* (2015) that with effective therapy, anaemia improved in TB patients after the completion of initiation phase of therapy. Similarly, the decline at 6-month into treatment may suggest that the continuation phase therapy may have a suppressing effect on erythropoiesis in line with the findings of Oyer and Schlossberg, (2011) that pharmacological agents (drugs) used for TB treatment may cause haematological changes.

The neutrophil-lymphocyte ratio (NLR) is a convenient marker of inflammation because it is a readily calculable laboratory marker used to evaluate systemic inflammation (Yoon *et al*., 2013). This explains the significantly higher NLR at pre-treatment compared to 2-months and 6-month treatment obtained in this study since TB infection is marked by inflammation. The laboratory and clinical utility of NLR has been established in various studies. de-Jager *et al* (2010) demonstrated that NLR was superior to C-reactive protein level, white blood cell (WBC) and neutrophil counts for predicting bacteraemia in an emergency care unit. Yoon *et al* (2013) also discovered that the NLR have stronger discriminative power for predicting bacteraemia compared to neutrophilia or lymphocytopenia alone.

The significant decrease observed at 2-month and 6-month into treatment agrees with the findings of Iqbal *et al* (2014) of a significant decrease in NLR in patients undergoing

treatment because Neutrophilia and Lymphopenia improves with settlement of inflammation and appropriate treatment. According to Ayed *et al* (2017), the peripheral blood neutrophil to lymphocyte ratio (NLR) has been reported to correlate with the prognosis of many acute or chronic infectious diseases. This was confirmed in their study in which Mean value of NLR was significantly higher in the Poor Prognosis group. They concluded that high NLR was an independent risk factor for predicting poor prognosis in Extra-Pulmonary TB patients.

According to Iqbal *et al* (2014) Monocyte-Lymphocyte ratio (MLR) gives an index of the relationship of the processes of tuberculosis infection to those of resistance because increase of the monocytes (which are the target cells for mycobacterial proliferation) is an index of dissemination of the disease and the increase of the lymphocytes (which provide resistance to the spread of infection causing mycobacterial clearance) is an index of resistance. This implies that MLR is high when the disease is active and normal or low when the patient had good combative powers. This makes it a good prognostic tool in TB infection (Naranbhai *et al*., 2014; Zhang *et al*., 2018). This study shows a significant increase in MLR at pre-treatment when the infection was at its peak compared to 2-month and 6-month treatment. There was a significant decline at 2-month into treatment and the decrease continued to 6-month into treatment showing a good response to therapy. This finding agrees with that of Iqbal *et al* (2014). They found that the MLR significantly decreased after initiation phase and two month of maintenance phase of treatment from the baseline value. They concluded that Tuberculosis is associated with increased MLR, which declines with anti-tuberculous therapy. Manna *et al* (2017) also returned a similar finding. They discovered that patients with active TB disease had a very high MLR, as compared to cured TB patients and additional analysis showed that the MLR value decreased after anti-mycobacterial therapy. It can be concluded from our finding in relation to previous similar findings that MLR can be considered as an independent prognostic marker and a reliable tool to evaluate treatment success in TB infection.

Body mass index (BMI) or Quetelet index is a measure of the body fat based on height and weight. It is a more accurate marker of nutritional status than weight because it also takes height into account (Sultan *et al*., 2012). According to Zhang *et al* (2017) Body mass index (BMI) has been shown to be associated with host susceptibility to several infections. Low body mass index (BMI) has been shown to be associated with host susceptibility to active TB development (Semunigus *et al*., 2016). In addition, obesity (BMI $\geq$ 30 kg/m2) and overweight (25 kg/m2 $\leq$ BMI < 30 kg/m2) were observed to be significantly associated with

decreased risk of developing active TB as compared with normal-weight (18.5 kg/m2 ≤ BMI < 25 kg/m2) (Leung, 2007). In this study a statistically significantly lower BMI (19.81 kg/m^2) was found at pre-treatment than afterwards which suggests a tending towards under-weight This may not be unconnected with the findings that under-nutrition is associated with an increased risk of tuberculosis (TB) (Kim *et al*., 2017). According to Raviglione (2007), TB is associated with poverty which in turn drives malnutrition. Lonnroth *et al* (2010) as well as Casha and Scarci (2017) reported a strong association between TB and BMI in pulmonary TB According to Semba *et al* (2010) a successful TB treatment should result in weight gain among underweight individuals through restoring muscle and fat mass, depending on the nutritional intake, thus the significant increase in BMI from 2-month to 6-month into treatment in this study suggests that the subjects gained in BMI with treatment which must have resulted from a reduction in TB load and cure for the infection. This corroborated with the findings of Kim *et al* (2017) that incidence of TB decreased as BMI was increased and high BMI might be associated with decreased risk of TB.

This study showed that TNF-α level significantly increased at 2-month into treatment and declined at 6-month into treatment to a level significantly above the pre-treatment value. The initial increase observed at 2-month into treatment, could be explained by the fact that TNF-α is a pro-inflammatory cytokine. Tumor necrosis factor (TNF-α) coordinates the inflammatory response via induction of other cytokines (IL-1 and IL-6) and the recruitment of immune and inflammatory cells. This may explain why a similar pattern of response was obtained for IL-6. According to Mootoo *et al* (2009), TNF-α is an essential component of the innate defence mechanism of the host against pathogenic challenge; it plays a major role in the pathology of certain diseases such as TB. The implication of an increased TNF-α at 2-month into treatment is macrophage activation with an enhanced capacity of macrophages to phagocytose and kill mycobacteria, cell recruitment to site of infection as well as formation and maintenance of granuloma all of which are helpful in the elimination of the pathogen (Robinson *et al*., 2012). The haemostatic implication could be an increase in platelet adhesion molecule expression as TNF-α has been shown to play a role in the up-regulation of adhesion molecules (Zganiacz *et al*., 2004; Keane, 2005). The decline at 6-month into treatment could be due to the fact that TNF-α expression is strictly controlled, since its super-production can mediate damaging effects (Cavalcanti *et al*., 2012). This finding also correlates with the elevation of anti-inflammatory markers/cytokines (such as IL-10) which have been reported by Shalev *et al* (2011) to inhibit the inflammatory roles of TNF-α at the same period.

Therefore this decrease at 6-month into treatment implies that with adequate adherence to therapy, there is a waning of the inflammatory process at the 6th month as the TB cure is completed.

In this study, the IL-6 increased significantly at 2-month into treatment and decreased significantly at 6-month into treatment. The value at 6-month though reduced was higher than the pre-treatment value. This observed increase in IL-6 at 2-month into treatment could be due to the fact that it is a pro-inflammatory cytokine mainly secreted by monocytes which according to our study was elevated prior to initiation of therapy. According to Kishimoto (2010), it is a pleitropic pro-inflammatory cytokine with a wide range of biological activities in immune regulation, haematopoiesis, inflammation and oncogenesis. It is of critical importance for acquired immunity against *M. tuberculosis* infection (Martinez *et al*., 2013).

The haemostatic implication of an increased IL-6 is a release of platelet because when IL-6 reaches the bone marrow it promotes megakaryocyte maturation (Tanaka *et al*., 2014). This agrees with the finding of persistent increase in platelet count up till 6-month into treatment. Also the increase in IL-6 among other factors could also have resulted in the reduction in PCV observed in the 6th month. This is because IL-6 is involved in the regulation of serum iron via control of its transporter. It does this by inducing hepcidin production which blocks the action of iron transporter ferroportin 1 on gut and thus reduces serum iron levels (Nemeth *et al.*, 200). This is responsible for the hypoferremia and anaemia associated with chronic inflammation. These changes in platelet count and red blood cell with increase in IL-6 can be used for evaluation of inflammatory severity in routine clinical laboratory examinations (Tanaka *et al*., 2014). The significant decrease at 6-month into treatment could be due to the reduction in inflammation as anti-inflammatory markers predominates and down-regulates the pro-inflammatory cytokines such as IL-6 (Shalev *et al*., 2011). The down-regulation is very necessary because dysregulated continual synthesis plays a pathological effect on chronic inflammation and autoimmunity, thus IL-6 expression is strictly controlled by transcriptional and post-transcriptional mechanisms and expressed at low levels except during infection, trauma or other stress (Tanaka *et al*., 2014).

It is worthy of note that the findings with TNF-α and IL-6 which are known pro-inflammatory markers gives a strong basis to conclude that 2-month into treatment is the peak of inflammation in TB subjects and the pro-inflammatory activities are down-regulated at 6-months into treatment in this study.

The IL-10 level was significantly lower at pre-treatment and at 2-month into treatment, but increased significantly at 6-months into treatment. Interleukin-10 is a potent immune-modulatory cytokine that affects innate and acquired immune responses and promotes TB disease progression which has important diagnostic and/or therapeutic implications. Thus the significant reduction in IL-10 at pre-treatment and 2-month compared to 6-month into treatment is very informative and with obvious beneficial effect to the subjects because it has been found that an increased level or over-production in TB patients has been associated with immune-suppression and greater susceptibility to the disease (Cooper *et al*., 2011; Sakomoto, 2012; Guimarães *et al*., 2012). The beneficial effect of a lowered IL-10 level was corroborated by Beamer *et al* (2009), who demonstrated that an increased production exacerbates TB disease and provides supportive evidence that IL-10 can be a bio-marker of TB disease progression in humans. They suggested that IL-10 production during infection with tuberculosis leads to the poor T cell responses and impaired control of infection. Cavalcanti *et al* (2012) supported this assertion by stating that an increase in IL-10 seems to support the survival of mycobacterium in the host.

Similarly, the increase in the level of IL-10 at 6-month into treatment is also beneficial to the subjects as modulation of inflammation and pro-inflammatory cytokines is necessary to avoid pathologic consequences that will result from an unregulated/unlimited inflammatory process. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that can dampen an overly exuberant immune response and potentially prevent tissue damage. It is considered primarily an inhibitory cytokine, important to an adequate balance between inflammatory and immunopathologic responses (Cavalcanti *et al.*, 2012). Cavalcanti *et al* (2012) asserted that elevated IL-10 in plasma of TB patients modulates excess pro-inflammation in TB patients. In the same vein, Shalev *et al* (2011) went ahead to specify that IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines such as IL-2, TNF-α, IL-6, IFN-Y, IL-1β etc and the reduction of these cytokines minimizes inflammation (Bijjiga and Martino, 2013).

Summarily, it can be deduced from these that, IL-10 manages a delicate balance between suppressing and activating host responses to pathogens because too much increase at one end may over-control otherwise protective T-cell responses, leading to chronic infection and at the other end, too little or no IL-10 may tend towards fatal host-mediated pathology (Redford *et al*., 2011). This makes it considerable to conclude that the reduction in IL-10 at pre-treatment and 2-month into treatment was to work against TB disease progression and susceptibility of the patients to the disease while an increase at 6-month was to help dampen

the immune and inflammatory response by inhibiting the synthesis and down-regulating the activities of the pro-inflammatory cytokines such as IL-6 and TNF-α which in our study was significantly reduced at 6-month into treatment.

It was discovered that the level of TGF-β was lower at pre-treatment and 2-month into treatment and significantly higher at 6-month into treatment. A prominent role of TGF-β in macrophage deactivation and suppression of T-cell responses to *Mycobacterium tuberculosis* is well established (Mianda *et al.,* 2012). This function makes an increased level harmful and a decreased level beneficial at the early stage of TB infection (pre-treatment and 2-month into treatment) where a prompt immune response and inflammatory process is necessary to overcome the bacterial load and work at eliminating the infection. However, the significant increase at the tail end of infection and treatment (6-month into treatment) as with IL-10 is necessary because TFG-β also inhibits secretion of many other cytokines such as TNF-α and IFN-γ (Wahl *et al.,* 2006). Thus it plays a role in modulation of inflammation in TB subjects.

In this study, the level of IL-2 increased significantly at 2-month into treatment and then declined at 6-month treatment. IL-2 has been shown to have multiple and sometimes opposing functions during an inflammatory response and in its dual and contrasting functions, it contributes to both the induction and termination of inflammatory immune responses (Hoyer *et al.,* 2008). Thus the significant increase at 2-month into treatment could be part of its contribution to the induction of inflammatory response and in line with its established role as part of the body's natural response to microbial infection (Liao *et al*., 2011). On the other hand the decline at the 6-month into treatment could be due to the waning of the inflammatory process that has already been established to occur at the 6-month into treatment. It could also be probably because the expression and secretion of IL-2 is tightly regulated and functions as part of both transient positive and negative feedback loops in mounting and dampening immune responses (Malek and Castro, 2010). The reason for the level at 6-month being significantly higher than the pre-treatment level could be as a result of the role of IL-2 in the termination of inflammatory immune response (Hoyer *et al*., 2008).

A non-significant decrease in PAF was found from the second month till the 6 month into treatment. Platelet activating factor (PAF) is important to the process of haemostasis because it causes platelets to aggregate and blood vessels to dilate (Mclntyre *et al*., 2009). High PAF level are associated with variety of conditions involving inflammation and it can trigger inflammatory and thrombotic cascades and mediate molecular and cellular interactions

(cross-talks) between inflammation and thrombosis. This aligns with the positive correlation observed between TNF-α (an inflammatory marker) and PAF as well as a significant but weak negative correlation between TGF-β (an anti-inflammatory marker) and PAF. This link between PAF and inflammation makes it unclear the reason for the no statistical significant change with increase and decrease in inflammation. Additionally PAF is synthesized by two different pathways (*de novo* pathway and remodeling pathway) and the remodelling pathways is activated by inflammatory agents and it is thought to be the primary source of PAF under pathological condition, thus the non-significant change could also mean that the threshold or extent of inflammation was not sufficient to activate the remodeling pathway for PAF synthesis.

This study found a significant increase in P-selectin (P-SEL) at 2-month into treatment and then a significant decrease at 6-month into treatment. P-selectin functions as a cell adhesion molecule and a marker of platelet activation and degranulation (Kullaya *et al.*, 2018). According to Zganiacz *et al* (2004) and Keane (2005), TNF-α has been shown to play a role in the up-regulation of adhesion molecules of which P-selectin is one. Thus the significant increase in the level of P-selectin at 2-month into treatment which is the peak of inflammatory process in our study, points to the role of inflammation in up-regulation of the activity of P-selectin. This may also be linked to the weak significant positive correlation observed between IL-6 and P-selectin as well as the non-significant positive correlation between TNF-α and P-selectin. Functionally, P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury during inflammation and aggregation of platelet at areas of vascular injury (Cleator *et al*., 2006). The principle behind this action is that in quiescent platelets, P-selectin is located on the inner walls of α-granules, thus upon platelet activation, there is a "membrane flipping" where the platelets releases α-granules and dense granules and the inner walls of the granules (where the P-selectin is located) are exposed on the outside of the cell, the exposed P-selectin then promotes platelet aggregation through platelet-fibrin and platelet-platelet binding (Cleator *et al.*, 2006). The significant decline at 6-month into treatment shows that the activity of P-selectin wears off as the inflammatory cytokines recedes and anti-inflammatory markers dominates.

This study showed a significant increase in Thrombopoietin hormone (TPO) from the second month till 6-month into treatment where there was a slight but not significant decline. The reason for the significant increase at 2-month into treatment could mean that the hormonal increase is tied to the increase in inflammation and pro-inflammatory cytokines which peaks

at 2-month into treatment. This thinking is re-enforced by the finding by Kaushansky (2006) that in the liver, the production of TPO is augmented by IL-6. This is supported by the significant positive correlation between IL-6 and TPO in our study. Thus it can be conclusively assumed that an increase in IL-6 at 2-month into treatment resulted to an increased TPO production. The increase could also have a direct relationship with the increased platelet count as it is known to regulate the production of platelets by stimulating the production and differentiation of megakaryocytes.

The slight though non-significant decline at 6-month into treatment compared to 2-month into treatment may suggest that the process of reduction in TPO has set in with the down-regulation of pro-inflammatory cytokines observed at 6-month into treatment especially IL-6 which as earlier stated plays a role in the production of TPO. If this be the case then the rate of decline of TPO is not proportionate with rate of decline of inflammatory cytokines, thus the non-significant decline. It could also be due to the negative feedback resulting from a further significant increase in the platelet count at 6-month into treatment. It is noteworthy that the negative feedback of TPO is different from that of most hormones in endocrinology, in that the effector regulates the hormone directly. This is because TPO is bound to the surface of platelets by a receptor (Kaushansky, 2006). The implication of this is that the rising and dropping platelet count regulates the TPO levels. This means that low platelet lead to higher degree of TPO exposure to the undifferentiated bone marrow cells, leading to differentiation into megakaryocytes while an increased platelet count as in our finding lead to reduction and less availability of TPO to megakaryocytes, this may have initiated the decline at 6-month into treatment though yet to be statistically significant.

A significant increase in GP IIb/IIIa was seen after 2-months of treatment, followed by a significant decline after 6-months of treatment. The increase in GP IIb/IIIa complex after 2-month into treatment could also be a response to inflammation as TNF-α has been earlier stated to play a role in the up-regulation of adhesion molecules (Zganiacz *et al*., 2004; Keane, 2005). This argument is supported by our finding of a significant positive correlation between GP IIb/IIIa and TNF-α and negative correlation between GP IIb/IIIa and TGF-β. Thus it could be that inflammation up-regulated the activity of Gp IIb/IIIa as obtained with P-selectin. The implication of an increased GP IIb/IIIa is an enhanced platelet aggregation and endothelial adherence (Shattil, 1999). This defines a pro-coagulant effect/state. The mechanism is that GP IIb/IIIa is capable of binding fibrinogen, resulting in many platelets aggregating as they connect to the same strands of fibrinogen resulting in a clot. The

coagulation cascade then follows to stabilize the clot as thrombin (factor IIa) converts the soluble fibrinogen into insoluble fibrin strands which are then cross-linked by factor XIII to form a stabilized blood clot. The significant decline in GP IIb/IIIa at 6-month into treatment could also be due to the down-regulation of inflammatory cytokines as earlier established in this study.

The findings of this study showed that PF-4 increased at 2-month into treatment but decreased significantly at the 6-month into treatment. An increased PF-4 at 2-month into treatment suggests increased platelet activation. Elevated concentration of platelet factor-4 could be used as an indicator for platelet activation since they are usually released from alpha granules of activated platelets. Elshamaa *et al* (2007) proved that PF-4 plays an important role in inflammation and wound repair and it is a strong chemo-attractant for neutrophils which is very crucial in the inflammatory process. Thus up-regulation and down-regulation of inflammation may be the reason for the non-significant increase at 2-month into treatment and decrease at 6-month into treatment. This is based on the fact that the increase at 2-month and decrease at 6-month into treatment follows the same pattern as the changes in IL-6 and TNF-α (pro-inflammatory cytokines). There was also a significant weak positive correlation between PF-4 and TNF-α. The implication of an increase in PF-4 is promotion of blood coagulation because it moderates the effect of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation.

The malaria infected tuberculosis patients in this study were asymptomatic. This confirms previous reports that malaria is hyper-endemic in Nigeria and that majority of malaria infections in individuals living in endemic regions are asymptomatic (Oseghale *et al*., 2012). According to Langhorne and Quin (2002), malaria has known immunomodulatory effect and alters the balance of circulating cytokines that are instrumental to the control of mycobacterial infection. Furthermore, Olaniyan *et al* (2018) state that *Plasmodium falciparum* co-infection with Mycobacterium tuberculosis could generate both innate and acquired immunity involving inflammatory responses which could affect the plasma levels of cytokines. Pro- and anti-inflammatory cytokines are involved in the malarial pathogenesis and the outcome of malaria infection is determined by the balance in induction and counter-regulation of both pro- and anti-inflammatory cytokines (Harischandra *et al*., 2015), because overproduction or inappropriate regulation of both pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, gamma interferon (IFN-Y), and tumor necrosis factor (TNF) and

anti-inflammatory cytokines, such as IL-10, IL-4, and transforming growth factor (TGF-β), may also lead to localized and systemic inflammation and has been associated with symptomatic and severe malaria.

A significantly higher level of TNF was observed in TB subjects co-infected with malaria (TB/MP+) compared to TB subjects not infected with malaria (TB/MP-) at 2-month into treatment. Tumor necrosis factor-alpha (TNF-α) has been shown to have potent anti-parasitic activity and high levels is associated with malaria pathology such as fever while a sustained high levels was associated with rapid clearance of fever and parasites (Depinay *et al*., 2011). Thus the increase at 2-month into treatment could have been a mechanism to combat the malaria parasite. Similarly, Robinson *et al* (2009), noted that TNF is a major effector cytokine that is implicated in mediating both protection and pathogenicity during malaria infection. Moreover, in line with the finding at 2-month into treatment, Olaniyan *et al* (2018) found significantly higher plasma TNF alpha in *Mycobacterium tuberculosis* and *Plasmodium falciparum* co-infected patients than *Mycobacterium tuberculosis* mono-infected patients. Conversely this study also found a significant lower level of TNF-α in TB/MP+ compared to TB/MP- at 6-month into treatment and a non-significantly lower level was found in TB/MP+ at pre-treatment. Depinay *et al* (2011) posits that TNF- α have both beneficial and detrimental effect and this may suggest that the varying levels observed at the three phases was to create a balance in TNF-α response in TB/MP+ subjects. Though the negative correlation found between MPC and TNF was not statistically significant, it has been shown to play a role in limiting parasite density and providing protection from mild symptomatic malaria episodes (Robinson *et al*., 2009).

The finding of this study showed a significantly lower level of IL-6 in TB/MP+ at pre-treatment and 6-month into treatment as well as a significantly higher level in TB/MP+ at 2-month into treatment compared to TB/MP-. This finding when analysed alongside the increased TNF-α level at 2-month into treatment suggests that there is a higher level of inflammation at 2-month into treatment in TB/MP+ subjects compared to other times. This finding may be due to the peak of inflammation in TB patients at 2-month into treatment which may have been potentiated by malaria infection since both are pro-inflammatory cytokine. This agrees with the findings of Hochman and Kami (2009) of an increase in pro-inflammatory response with malaria infection, and malaria has long been associated with high circulating levels of inflammatory cytokines such as tumour necrosis factor (TNF)-α,

interleukin (IL)-1 and IL-6 and a link has been established between TNF-α, IL-6 and the severity of the disease in human malaria infection. According to Robinson *et al* (2009) an increased plasma IL-6 level was associated with fatal outcomes in malaria infection, thus the reduction we found at 6-month into treatment may be a sign of good prognosis in the TB/MP+ infected patients.

In this study IL-10 was significantly lower in TB/MP+ compared to TB/MP- at pre-treatment and 2-month into treatment. This seems to suggest that malaria infection suppresses anti-inflammatory response in TB subjects at pre-treatment and 2-month into treatment. This disagrees with the findings of Chukwuanukwu *et al* (2016) that TB and malaria co-infection caused a marked increase in the production of the anti-inflammatory cytokines IL-10 and IL-4 in response to TB antigen suggesting that malaria co-infection promotes an anti-inflammatory response against *M. tuberculosis* which might have important consequences for disease progression. Similarly, Olaniyan *et al* (2018) stated that there was a significantly higher plasma IL-10 in *Mycobacterium tuberculosis* and *Plasmodium falciparum* co-infected patients than *Mycobacterium tuberculosis* mono-infected patients. Moreover, according to Yoshida *et al* (2000) early increase in IL-10 production has been associated with susceptibility to malaria infection, thus the finding of a lowered level could be an adaptive mechanism to reduce susceptibility. Also since IL-10 has consistently been shown to be associated with favourable outcome in malaria (Casals-Pascual *et al*., 2006), the non-significant difference observed between TB/MP+ and TB/MP- at 6-month into treatment may be due to an equilibrated up-regulation of anti-inflammatory markers (IL-10) in both subjects with resolution of TB infection and completion of treatment.
A non-significant positive correlation was observed between Median Parasite Count (MPC) and IL-10. This aligns with the findings of Medina *et al* (2011) that the concentration of IL-10 was higher in the group with high parasite density as well as that of Casals-Pascual *et al* (2012) that IL-10 levels were very closely associated with parasitemia. This also indicates that IL-10 has an important regulatory role in malaria infection, controlling the intensity of the immune response.

This study showed a significantly lower level of TGF-β in TB/MP+ at 6-month into treatment. This may suggest a down-regulation of the anti-inflammatory and immunoregulatory role of TGF-β by malaria infection in TB subjects since TB subjects without malaria has a higher level of this cytokine. Also the reduced level of IL-10 and TGF-

β corroborates that of Hansen and Schofield (2010) that the low levels of regulatory cytokines, such as TGF-β and IL-10, have been correlated with acute malaria.

Collectively, the differences in the above cytokines in TB/MP+ corroborates the report of Robinson *et al* (2009) that a critical balance between early pro- and anti-inflammatory cellular responses is crucial both for effectively controlling parasitemia and for preventing pathology in malaria infection.

Furthermore, a significantly higher level of IL-2 was found in TB/MP+ at 2-month into treatment compared to TB/MP-. This is expected as IL-2 has been known to play an antagonistic role in malaria infection. Thus an increase in IL-2 is expected in line with this role of combating malaria infection. This finding may also be due to the peak of inflammation in TB patients at 2-month into treatment which may have been potentiated by malaria infection since IL-2 is a pro-inflammatory cytokine.

The finding of this study showed a significantly reduced PF4 in TB/MP+ compared to TB/MP- at 6-month into treatment. PF4 belongs to growing list of chemokine molecules called kinocidins, which have a remarkable capacity to function as both chemotactic and antimicrobial molecules (Yeaman and Yount, 2007). They have been shown to have an important *in vivo* malaria-protective role that involves control of parasite growth during the early stage of erythrocytic malarial infection (McMorran *et al*., 2014). This mechanism of platelet-associated parasite killing involves the binding of platelets preferentially to parasite-infected red cells and release of PF4 from the platelets leading to intra-erythrocytic accumulation of PF4 which are subsequently absorbed into the parasite where it lyses the parasite food vacuole and kills the parasite (McMorran *et al.*, 2012; Love *et al.*, 2012; Kho *et al*., 2018). It can thus be adduced that the reduction in PF4 in TB/MP+ could be due to this role of PF4 in which case it is being used up in the process of platelet-associated parasite killing leading to a reduction in the level of PF4 available in circulation. Our finding does not align with that of Aisha *et al* (2012) that found a significant increase in PF4 in malaria parasite infections. This may explain the significant moderate positive correlation between MPC and PF4 in this study. Aisha *et al* (2012) also stated that assessment of PF4 concentrations in patients with parasitic infections can be used as an indicator for platelet activation because parasitic infections may cause platelet activation within few seconds.

At pre-treatment and 6-month into treatment, a significant higher level of P-selectin was seen in TB/MP+ compared to TB/MP-. According to Chang *et al* (2003) there is a marked up-

regulation of P-selectin during malaria infection and this increased P-selectin expression which may be due to increased endothelial cell expression, activated platelet expression, or both contributes to malarial pathogenesis. The finding of this study re-emphasizes the indispensable role of P-selectin in malaria parasite infection as an important adhesion molecule allowing parasitized erythrocytes to roll and adhere in vitro to endothelial cells and important in mediating the adherence of platelets to the activated endothelium during malaria infection (Massberg *et al.*, 1998). Also a significant moderate positive correlation was found between MPC and P-selectin which further confirms the relationship between malaria infection and P-selectin activity.

The finding showed a significantly higher level of GP IIb/IIIa in TB/MP+ at pre-treatment and a lower level in TB/MP+ subjects at 6-month into treatment as well as a lower but non-significant level in TB/MP+ at 2-month into treatment. This may suggest that malaria infection induces an increase in the level of GP IIb/IIIa at treatment naive which reverses with anti-tuberculosis therapy at 2-month and 6-month into treatment.

This study found a significantly higher level of PAF in TB/MP+ at pre-treatment and a significant lower level at 2-month into treatment as well as a non-significant lower level at 6-month into treatment. According to Davis *et al* (1995) increased availability of PAF may reflect parasite burden, thus we can assert that malaria parasite infection increased the PAF level at pre-treatment. However, the reduction from 2-month into treatment suggests that the malaria-induced increase in PAF at pre-treatment is reversed with the initiation of TB therapy.

Generally, haematological changes are some of the most common complications in malaria infection and the changes involve red blood cells, leucocytes and thrombocytes (Maina *et al*., 2010; Bakhubaira, 2013; Wolfswinkel *et al*., 2013; Warimwe *et al*., 2013). The findings of this study shows that at pre-treatment, there was a significant reduction in total white cell count, neutrophil count, lymphocyte count and neutrophil-lymphocyte ratio in TB/MP+ compared to TB/MP- patients. According to previous studies, Malaria-induced changes in the differential white cell counts are very diverse and contradictory and include leucopoenia, lymphopaenia, lymphocytosis, the presence of atypical lymphocytes, monocytosis, neutropenia, neutrophilia, immature neutrophils (band cells), eosinopaenia, eosinophilia and leukemoid reactions (Taylor *et al*. 2008; Gonzalez *et al*. 2009). The reduction in lymphocyte count in this study agrees with that of Rodrigues-da-Silva *et al* (2014) and Berens-Riha *et al*

(2014). The decreased lymphocyte levels during malaria co-infection have been attributed to the reallocation of cells to deep lymphoid organs or by parasite induced apoptosis of human mononuclear cells (Balde *et al*. 2000). Also the significant reduction observed in white blood cell counts, neutrophil count, lymphocyte count agrees with the work of Kotepui *et al* (2014), but their finding of a significant higher NLR disagrees with our finding. Francis *et al* (2014) also found a decreased neutrophil count. The decrease in neutrophil count is in contrast with some previous studies by Berens-Riha *et al* (2014) as well as Maina *et al* (2010) who suggested that the increase might be due to activated neutrophil production or release from the marrow or suppressed peripheral removal.

The eosinophil count was significantly higher in TB/MP+ both at pre-treatment and at 6-month into treatment. This is expected since eosinophils are known to play a major role in parasitic infections and malaria-TB co-infection may not be an exception. The major function of eosinophil as a cytotoxic cell is against parasitic infections and eosinophils can kill a wide variety of parasitic organism especially in their larval stages, by depositing cationic proteins on the surface of the parasite (Wardlaw, 1994). Our finding agrees with a previous study by Tangpukdee *et al* (2008). Furthermore, induction of eosinophils has been attributed to various factors such as higher release of eosinophils caused by *Plasmodium* or a direct response to the parasite, stimulation by cytokines or other mediators produced during malaria attack. However, our finding also disagrees with that of Francis *et al* (2014), Kotepui *et al* (2014) and Rodrigues-da-Silva *et al* (2014) that found a low eosinophil count. Rodrigues-da-Silva *et al* (2014) attributed the lowered eosinophil count in their study to malaria suppression of eosinophil production and release from the bone marrow or enhanced the peripheral removal of these cells.

Packed cell volume (PCV) was significantly lower in TB/MP+ at 6-month into treatment. This agrees with the findings of Francis *et al* (2014). The mechanism of reduction in PCV during malaria co-infection is not clearly understood, however it may result from the fact that the parasites' primary target is the red blood cell resulting in RBC destruction, accelerated removal of both parasitized and non-parasitized and bone marrow dysfunction. Malaria causes the excessive destruction of red blood cells during the parasites life cycle because there is the parasitization of red cells by the malaria parasite which leads to shortened survival or death of erythrocytes (Ajibola *et al*., 2012). Anaemia is a very common presentation of malaria due to direct depression of erythropoiesis by malarial infection and the actual parasitization of red cells by the malaria parasite leading to shortened survival or

death of erythrocytes. For the above reasons, packed cell volume and haemoglobin values are usually reduced when measured in malaria positive patients (Iwunze *et al.*, 2015).

The platelet count was significantly lower in TB/MP+ at pre-treatment. Malaria infections are commonly accompanied by a thrombocytopenia (Lacerda *et al.*, 2011) or loss of platelets, the severity of which closely mirrors the increasing parasite mass (McMorran *et al*., 2009). The underlying causes of the reduction in platelet count in malaria infection have been variously attributed to systemic platelet activation, immune-mediated clearance and vascular pooling (de-Mast *et al*., 2010; Coelho *et al*., 2013). The reduction in platelet count in TB/MP+ in this study may also be related to the finding of Kho *et al* (2018) that Platelet-erythrocyte complexes (which results from the preferential binding of platelet to infected erythrocytes more than uninfected erythrocytes) makes up a major proportion of the total platelet pool in patients with malaria and may therefore contribute considerably to malarial thrombocytopenia. Similarly, the reduction in platelet count in TB/MP+ may also be linked to the role of platelet in innate control of Plasmodium infection in human malaria that involves control of the growth of intra-erythrocytic *Plasmodium* parasites by directly binding to infected erythrocytes and killing the parasite inside via the action of PF4 (McMorran *et al.*, 2013; Kho *et al*., 2018). This process results in the utilization of platelet in parasite killing that result in the decrease in peripheral circulation. Additionally, previous studies have suggested that thrombocytopenia is a poor prognostic marker in malaria (Moerman *et al*., 2003), thus the finding of a lowered platelet count at pre-treatment suggests poor prognosis which may have improved after 2-month and 6-month into treatment, thus the non-significant difference observed in the study. However, our findings disagrees with that of Aisha *et al* (2012) that found an increase in platelet count which they attributed to platelet activation resulting from malaria parasite infection because in addition to their well-defined role in haemostasis, platelets are increasingly implicated in immunological processes, including direct pathogen-killing functions (Yeaman, 2010; de-Stoppelaar *et al*., 2014; Ali *et al*., 2017) as earlier described. We also observed a significant moderate negative correlation between MPC and platelet count. This agrees with the findings of Kho *et al* (2018) that patient platelet counts and platelet-associated parasite killing correlated inversely with patient parasite loads. It also agrees with the study of Jeremiah and Uko (2007) that found an inverse relationship between parasite density and platelet count as well as that of Rodrigues-da-Silva *et al* (2014) that also observed a negative correlation between platelet counts and malaria parasitaemia.

This study showed a male preponderance, with TB-infected male to TB-infected female ratio of 1.4:1. This is similar to the findings of Nagpal *et al* (2015) that also obtained a male to female ratio of 1.5:1. This may suggest that males are more susceptible to tuberculosis than women as reported in a previous study (Heranadez-Garduano and Elwood, 2008). Also studies have reported that sex differentials in prevalence rates begin to appear between 10 to 16 years of age and remain higher in males than females thereafter. This was also corroborated by the WHO Global TB report (2018) that found that out of the globally estimated 10.0 million people (range, 9.0–11.1 million) that developed TB disease in 2017; 5.8 million were men, 3.2 million women and 1.0 million were children. Though the reason for the higher male prevalence and incidence is poorly understood, Shepherd and Alasdair (2014), posits that gender can affect *M. tuberculosis* exposure because of differences in social roles, risk behaviors, and activities. Males may travel more frequently, have more social contacts, spend more time in settings that may be conducive for transmission, such as bars, and engage in professions associated with a higher risk for tuberculosis, such as mining (Oni *et al.,* 2012; Narasimhan *et al.,* 2013).

Assessment of inflammatory cytokines in male and female TB subjects is needful because gender is a major factor influencing the regulation of inflammatory factors; possibly due to a specific hormonal balance or adipose tissue distribution in women (Popko *et al*., 2010). Female sex exerts a permissive influence over inflammatory responses, a phenomenon thought to be driven principally by the activity of female sex hormones (Mendelsohn and Karas, 2005). Several mechanisms could explain the sex differences in inflammatory markers. The well-documented sex differences in body fat distribution and systemic sex hormone concentrations could represent major factors in the sex dimorphism. In particular, women generally have a higher percentage of body fat than do men and many studies have shown that an excessive accumulation of fat in the abdominal region, especially visceral obesity, was often associated with a prothrombotic and pro-inflammatory state (Ross *et al*., 2002). Fish (2008) noted that as a general rule, females exhibit more-robust immune responses to antigenic challenges, such as infection and vaccination, than males which is mediated to a large extent by sex hormones. The role of sex hormones in this gender bias is supported in tuberculosis infection by the fact that it does not arise until puberty (Marais *et al*., 2004). However, Casimir *et al* (2010) agreed that gender influences clinical presentations and markers in inflammatory diseases, but contested the general notion of hormonal involvement in the gender difference in inflammatory markers and opined that the persistence

of this gender dimorphism over the whole lifetime casts doubts on its direct relationship with the individual hormonal status.

In this study, both TNF-α and IL-10 were significantly higher in male TB subjects than female TB subjects at 2-month into treatment. This confirms the generally observed trends of increased production of some cytokines in males compared with females (Casimir *et al*., 2010). This agrees with the findings of Cartier *et al* (2009) that tumor necrosis factor-alpha (TNF-α) concentrations have been reported to be lower in women than in men, as well as that of An *et al* (1999) that plasma IL-6 and TNF-α concentrations have been found to be lower in women than in men, possibly because of the inhibitory effect of estrogens on the expression of inflammatory marker genes. Even in animal study this finding holds true as reported by Inmaculada *et al* (2011) that TNF-α were also suppressed in female compared with male mice. Aside from these there are also some contradicting research findings. Wegner *et al* (2017) revealed greater in vivo pro-inflammatory responses in women compared with men, with significantly higher increases in plasma TNF-α and IL-6 concentrations which could be due to the finding that both IL-6 and TNF-α are secreted by adipocytes and their concentration correlates with the percentage and distribution of fat tissue in the body (Popko *et al*., 2010). Auda *et al* (2013) found no significant difference between serum IL-10 concentration in male TB patients and female TB patients while Olaniyan *et al* (2018) found no significant age and gender influence on the plasma values of IL-10 and TNF alpha.

This study found a contradiction in the gender comparison of IL-6 at pre-treatment and 2-month into treatment. While it was significantly higher in male TB subjects at pre-treatment, it was significantly higher in female TB subjects at 2-month into treatment. However, most studies reported a higher level in males than females. This includes the study by Sperry *et al* (2008) that found that IL-6 serum levels were statistically higher in males relative to females and this higher level of IL-6 expression in males remained statistically significant over time even after controlling for differences in age. Cartier *et al* (2009) also found that IL-6 was significantly lower in women than males. This reduced level of IL-6 in females may be possibly because of the inhibitory effect of estrogens on the expression of inflammatory marker genes (An *et al*., 1999). Casimir *et al* (2010) summed it up that higher levels of cytokines in males could be explained by differences in the complex relationship between inflammatory mediators with a possible role of IL-6 that could be more potent in males than in females. Conversely, the findings of Wegner *et al* (2017) revealed greater in vivo pro-

inflammatory responses in women compared with men, with significantly higher increases in plasma IL-6 concentrations. This finding which for non-obvious reasons seem to tarry with our finding at 2-month into treatment could be due to the fact that markers of inflammation strongly correlate with measures of adiposity, and this association has been reported to be generally stronger in women than in men, especially for IL-6 (Thorand *et al*., 2006). Popko *et al* (2010) also reported that both IL-6 and TNF-α are secreted by adipocytes and their concentration correlates with the percentage and distribution of fat tissue in the body. This gives women an advantage over men in the production of these cytokines.

This study found a significant higher level of P-selectin in male TB subjects at pre-treatment, 2-month and 6-month into treatment. This means that irrespective of treatment status, this adhesion molecule is always higher in male TB subjects than female TB subjects. This may be explained by the findings of Jima *et al* (1996) that lower plasma levels of P-selectin was observed in premenopausal women compared to those in men. They also found that increase in estradiol led to a decrease in P-selectin in those women. They concluded that estradiol down-regulate the expression of P-selectin in women. Immaculada *et al* (2011) also found lower leukocyte recruitment in female subjects which they linked to changes in endothelial adhesion molecule (such as P-selectin) expression. They supported their finding with the view that female sex hormones exert a modulating effect on the expression of P-selectin on endothelial cells during leukocyte recruitment.

Thrombopoietin hormone (TPO) was significantly higher in male TB subjects at 2-month into treatment than female TB subjects in our study. This agrees with the findings of Butkiewicz *et al* (2006) that Thrombopoietin concentration is gender-dependent and is lower in women than in men.

This research findings showed a significant higher values of total white cell count (TWBC), neutrophil count (NEUT), Lymphocyte count (LYM), Monocyte count (MONO), eosinophil count (EOS) and MLR in male TB-infected subjects compared to female TB-infected subjects at 2-month into treatment. Inmaculada *et al* (2011) in their report have demonstrated the existence of a sex difference in leukocyte recruitment under inflammatory conditions and implicated female sex hormones in mediating this effect. Similarly, several studies have demonstrated that sex hormones (particularly estrogen) suppress the leukocyte recruitment that is evident in innate immune responses in experimental models of inflammation using both in vivo and in vitro models (Card *et al*., 2006). Aside from recruitment, neutrophil

activation also appears to be greater in males and is potentiated by testosterone and limited by estrogen (Deitch *et al*., 2006).

Both PCV and NLR were significantly higher in male TB subjects at pre-treatment. The higher PCV was similar to those published in literature (Bohler *et al*. 2008). This rise in blood parameters has been attributed to the hormone androgen, which stimulates erythropoiesis and increases the number of circulating erythrocytes and consequently, PCV and Hb (Oladele *et al*., 2001). The haematocrit (PCV) in healthy individuals varies predictably with the haemoglobin content of the blood it is measured in, and can be used interchangeably with haemoglobin level to compare the same value in populations of healthy individuals (Murphy, 2014). This sex difference is constitutive, and women do not attempt to achieve male levels in health (Murphy *et al.*, 2010; Tilling *et al.*, 2013). Androgens raise the haemoglobin level in males and females, and oestrogen lowers it (Coviello *et al*., 2008). Thus the sex difference in mean venous haemoglobin levels and red cell mass is generally considered to be caused by a direct stimulatory effect of androgen in men in the bone marrow in association with erythropoietin, a stimulatory effect of androgen on erythropoietin production in the kidney, and an inhibitory effect of oestrogen on the bone marrow in women (Shahani *et al*., 2009; Jelkmann, 2011). However, the rationale for the higher value of NLR in male TB subjects is unclear.

This study found a significantly higher platelet count in male TB subjects at pre-treatment but a significantly higher count in females at 2-month into treatment and a non-significantly higher count in females at 6-month into treatment. In line with our finding of a higher platelet count in female TB subjects at 2-month and 6-month into treatment, Butkiewicz *et al* (2006) stated that platelet count is gender dependent and that in women, platelet count is higher than in men, which seems to reflect different hormonal profiles or a compensatory mechanism associated with menstrual blood loss. Interestingly, both older and current research findings support this line of thought which suggests that it isn't a new finding but an affirmation of what has been documented in literature (Breet *et al*., 2011). Previous study by Campelo *et al* (2012) asserts that sex hormones namely testosterone and estrogen have potential influence and regulatory role in platelet function. However this generally accepted finding seems to be reversed before the initiation of anti-tuberculosis therapy at treatment naive wherein the male TB subjects had a higher platelet count. The reason for this deviation is not clear.

The TB subjects in this study were classified into different age groups as young adults (18-30 years), middle-aged adults (31-50 years) and older adults (>50 years) according to Petry (2002).

It has been well established that the immune system is compromised in aged individuals (Ponnappan and Ponnappan, 2011). While changes occur in both arms of immunity, innate and adaptive, studies have demonstrated that certain specific immune responses are diminished, leaving others unaffected or exacerbated. This decrease in immunity that occurs for the large part, often referred to as ‘‘immune senescence,’’ has been attributed to be the basis of increased frequency and severity of infections, lowered immune surveillance of malignant cells, and decreased efficacy of vaccination in the elderly (McElhaney and Effros, 2009). Also as a part of the age-associated deterioration of the immune system there is a chronic pro-inflammatory state even in the absence of clinically-apparent disease. This state defined as “inflamm-aging” is characterized by elevated circulating levels of pro-inflammatory factors (IL-1β, IL- 6, TNFα, and prostaglandin E2) and anti-inflammatory mediators, (IL-1 receptor antagonist, soluble TNF receptor, IL-10, transforming growth factor beta, acute phase proteins, C-reactive protein, and serum amyloid A) and contributes to the decreased ability of the elderly to mount an appropriate immune response following an infectious challenge (Trzonkowski *et al*., 2004). One of the most prominent aspects of “inflammaging” is in presence of elevated circulating levels of the pro-inflammatory cytokine, interleukin (IL)-6 (Franceschi *et al*., 2000). Similarly, specific cytokines and chemokines, which are signal molecules produced by innate immune cells, have been reported to substantially alter with age, especially pro-inflammatory cytokines such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF-α), and TGF-β, leading to chronic inflammation, and thus contributing to the inflamm-aging phenotype, often observed in the elderly (Bruunsgaard *et al*., 2003). Increases in pro-inflammatory cytokines have been attributed to be the underlying basis of the progression of degenerative geriatric diseases that often accompany advanced age.

In this study we found that TNF-α was significantly higher in young adults (18-30 years) compared to middle-aged adults (31-50 years) and older adults (>50 years) at 6-month into treatment. This suggests a decrease in TNF-α level with increasing age as against the general notion that inflammatory markers increase with age as earlier discussed. Also Peters *et al* (2009) stated that with regard to inflammatory cytokines and chemokines, studies in humans

have demonstrated a significant increase in levels of TNF-α, IL-6 and IL-1 in monocytes obtained from peripheral blood of older subjects. However, Olaniyan *et al* in their own study found that there was no significant age influence on the plasma values of IL-10 and TNF alpha.

This study also found that at pre-treatment the level of IL-6 was significantly higher in older adult TB subjects (>50 years) compared to young and middle aged adult TB subjects (18-30 years and 31-50 years). But conversely, at 6-month into treatment the level of IL-6 was significantly lower in older adult TB subjects (>50 years) compared to middle-aged and younger adult TB subjects (31-50 years and 18-30 years). Some researchers have reported an increase, decrease, or no effect of age on interleukin-6 (IL-6) production. Differences in experimental conditions and the health status of subjects may explain these contradicting results. The increase in IL-6 in older TB subjects in our study agrees with the finding of Ferrucci *et al* (2005) that there is strong evidence that IL-6 serum concentration increases with age. Many of the changes in immune function seen with advanced age may be the consequence of increased IL-6 production, and Maggio *et al* (2006) noted that the IL-6 pathway appears to be profoundly implicated in the pathophysiology of physical function decline and chronic diseases that often affect older persons. All these findings points to the role of IL-6 in old age and various reasons have been adduced for this increase in IL-6 with age. While some researchers hypothesized that age-related change in the endocrine system somehow "relax" the strict control over IL-6 expression, resulting in its increased production with age (Straub *et al.*, 1998). Other researchers, such as Roubenoff and colleagues (1998) suggested that IL-6 elevation is related to both age and inflammation and therefore the increase in IL-6 may not be solely a phenomenon of advancing age. Ershler and Keller (2000) linked the increase to hormonal influence. They stated that among several factors that down-regulate IL-6 gene expression are estrogen and testosterone. Thus after menopause (in female) or andropause (in males) which results to reduction in estrogen and testosterone, IL-6 levels are elevated, even in the absence of infection, trauma, or stress. However, Alison *et al* (2001) had a different finding, that circulating IL-6 levels were not significantly different between young and elderly subjects. They argued that in healthy subjects, increased production of IL-6 is not a normal consequence of aging. They went further to assert that previously reported higher IL-6 levels in elderly subjects might reflect an underlying, undiagnosed disease state and concluded that IL-6 may be elevated in most elderly persons due to the presence of disease which are common in elderly persons.

The reduction in IL-6 among older adults at 6-month into treatment in this study is similar to what was obtained for TNF-α. This may suggest that the generally acknowledged increase in pro-inflammatory cytokines in elderly people is reversed in older adult TB subjects after treatment probably by the generalized down-regulation of inflammatory markers at 6-months follow-up in TB subjects. Further research is needed to reach a definite conclusion on this.

IL-10 was significantly higher in older adult TB subjects (>50 years) at pre-treatment compared to younger adults and middle-aged adult TB subjects (18-30 years and 31-50 years). There isn't any clear explanation for this finding except to assume that there is an increased level of anti-inflammatory marker (IL-10) in elderly TB subjects prior to initiation of therapy. But at 2-month into treatment, it was significantly higher in younger adults (18-30 years) compared to middle-aged (31-50 years) and older adult TB subjects (>50 years). This agrees with previous findings that anti-inflammatory cytokine levels (IL-10 and IL-4) decrease with age (Kuzumaki *et al*., 2010). Aside this, Olaniyan *et al* (2018) in their research found no significant age influence on the plasma values of IL-10 and TNF alpha.

The findings showed that TGF-β was higher in younger adult TB subjects (18-30 years) at 6-month into treatment compared to middle-aged adults (31-50 years) and older adult TB subjects (>50 years). This decrease with age may be explained in the light of the role of TGF-β. According to Kraan (2014), age is the most important risk factor for primary osteoarthritis and members of the TGF-β superfamily play a crucial role in chondrocyte differentiation and maintenance of healthy articular cartilage. Thus the increased incidence of osteoarthritis with age implies a tendency for a decrease in TGF-β.

The level of IL-2 at pre-treatment was significantly lower in older adult TB subjects (>50 years) than middle-aged and younger adult TB subjects. While at 2-month into treatment it was significantly higher in older adult TB subjects (>50 years) compared to middle-aged adults (31-50 years) and younger adult TB subjects (18-30 years). These conflicting findings can generally be linked to the fact that aging is associated with dys-regulation of the immune and inflammatory responses, including changes in the regulation and production of cytokines.

The finding of this study showed that GP IIb/IIIa was significantly higher in younger adult TB subjects (18-30 years) than middle-aged adult TB subjects (31-50 years) and older adults TB subjects (>50 years) at pre-treatment and 6-month into treatment while PF4 was significantly higher in middle-aged adult TB subjects at 2-month into treatment. A previous

study had shown that age is associated with an increase in platelet aggregability (Campelo *et al*., 2012). Esan (2016) reported that increased plasma levels of two platelet alpha-granule constituents, ß-Thromboglobulin and platelet factor-4 (PF-4), have been found in individuals over 65years of age in comparison with younger individuals. This was supported by the finding of Franchini (2006) that aging results in significant increases in the release of plasma β-Thromboglobulin (β-TG), plasma platelet factor-4 (PF-4), platelet aggregation and plasma fibrinogen. These findings do not align with the finding of this study.

Just as the immune system develops and matures with age, various haematological parameters have also been shown to change and develop with age (Mahlknecht and Kaiser, 2010), although other factors such as genetics, sex, altitude, and life style may affect this process. According to different studies the hemoglobin concentration, red blood cell count and hematocrit value begin to decrease in men in their sixth decade and in women in their seventh decade and the change are more prominent with advancing age, especially in men (Dey, 2006). This can be correlated with some hormonal changes in females and poor nutrition resulting in vitamin B12 and folic acid deficiency in old age which might be the cause of haematological changes and aging (Padalia *et al*., 2014). Also, according to Esan (2016) there is a continuous decrease in the volume of the hematopoietic marrow with age.
This study shows that the total white blood cell count (TWBC) was significantly higher in older adult TB subjects (>50 years) compared to middle-aged adults (31-50 years) and younger adult TB subjects (18-30 years) at pre-treatment. According to Aminzadeh and Parsa (2011), total white blood cells (WBCs) decrease slightly in the elderly but in response to an acute infection, the number of WBCs increases and in sepsis the increase is very dramatic. This supports our finding since the subjects were TB infected. However there are varying findings also. Beutler *et al* (2011) and Esan (2016) found no consistent, significant variation in the total white blood cell count in older subjects; rather normal total white blood cell count and neutrophil counts were found in nonagenarian and centenarian populations. Esan (2016) also reported that leukocyte count does not rise as high in response to infection in elderly individuals as in young people.

This study also found a significant higher neutrophil count in older adult TB subjects (>50 years) at pre-treatment compared to middle-aged adults (31-50 years) and young adult TB subjects (18-30 years). Previous studies have shown significant changes in neutrophil function but not in neutrophil count. According to Fortin *et al* (2008), while neutrophil counts

in the blood of the elderly appear to be comparable to those in healthy young individuals, neutrophil function is significantly compromised in older humans and the reported decline during aging in neutrophils appears to encompass major functional attributes and include lowered chemotaxis, decreased phagocytosis of microbes, and reduced generation of superoxide in response to pathogen-associated molecules. Ponnappan and Ponnappan (2011) noted that in the light of increased bacterial infections and chronic inflammatory conditions that often accompany aging, defects in the functional response, but not in the numbers, of neutrophils has long been demonstrated in human aging. Esan (2016) posits that the recruitment of neutrophils in response to exogenous stimuli is slightly decreased, but the response to infection does not appear impaired, thus neutrophil function is not significantly decreased with age of the subject.

This study showed that at pre-treatment, eosinophil count was significantly higher in older adult TB subjects (>50 years) compared to middle-aged adult TB subjects (31-50 years), while at 2-month into treatment it was significantly higher in middle aged adult TB subjects (31-50 years) compared to young adult TB subjects (18-30 years). According to Mathur *et al* (2008) as opposed to neutrophils, there is a paucity of information on cellular function with advancing age, with regard to eosinophils and basophils. However, they reported age-related changes in peripheral blood eosinophil "effector" functions.

The finding of this study shows that PCV was significantly lower in older adult TB subjects (>50 years) compared to middle aged adult (31-50 years) and young adult TB subjects (18-30 years) at pre-treatment. Similarly at 2-month into treatment, it was also significantly lower in older adult TB subjects (>50 years) compared to young adult TB subjects (18-30 years). This confirms a reduction in PCV with age in TB subjects. Generally, anaemia has become a common concern in geriatric health (Mahlknecht and Kaiser, 2010). According to Izaks and Westendorp (2003), the evaluation of haemoglobin or PCV levels in the elderly is a complex task, since it is difficult to assess whether a value out of the normal range in a given individual is the result of an underlying disease or whether it is a phenomenon of the expression of age. However, numerous factors have been described as affecting blood counts in the elderly. These includes; reduced numbers of haematopoietic stem cells, the finite number of cell divisions, a defect in progenitor cell proliferation, the inability to sufficiently mobilize such progenitors, the lack of hormonal stimulation or the reduced response to hormonal stimulation and a considerable decline in oxygen need due to the diminishing body mass and/or physical activity (Nilsson-Ehle *et al.*, 2000).

The neutrophil-to-lymphocyte ratio (NLR) which has been used as a surrogate marker of systemic inflammation was also significantly higher in older adult TB subjects in our study at pre-treatment. This also confirms the prominence of inflammation in TB subjects with age.

The Monocyte-lymphocyte ratio (MLR) was significantly lower in older adult TB subjects (>50 years) at 2-months compared to middle-aged adult TB subject (31-50 years). A lower MLR implies a good combative power against TB. The reason for this in the older adults who ought to have a weaker immune system compared to the middle aged adult is not obvious.

A significant higher platelet count was found in older adult TB subjects (>50 years) compared to middle-aged adult TB subjects (31-50 years) at 2-month into treatment. According to Segal and Moliterno (2006), Biino *et al* (2013) and Mohebali *et al* (2014), age is a major factor giving rise to inter-individual variation in platelet count and function. This notwithstanding, the finding of an increased platelet count in older adult TB subjects is a deviation from previous findings such as that by Jones (2016) that found a relatively stable Platelet count during middle age (25–60 years old) that falls in older people. They posited that the changes seen in platelets during ageing are likely to result from changes in hematopoietic tissue, alterations in platelet production and a reaction to the environmental changes within the blood or vasculature. Other researchers also found that Platelet count is stable up to about 60 years old and then falls (Biino *et al*. 2011; Troussard *et al*., 2014). Also Biino *et al* (2011) found that in a given population, a 10-year increase in age corresponds to a 9 x 10^9/l decrease in platelet count, while Biino *et al* (2013) found that platelet count in old age was reduced by 35% in men and by 25% in women with respect to early infancy. However, Esan (2016) reported that platelet count does not change with age. All these findings disagree with the findings of this study. It is however suspected that infection with tuberculosis and administration of intensive phase of therapy for 2-months may be a factor that defines the differences in finding.

5.2. Conclusions

Inflammation in TB subjects is highest at 2-month into treatment as indicated by an increase in TNF-α, IL-6 and IL-2 and is down-regulated at 6-month into treatment as indicated by a decrease in these parameters, while anti-inflammatory response is highest at 6-month into treatment as indicated by an increase in regulatory cytokines (IL-10 and TGF-β). The levels of P-selectin, GP IIb/IIIa, PF-4 and TPO are influenced by inflammatory cytokines because they change accordingly with changes in TNF-α, IL-6 and IL-2 in TB subjects. Thus

Inflammation shifts the haemostatic activity towards procoagulant state by moderating the levels of some haemostatic parameters such as P-SEL, GP IIb/IIIa, PF-4 and TPO in TB subjects.

Tuberculosis infection is marked by an initial increase in white cell parameters (TWBC, NEUT, LYM, MONO, EOS, NLR and MLR) in TB subjects after infection (before initiation of therapy) that decreases with anti-tuberculosis treatment. This implies that they could be a good prognostic index in TB subjects. Most WBC parameters (TWBC, NEUT, LYM, NLR, and EOS) were exclusively increased in TB/malaria co-infected subjects before therapy but not after initiation of therapy except EOS that was also increased in TB/MP co-infection at 6-month into treatment. Packed cell volume (PCV) is increased by initiation phase therapy and decreased by continuation phase therapy. Tuberculosis infection is normally marked by a decreased Body Mass Index tending towards under-weight but increases with initiation of treatment.

In comparison to TB subjects not infected with malaria, inflammation is higher in TB subjects infected with malaria at the second month of treatment as indicated by a higher level of TNF-α, IL-6, and IL-2 vis-a-vis the case at pre-treatment and 6-month into treatment where there is a reduced level of TNF-α and IL-6. P-selectin levels increased significantly in MP-infected TB subjects at pre-treatment and at 6-month into treatment. GP IIb/IIIa and PAF level increases with MP infection in TB subjects before therapy and reverses with initiation of intensive phase therapy and continuation phase therapy.

Males are predominantly infected with TB infection with Male/female ratio of 1.4:1. P-selectin level is consistently higher in male TB subjects irrespective of treatment status and duration of treatment. Majority of the parameters (TNF-α, IL-10, TPO, and WBC parameters) were higher in males TB subjects at 2-month into treatment and does not differ with the female values at other times. Initiation of anti-tuberculosis therapy reverses the trend of a higher platelet count in males seen at pre-treatment. Differences in age groups lead to variations in some parameters at different times in the course of infection and treatment of TB subjects.

5.3. Recommendations

Based on the high pro-inflammatory and pro-coagulant state established in this study at the second month into treatment, it is recommended that TB patients should be closely monitored

with necessary laboratory investigations in the first 2 month to rule out the risk of thrombotic complications.

Further studies on the role of gender-based hormonal variations in the modulation of these parameters in TB subjects is recommended especially at 2-month into treatment where a predominantly increased level of most of the parameters was observed in male TB subjects compared to the females.

Contribution to knowledge

Changes in the levels of P-selectin (P-SEL), Platelet glycoprotein IIb/IIIa (GP IIb/IIIa), Platelet factor-4 (PF-4) and Thrombopoietin hormone (TPO) align with the alteration in inflammatory cytokines (TNF-α, IL-6 and IL-2) in TB subjects.

The risk of thrombotic complications is highest in the first two months of TB treatment because of the high pro-inflammatory response that creates a pro-coagulant state.

P-selectin level is consistently higher in male TB subjects irrespective of treatment status and duration of treatment

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APPENDIX 1

SAMPLE SIZE DETERMINATION USING G*POWER

F tests - ANOVA: Repeated measures, within factors

Analysis:	A priori: Compute required sample size	
Input:	Effect size f	= 0.25
	α err prob	= 0.05
	Power (1-β err prob)	= 0.90
	Number of groups	= 1
	Repetitions	= 3
	Corr among rep measures	=0.5
	Nonsphericity correction ε	=0.5
Output:	Noncentrality parameter λ	=10.875000
	Critical F	= 4.009868
	Numerator df	= 1.000000
	Denominator df	= 57.000000
	Total sample size	= 58
	Actual power	= 0.900028

APPENDIX 2

ETHICS APPROVAL LETTER

APPENDIX 3

LETTER OF PERMISSION TO COLLECT SAMPLE

APPENDIX 4

INFORMED CONSENT FORM

I, Okeke, Chizoba Okechukwu a PhD student of the Department of Medical Laboratory Science, Nnamdi Azikiwe University, Nnewi Campus is carrying out a study on "Haemostatic and inflammatory markers changes in tuberculosis treatment naïve subjects and following directly observed treatment short course in Abakaliki."

Purpose of study

Hypercoagulable state and marked inflammation has been reported in active tuberculosis. This study therefore is aimed at assessing the changes in inflammatory and haemostatic monitors of pulmonary tuberculosis infected subjects with DOTS administration.

Method of recruitment

Consenting subjects shall be randomly selected for this study. For me to effectively achieve my objectives, I shall collect 8ml of blood from each participant, which will be used only to conduct tests that are necessary and specified in this research work.

Participation/risk/benefit/confidentiality

Participation is strictly voluntary and you may choose to withdraw your participation in the research at any time without any victimization of any sort. No risk is involved in participating in this study and no payment of any kind is involved. Strict confidentiality of any information supplied and that obtained from the blood analysis will be maintained.

Declaration of consent

I have been fully informed about the protocol of this study. I know about the purpose, risks and benefits of this research. I also understand that my participation is voluntary and has the right to decline at any stage of the study without being victimized for doing so.

I am willing to participate in this study.

…….

Participant's signature and Date

For enquiries and complaints please contact;

Okeke Chizoba O.

(07033484245)

Department of Medical Laboratory Science N.A.U Nnewi Campus.

APPENDIX 5

RESEARCH QUESTIONNAIRE

Dear participant,

I am Okeke Chizoba O., a PhD student of the Department of Medical Laboratory Science, Nnamdi Azikiwe University, Nnewi Campus. I am carrying out a research on the "**Haemostatic and inflammatory markers changes in tuberculosis treatment naïve subjects and following directly observed treatment short course in Abakaliki."** The information requested below is purely for the purpose of this research work and absolute confidentiality is assured.

SECTION A: SOCIO-DEMOGRAPHIC DETAILS

1. Research ID/no:…….

2. Age (years) ……

3. Gender: (a) Male [ ] (b) Female [ ]

4. Weight (kg) ……

5. Height (metres)……..……..

6. Blood pressure (mmHg): Systolic…… Diastolic…….

7. Occupation……..……..

8. Marital status: (a) Single [ ] (b) married [ ] (c) Divorced [ ] (d) Widowed [ ]

9. Educational status: (a) Tertiary [ ] (b) Secondary [ ] (c) Primary [ ] (d) none [ ]

SECTION B: CLINICAL PRESENTATION

10. How long have you been infected with TB …….

11. How long have you been on treatment(a) 2 months [ ] (b) 4 months [ ] (c)6 months [ ] (d) > 6 months [ ]

12. Have you been transfused with blood in the last three months (a) Yes [ ] (b) No [ ]

13. Have you had any major bleeding episode in the past? (a) Yes [ ] (b) No [ ]

14. If yes do you have any known bleeding disorder? (a) Yes [ ] (b) No [ ]

15. If yes mention the bleeding disorder……..

16. Do you smoke cigarettes? (a) Yes [ ] (b) No [ ]

17. Do you take alcohol? (a) Yes [ ] (b) No [ ]

18. Do you have any co-infection (Such as HIV, HBV, HCV etc)? (a) Yes [ ] (b) No [ ]

19. If yes mention the co-infection ……

20. Are you on any oral contraceptives (females only)? (a) Yes [ ] (b) No [ ]

21. Are you pregnant (females only)? (a) Yes [ ] (b) No [ ]

22. Are you on any supplements or herbal drugs? (a) Yes [ ] (b) No [ ]

23. If yes mention ……

24. Are you on any hormone replacement therapy? (a) Yes [ ] (b) No [ ]

25. Are you taking Aspirin or any anticoagulant drugs? (a) Yes [ ] (b) No [ ]

26. Do you suffer from any known clinical disease (such as Diabetes, Cancer, Renal or Liver disease etc). (a) Yes [ ] (b) No [ ]

SECTION C: SIGNS/SYMPTOMS

27. Tick the boxes indicating the symptoms you experience;

(a) loss of weight [ ]
(b) Loss of appetite [ ]
(c) Fever [ ]
(d) Weakness/fatigue [ ]
(e) Night sweat [ ]
(f) Persistent/chronic cough [ ]
(g) Chest pain [ ]
(h) Blood-tinged sputum [ ]

APPENDIX 6

REAGENT PREPARATION

Kit components for TNF-α, IL-10, IL-6 and IL-2

Coating antibody (lyophilized), standard (lyophilized), Biotinylated detection antibody (lyophilized), SPP conjugate (lyophilized), BSA stock solution (10%), Cytokine stabilization buffer (CSB), Tween-20, TMB substrate solution, Stop solution, ELISA plates and Adhesive cover slips.

Reagent and sample preparation for TNF-α, IL-10, IL-6 and IL-2

All samples were brought out from storage at -80^0C and allowed to thaw at room temperature. A 1/20 volume of Cytokine stabilization buffer (CSB) was added to the pure serum samples to inhibit the degradation of cytokines. The reagents were also brought to room temperature and reconstituted according to the manufacturer's instructions as follows; The lyophilized SPP conjugate and lyophilized Biotinylated detection antibody were reconstituted by injecting 500µl of sterile distilled water into the vials respectively and the solution mixed gently for approximately 15 seconds and allowed to stand at room temperature for 5 minutes. For one ELISA plate, 100µl of each was gently and thoroughly mixed with 10ml Dilution buffer. The lyophilized coating antibody was reconstituted by injecting 250µl of sterile distilled water into the vial and the solution mixed gently for 15 seconds and allowed to stand for 5 minutes at room temperature. For one ELISA plate, 50µl was gently but thoroughly mixed with 5ml of Phosphate buffered saline (PBS). The lyophilized standard was reconstituted by injecting 500µl of sterile distilled water into the vial and the solution was mixed gently for 15 seconds and allowed to stand for 5 minutes at room temperature. Serial dilutions were made of the reconstituted standard using the dilution buffer in seven tubes (295, 147.5, 73.75, 36.87, 18.43, 9.2, 0 pg/ml).

Kit components for TGF-β, P-selectin, GP IIb/IIIa, PAF, PF-4 and Thrombopoietic

Micro ELISA plate, Reference standard, Concentrated Biotinylated Detection Antibody, Concentrated HRP Conjugated, Reference Standard and sample diluents, Biotinylated Detection antibody Diluent, HRP Conjugate Diluent, Concentrated Wash Buffer, Substrate Reagent, Stop Solution and Plate Sealer.

Reagent and sample preparation for TGF-β

All the reagents and samples were brought to room temperature (18-25^{0}C) before use. TGF-β in biological samples usually exists in unactivated forms. So it must be activated before testing. Thus the samples were heat activated by diluting samples with Sample Diluent at a ratio of 1:10 and placed in the Water bath at 80^{0}C for 8 minutes, taken out and allowed to cool for 5 minutes and tested within 2 hours. The reagents were prepared according to the manufacturer's instructions as follows; The 100x Concentrated HRP Conjugate was diluted to 1x working solution for each plate by mixing 100µl of the stock with 9900µl of HRP Conjugate Diluent. The 100x concentrated Biotinylated Detection antibody was centrifuged and diluted to 1x working solution for each plate by mixing 100µl of the stock with 9900µl of Biotinylated Detection antibody Diluent. The Wash Buffer was prepared by diluting 39ml of Concentrated Wash Buffer with 720ml of deionized water. The Reference standard was centrifuged at 10,000xg for 1 minute and 1.0ml of Reference Standard and sample Diluent and allowed to stand for 10 minutes, inverted gently several times and afterwards mixed thoroughly with a pipette. This reconstitution produces a working solution of 2000pg/ml. Serial dilutions were made in seven tubes using the Reference Standard and Sample Diluent producing dilution gradients of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/ml.

Reagent and sample preparation for P-selectin, GP IIb/IIIa, PF-4, TPO and PAF

All the reagents and samples were brought to room temperature (18-25^{0}C) before use. The reagents were prepared according to the manufacturer's instructions as follows; The 100x Concentrated HRP Conjugate was diluted to 1x working solution for each plate by mixing 100µl of the stock with 9900µl of HRP Conjugate Diluent. The 100x concentrated Biotinylated Detection antibody was centrifuged and diluted to 1x working solution for each plate by mixing 100µl of the stock with 9900µl of Biotinylated Detection antibody Diluent. The Wash Buffer was prepared by diluting 39ml of Concentrated Wash Buffer with 720ml of deionized water. The Reference standard was centrifuged at 10,000xg for 1 minute and 1.0ml of Reference Standard and sample Diluent was added and allowed to stand for 10 minutes, inverted gently several times and afterwards mixed thoroughly with a pipette. This reconstitution produces a working solution of 10ng/ml. Serial dilutions were made using the Reference Standard and Sample Diluent producing dilution gradients of 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0 ng/ml.