Sudan University of Sciences and Technology
College of Graduate Studies

Estimation of Tumor Necrosis Factor alpha and C-Reactive Protein Serum Levels in Sudanese Active Pulmonary Tuberculosis Patients Attending Aboanga Hospital, in Khartoum State, Sudan

تقدير مستوى عامل نخر الورم ألفا وبروتين سي التفاعلي في مصل المرضى السودانيين المصابين بالسل الرئوي النشط المترددين على مستشفى أبو عنجة بولاية الخرطوم، السودان

A Dissertation Submitted in Partial Fulfillment for the Requirements of M.Sc. Degree in Medical Laboratory Science (Microbiology)

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الآية

بسم الله الرحمن الرحيم

قال تعالى:

﴿ اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ ۚ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ ۖ الْمِصْبَاحُ فِي زُجَاجَةٍ ۖ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِن شَجَرَةٍ مُّبَارَكَةٍ زَيْتُونَةٍ لَّا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ ۚ نُّورٌ عَلَىٰ نُورٍ ۗ يَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاءُ ۚ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ ۗ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ ﴾

سورة النور (الآية 35)

DEDICATION

To my beloved parents who made me what I'm today

To my respectful brothers and sister

To whom will find it beneficial work

To those who have made it possible …. Teachers

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First of all, I thank great full to **ALMOGITH ALLAH** who give me ability and strongly to complete this research. I would like to thank my supervisor **Dr. Hind Haidar Ahmed** who gave me a lot of her time and helped me to complete this work. Special thanks to **Prof. Muawia Mukhtar** and **Dr. Mona Omer** and medical staff of **Al Riada laboratory** for their assistant and help.

ABSTRACT

Mycobacterium tuberculosis (M. tuberculosis) is widely distributed pathogen especially in developing countries which cause primary and secondary pulmonary tuberculosis and if untreated, it causes extra pulmonary tuberculosis. This is analytical case control study was aimed to estimate the Tumor Necrosis Factor alpha (TNF-α) and C-reactive protein (CRP) serum levels in Sudanese active tuberculosis compared to healthy control group in Khartoum state during the period from March 2021 to March 2022.

Blood specimen was collected from each subject, 44 of them were active pulmonary tuberculosis patients, and other 44 were apparently health control. TNF-α concentration was measured by Enzyme Linked Immunosorbent Assay (ELISA) in Institute of Endemic Disease and C-reactive protein concentration was measured by DIRUI Auto-Chemistry Analyzer CS-T180 in Al-Riada medical laboratory. The data was analyzed using SPSS Version 20.

Active tuberculosis patients and apparently health control were enrolled in this study, their ages varied from 17-70 years, 44 subjects were active pulmonary tuberculosis, 33 of them were males and 11 were females with mean age (41±18.3 STD) years. The other 44 subjects were apparently healthy control 33 were males and 11 were females with mean age (41±18.3 STD) years. The mean level of TNF-α in active tuberculosis patient is higher (44.4 pg/ml), compared to control group (9.7 pg/ml) with statistical significant difference between case and control group (*p*-value 0.000). There was no statistical significant difference between TNF-α level and age, sex among cases subjects (*p*.value= 0.902, 0.564), respectively. The mean level of CRP in active tuberculosis patient is higher (53.9 mg /L) compared to control group (3.5 mg /L) with statistical significant difference between case and control group (*p*-value 0.000). There was no statistical significant difference between CRP level and age, sex among cases subjects (*p*.value = 0.406, 0.769), respectively.

This study concluded that TNF-α concentration may be a useful diagnostic marker for active tuberculosis infection in combination with CRP level.

المستخلص

بكتيريا السل الرئوي هي باكتيريا واسعه الانتشار وبالأخص في الدول النامية والتي تسبب السل الاولي والثانوي و اذا لم تتم معالجته يتسبب في السل المنتشر خارج الرئة. هدفت هذه االدراسة; دراسة الحالة الضابطه التحليلية إلى قياس مستوى عامل نخر الورم ألفا وبروتين سي التفاعلي في مصل المرضى المصابين بالسل الرئوي النشط بالمقارنة مع افراد اصحاء في ولاية الخرطوم في الفترة ما بين مارس 2021ومارس 2022.

سحبت عينه دم من كل فرد حيث ان 44 شخص منهم مصاب بالسل الرئوي النشط و44 الآخرين كانوا أصحاء .تم قياس تركيز المادة الخلوية عامل نخر الورم **ألفا** عن طريق فحص الممتز المناعي المرتبط بالإنزيم في مركز أبحاث معهد الامراض المستوطنة، كما تم قياس تركيز بروتين سي التفاعلي عن طريق محلل الكيمياء الذاتي DIRUI–CS–T180 في معمل الريادة الطبي الحديث. حللت البيانات باستخدام الحزمة الإحصائية للمجتمع نسخة 20.

شارك في هذه الدراسة مرضى مصابين بالسل الرئوي النشط وافراد اصحاء تتراوح أعمارهم بين 17–70 سنه منهم 44 فرد مصاب بالسل الرئوي النشط 33 منهم ذكور و11 اناث مع متوسط اعمار يتراوح بين (18,3±41) انحراف معياري والأشخاص المتبقين والذين كان عددهم 44 شخص هم افراد اصحاء 33 منهم ذكور و11 اناث مع متوسط اعمار يتراوح بين (18,3±41) انحراف معياري. كان هناك ارتفاع ذو دلاله إحصائية للوسط الحسابي للمادة الخلوية عامل نخر الورم ألفا في مرضى السل الرئوي النشط (44,4 بيكوجرام/مل) مقارنه بالأفراد الأصحاء (9,7 بيكوجرام/مل) حيث بغت القيمة الاحتمالية (0,000). كما لم يتأثر مستوى المادة الخلوية عامل نخر الورم ألفا بالفئه العمرية او الجنس القيمة الاحتمالية (0,902, 0,564) على التوالي. كذلك كان هنالك ارتفاع ذو دلاله إحصائية للوسط الحسابي لبروتين سي التفاعلي في مرضى السل الرئوي النشط (53.9 ملجم/ل) مقارنه بالأفراد الأصحاء (3,5 ملجم/ل) حيث بغت القيمة الاحتمالية (0,000). كما لم يتأثر مستوى بروتين سي التفاعلي بالفئة العمرية او الجنس القيمة الاحتمالية (0.406, 0.769) على التوالي.

خلصت هذه الدراسة الى ان مستوى عامل نخر الورم **ألفا** قد يكون مؤشر جيد لتشخيص السل الرئوي النشط وذلك بمصاحبة مستوى بروتين سي التفاعلي.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APCs	Antigen presenting cells
BACTEC	Becton Dickinson Microbiology Systems
BCG	Bacilli Calmette-Guerin
CD4	Cell Differentiation Molecules 4
CNS	Central nervous system
CMI	Cell-mediated immunity
CRP	C-reactive protein
CSF	Cerbrospinal fluid
CXCL	Type of chemokines
DAMPs	Danger-associated molecular patterns
DTH	Delayed-type hypersensitivity
EMB	Ethambutol
FQ	Fluoroquinolone
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1
HRCT	High resolution computed tomography
IFN-β	Interferon beta
IFN-α	Interferon alpha
IL	Interleukin
IGRA	Interferon gamma release assay
INH	Isoniazid
IFNγ	Interferon gamma

LTBI	Latent tuberculosis infection
LED	Light-emitting diode
LPAs	Line probe assays
LPS	Lipopolysaccharide
L-J media	Lowenstein-Jensen media
MHC	Major histocompatibility complex
MGIT	*Mycobacterial* Growth Indicator Tubes
MTBC	*Mycobacterium tuberculosis* complex
MDR-TB	Multidrug-resistant
M.tuberculosis	Mycobacterium tuberculosis
NAAT	Nucleic acid amplification test
PTB	Pulmonary Tuberculosis
POC	Point-of-care
PPD	purified protein derivative
PZA	Pyrazinamide
RAGE	Receptor for Advanced Glycation End products
RNIs	Reactive nitrogen intermediates
RR-TB	Rifampicin-resistant
REP	Rifampicin
R. Koch	Robert Koch
RNIs	Reactive nitrogen intermediates
STB	Skeletal tuberculosis
SLDs	Second- line anti-TB drugs
TNF-β	Tumor necrosis factor beta

TBM	Tuberculous meningitis
TST	Tuberculin skin test
TNF-α	Tumor necrosis factor
Th	T helper
TB	Tuberculosis
WHO	World Health Organization
WGS	Whole-genome sequencing
XDR-TB	Extensively drug-resistant

CHAPTER I
INTRODUCTION

1.1 Introduction

The causative agent of *M. tuberculosis* is an intracellular microorganism that lives in macrophages and lung epithelial cells. Cell-mediated immunity has a crucial role in the control of *M. tuberculosis* infection and ultimately determines whether *M. tuberculosis* infection is cleared, latent or active with *M. tuberculosis* consequences *M. tuberculosis* is now one of 10 most frequent causes of death and the top killer in infectious diseases due to the HIV/AIDS epidemics and the increased spread of multidrug-resistant TB (MDR-TB) Approximately one-third of the world's population has been infected by *M. tuberculosis*, but only 5–10% of them will eventually become ill with *M.tuberculosis*. However, persons with compromised immune systems, such as those living with HIV, malnutrition or diabetes, have a much higher risk of developing *M. tuberculosis* (Shen and Chen, 2018).

The natural history of tuberculosis begins with the inhalation of *M. tuberculosis* organisms; a period of bacterial replication and dissemination ensues, followed by immunologic containment of viable bacilli. The result of this process is asymptomatic latent tuberculosis infection, which is defined as a state of persistent bacterial viability, immune control, and no evidence of clinically manifested active tuberculosis. Currently, it is not possible to directly diagnose *M. tuberculosis* infection in humans; therefore, latent tuberculosis infection is diagnosed by response to in vivo or in vitro stimulation by *M. tuberculosis* antigens with the use of the tuberculin skin test or interferon-γ release assays. Studies suggest that active tuberculosis will develop in 5 to 15% of persons with latent infection during their lifetimes (Getahun *et al.*, 2015). Active disease can occur as primary tuberculosis, developing shortly after infection, or post primary tuberculosis, developing after a long period of latent infection. The principal means of testing for active tuberculosis is sputum analysis, including smear, culture, and nucleic acid amplification testing. Imaging findings, particularly the presence of cavitation, can affect treatment decisions, such as the duration of therapy (Nachiappan *et al.*, 2017). The development of the granuloma and its subsequent degeneration and necrosis, is the hallmark of infection caused by *M. tuberculosis*. These structures probably evolved as primitive particle responses, but in mammals they are facilitated by the emerging acquired immune response, in which cytokines and chemokines help control

their formation and integrity (Orme and Basaraba, 2014). Cytokines play a major role in the host defense process against *M. tuberculosis* infections. In this disease, cellular immunity and cytokines are intermediaries in the immune system and inflammatory responses. It seems that the acquired immune response in the pathway of T-helper lymphocytes can limit and stop bacterial growth. These cells secrete cytokines, such as interferon gamma and TNF-α, and stimulate macrophages to further produce more active oxygen and free radicals. As a result, cells are better able to kill microbes. (Mirzaei and Mahmoudi, 2018).

Also C-reactive protein plays an important role in innate immunity, opsonization by its properties, complement activation and immunoglobulins receptor binding. CRP is a protein of the acute systemic inflammation and is, therefore, a prime marker of inflammation. (Moutachakkir *et al.*, 2017). The incidence of active tuberculosis disease was estimated to be 10.4 million cases, with 1.7 million deaths during 2016. Rapid and accurate detection of active *M. tuberculosis* is essential for guiding treatment, yet case detection and reporting rates remain low, with 40% of estimated incident cases failing to be identified and reported. Thus, biomarkers are urgently required to detect active *M. tuberculosis* disease and differentiate it from latent *M. tuberculosis* infection (Chen *et al.*, 2020). In routine settings, not all presumed *M. tuberculosis* patients are willing or able to produce sputum for evaluation. When patients can produce sputum, *M. tuberculosis* diagnoses may be missed because sputum microbiologic testing is insufficiently sensitive, especially in settings where *M. tuberculosis* culture is not routinely available or affordable. Another challenge is that sputum collection generates cough aerosols that increase the risk of *M. tuberculosis* transmission; this risk may also give rise to fears of acquiring *M. tuberculosis,* thereby limiting engagement of both patients and health workers in sputum collection and reducing their success in obtaining sputum. The availability of a sensitive and easily detected, blood-based biomarker test for diagnose of active pulmonary tuberculosis would circumvent these operational challenges and potentially lower costs and enhance the yield of *M. tuberculosis* diagnostic evaluation. One of non-sputum-based test that could fit this profile is serum C-reactive protein. (Meyer *et al.*, 2020).

1.2 Rationale

Host immune response against *M. tuberculosis* is mediated by innate and cellular immunity, in which cytokines and Th1 cells play a critical role. In the process of control of the infection by *mycobacteria*, TNF-α seems to have a primordial function. This cytokine acts in synergy with IFN-gamma, stimulating the production of reactive nitrogen intermediates (RNIs), thus mediating the tuberculostatic function of macrophages, and also stimulating the migration of immune cells to the infection site, contributing to granuloma formation, which controls the disease progression. (Cavalcanti *et al.*, 2012).

The C-reactive protein (CRP) is a plasma protein of hepatic origin, belonging to pentraxin family and forms a major component of any inflammatory reaction. A key component of the innate immunity pathway, the concentration of CRP may rapidly increase to levels more than 1,000-folds above normal values as consequence to tissue injury or infection. Although functioning as a classical mediator of innate immunity, it functions via interaction of components of both humoral and cellular effector systems of inflammation. Initially considered as an acute-phase marker in tissue injury, infection and inflammation, it now has a distinct status of disease marker (Ansar and Ghosh, 2013). Also serum CRP levels are significantly correlated with disease severity in patients with active pulmonary tuberculosis (Mohammad *et al.*, 2012).

To my knowledge there is no published data about the evaluation of TNF alpha and CRP serum level among Sudanese active pulmonary tuberculosis patients, Therefore, this study will evaluate the utility of TNF-α and CRP as biomarker for early diagnosis of active pulmonary tuberculosis.

1.3 Objectives

1.3.1 General objective

1- To estimate TNF-α and CRP serum levels in active pulmonary tuberculosis patients, in Khartoum State -Sudan

1.3.2 Specific objectives

1- To measure TNF-α serum level among active pulmonary tuberculosis patients using ELISA technique.

2-To measure CRP serum level among active pulmonary tuberculosis patients by using DIRUI Auto-Chemistry Analyzer CS-T180

3- To compare between TNF alpha level among active pulmonary tuberculosis patients and healthy controls

4- To compare between CRP level among active pulmonary tuberculosis patients and healthy controls

5- To determine the association between TNF alpha and CRP serum levels with age and sex of patients with active pulmonary tuberculosis.

CHAPTER II

LITERATURE REVIEW

2.1 Tuberculosis

2.1.1 Historical Background

Palaeomicrobiology has detected the tuberculosis agent in animal and human skeletons that are thousands of years old. The German doctor Robert Koch was the first microbiologist to report in 1882 the successful isolation of the causative agent of tuberculosis, named one year later as *Mycobacterium tuberculosis*. This immense discovery, however, was not made from scratch, but involved the combining of previous scientific knowledge, chiefly the previous demonstration by the French doctor Jean-Antoine Villemin that tuberculosis was a transmissible disease, and two innovations a new staining procedure that allowed R. Koch to consistently observe the new organism in tuberculous lesions, and use of a solidified, serum-based medium instead of broths for the culture. These innovations allowed R. Koch not only to isolate *M. tuberculosis* from animal and patient specimens for the first time, but also to reproduce the disease in experimentally inoculated guinea pigs. It is thanks to R. Koch that one of the most lethal diseases in human history could be diagnosed, could be treated and cured after the discovery of streptomycin 65 years later, and could be efficiently prevented by isolation of cases. His microbiological innovations are now being renewed with molecular and improved culture-based detection being the twenty-first century weapons in the fight against this disease, which remains a major killer (Cambau and Drancourt, 2014).

2.1.2*Mycobaterium tuberculosis*

The organisms that belong to the genus *Mycobacterium* are aerobic (although some may grow in reduced oxygen). Non–spore forming (except for *Mycobacterium marinum*), nonmotile, very thin, slightly curved or straight rods. Some species may display a branching morphology. *Mycobacterium spp*. have an unusual cell wall structure. The cell wall contains N-glycolylmuramic acid instead of N-acetylmuramic acid, and it has a very high lipid content, which creates a hydrophobic permeability barrier. Because of this cell wall structure, *mycobacteria* are difficult to stain with commonly used basic aniline dyes, such as those used in Gram staining. Although these organisms cannot be readily Gram stained, they generally are considered Gram positive. However, they resist decolorization with acidified alcohol (3% hydrochloric

acid) after prolonged application of a basic fuchsin dye or with heating of this dye after its application (Patricia, 2017).

2.1.3 Causative agent of tuberculosis

Human tuberculosis (TB) is caused by members of the *Mycobacterium tuberculosis* complex (MTBC) (Rabahi, *et al.*, 2020) including *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium mungi*, *Mycobacterium orygis*, and *Mycobacterium pinnipedii*. All of these species are capable of causing tuberculosis (Patricia, 2017).

2.1.4 Epidemiology

Tuberculosis is an infectious bacterial disease caused by *M. tuberculosis* complex. A relatively small proportion (5% to 15%) of the estimated 1.7 billion people infected with *M. tuberculosis* will develop TB disease during their lifetime. However, the probability of developing TB disease is much higher among people with risk factors such as living with HIV, under-nutrition, diabetes, smoking and alcohol consumption. Although currently, four first-line drugs: isoniazid (INH), rifampicin (REP), ethambutol (EMB) and pyrazinamide (PZA) are recommended for the treatment of drug-susceptible TB (Getie *et al.*, 2021). However, the WHO estimates that nearly one third of all active tuberculosis cases go undiagnosed and unreported (Shapiro *et al.*,2021).

2.1.5 Disease transmission

M. tuberculosis is transmitted by infectious aerosols (Fennelly *et al.*, 2012). Persons with active pulmonary or laryngeal tuberculosis generate droplet nuclei that contain *M. tuberculosis* through coughing, singing, shouting, sneezing, or any other forceful expiratory maneuver that shears respiratory secretions from the airways, with coughing being the most efficient at generating infectious aerosols (Churchyard *et al.*, 2017).

2.1.6 Risk factors for TB

The risk of progression from exposure to the tuberculosis bacilli to the development of active disease is a two-stage process governed by both exogenous and endogenous risk factors. Exogenous factors play a key role in accentuating the progression from exposure to infection among which the bacillary load in the sputum and the proximity of an individual to an infectious TB case are key factors. Similarly, endogenous factors lead in progression from infection to active TB disease. Along with well-

established risk factors (such as human immunodeficiency virus (HIV), malnutrition, and young age), emerging variables such as diabetes, indoor air pollution, alcohol, use of immunosuppressive drugs, and tobacco smoke play a significant role at both the individual and population level. Socioeconomic and behavioral factors are also shown to increase the susceptibility to infection. Specific groups such as health care workers and indigenous population are also at an increased risk of TB infection and disease (Narasimhan *et al.*, 2013).

2.1.7 Tuberculosis pathogenesis and pathogenicity

Macrophages provide a major habitat for *M. tuberculosis* to reside in the host for years (Xu, *etal.*, 2014). After inhalation of *M. tuberculosis*, innate immune responses involving alveolar macrophages and granulocytes begin to combat the infection; in some persons, the bacilli are cleared, whereas in others, infection is established. Replication of bacilli in macrophages and regional lymph nodes leads to both lymphatic and hematogenous dissemination, with seeding of multiple organs, which may eventually give rise to extra pulmonary disease. Containment of bacilli within macrophages and extracellularly within granulomas limits further replication and controls tissue destruction, resulting in a dynamic balance between pathogen and host (Getahun *et al.*, 2015).

TB transmission occurs via inhalation of droplet nuclei. The most common site for the development of TB is the lungs (Cruz and Blake, 2013). At the time of initial infection, the distribution of inhaled droplet nuclei in the lung is determined by the pattern of regional ventilation. It thus tends to follow the most direct path to the periphery and to favor the middle and lower lung zones, which receive most of the ventilation. In immunocompetent hosts, it is theorized that alveolar macrophages ingest the *M. tuberculosis* organisms and may or may not destroy them, depending on the degree to which phagocytosing cells are nonspecifically activated, on host genetic factors and on resistance mechanisms in the bacteria. If bacteria are successfully cleared, then test results will remain negative on the tuberculin skin test (TST) or interferon gamma release assay (IGRA). When innate macrophage microbicidal activity is inadequate to destroy the initial few bacteria of the droplet nucleus they replicate logarithmically, doubling every 24 hours until the macrophage bursts to release the bacterial progeny. New macrophages attracted to the site engulf these bacilli, and the cycle continues. The bacilli may spread from the initial lesion via the lymphatic and/or circulatory systems to other parts of the body. After a period lasting

from 3 to 8 weeks the host develops specific immunity (cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) to the bacilli, and individuals typically show positive results on the TST or IGRA. The resulting *M. tuberculosis*-specific lymphocytes migrate to the site of infection, surrounding and activating the macrophages there. As the cellular infiltration continues, the center of the cell mass, or granuloma, becomes caseous and necrotic. Radiographically demonstrable fibrocalcific residua of the initial infection include a Ghon focus (a calcified granuloma in the lung) alone or in combination with a calcified granulomatous focus in a draining lymph node (Ghon complex). Infection and immune conversion are usually asymptomatic; any symptoms that do occur are self-limited. In a small proportion of those infected, erythema nodosum (a cutaneous immunologic response to an extracutaneous TB infection) or phlyctenular conjunctivitis (a hypersensitivity reaction) may develop (Long and Schwartzman ., 2014).

2.1.7.1 Pulmonary TB

2.1.7.1.1 Primary Infection

The *M. tuberculosis* in the droplet nuclei when inhaled by another individual is carried to the alveoli where they infect macrophages and multiply inside them. Within 2–6 weeks, the infection is followed by cellular immune response generated by CD4+ T-lymphocytes and infected macrophages release cytokines and chemokines. In 70–90% of instances, the immune response of an individual is strong enough, and it will fight off the *M. tuberculosis* and not become infected. During *M. tuberculosis* infection, the host immune response is able to contain the mycobacterial infection at the site of infection in approximately 30% of cases, but is unable to "sterilize" them. These foci later become associated with a state of LTBI with *M. tuberculosis* where persons are healthy, asymptomatic, and the infection is present in an enclosed environment in a non-transmissible state. The cellular immune response leads to the formation of a granuloma and the infection is curtailed. It also generates a delayed-type hypersensitivity reaction. Globally in 2014, approximately 1.7 billion individuals were estimated to be latently infected with *M. tuberculosis* and form a reservoir for future TB disease (Chandrasekaran *et al.*, 2017).

The cytokines produced by T helper (Th)1 cells (IFN-γ, IL-2 and TNF-β) correlate with protection, whereas the cytokines released by Th2 cells (IL-4, IL-5) and the anti-inflammatory cytokine IL-10 correlate with pathogenesis of tuberculosis (TB). However, the pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and IL-12p70)

are responsible for both protection and pathogenesis of *M. tuberculosis*, recognition of *M. tuberculosis* by phagocytic cells leads to antigen presentation, Th1 cell activation and production of cytokines that play a crucial role in the inflammatory response and the outcome of mycobacterial infections. The predominant protective response in *M. tuberculosis*-infected subjects is mediated by T helper 1 (Th1) type of cytokines, IL-2, IFN-γ and TNF-β. IFN-γ, in particular, is essential for the activation of macrophages and destruction of intracellular mycobacteria. On the contrary, the T helper type 2 responses, characterized by the secretion of cytokines, IL-4 and Il-5, are associated with the lack of protection and severity of tuberculosis. The Th2-type cytokine, IL-4, inhibits the *in vitro* production of IFN-γ, as well as the mycobactericidal mechanisms of macrophages leading to the cessation of granuloma formation and may therefore weaken host defense. Interleukin IL10 is a cytokine that modulates both innate and adaptive immunity, primarily by exerting anti-inflammatory effects IL-10 has also been shown to antagonize the pro-inflammatory cytokine response by downregulation of IFN-γ, TNF-α and IL-12 production and is strongly associated with reduced resistance and chronic progressive *M. Tuberculosis* Thus, IL-10 appears to play a major role in counteracting the host pro-inflammatory response to mycobacterial antigens. Furthermore, innate immune response-related pro-inflammatory cytokines with chemotactic activity, that is, IL-1 beta (IL-1β), IL-6, IL-8 and TNF-alpha (TNF-α) initiate events that curb mycobacterial growth by recruiting monocytes into the lesions and activating them to kill the pathogen .TNF-α is essential to control *M. tuberculosis* infection and cannot be replaced by other pro-inflammatory cytokines .However, it has been shown that although defective TNF-α production results in uncontrolled infection, overproduction of TNF-α may cause immunopathology. IL-6, which has both pro- and anti-inflammatory properties, is produced early during mycobacterial infection and at the site of infection. IL-6 may be harmful in *M. tuberculosis* infections, as it inhibits the production of TNF-α (Al-Attiyah *et al*., 2012).

2.1.7.1.2 Secondary (reactivated) tuberculosis

This can occur with weakening of immune responses. If person is unable to control the initial infection, it can progress to active primary disease as in children. If the infection is curtailed and goes into latency, with the weakening of the immune system, it can progress to disease even months or years later; by breakdown of granuloma and active uncontrolled replication of *M. tuberculosis* with resultant

disease in lungs and other organs The likelihood of getting infected with *M. tuberculosis* as well as the subsequent development of active disease depends upon number of factors like the infectivity of the source case, proximity and duration of contact, susceptibility of the host and various social, behavioral, economic, and environmental factors like undernutrition, overcrowding, indoor air pollution, smoking, and alcohol addiction (Chandrasekaran *et al.*,2017). According to the World Health Organization (WHO), approximately 2–3 billion people in the world are latently infected with *M. tuberculosis*, and 5%–15% of these people will suffer from reactivation of TB during their lifetime (Ai *et al.*, 2016).

2.1.7.1.3 Active pulmonary tuberculosis

Even in middle-to-high income countries where anti-TB treatment is more accessible, mortality of patients hospitalized for TB remains high (>5–10%). It is suggested that excessive inflammation contributes to disease severity and complications in active TB; however, the immunopathogenic processes involved have not been fully elucidated**.** Recent studies indicate that in addition to cell-mediated immunity, innate immune responses and the pro-inflammatory cytokines play significant roles in *M. tuberculosis* control**.** High mobility group box 1 (HMGB1), a nuclear DNA-binding protein, is one of the key danger-associated molecular patterns (DAMPs) that activate the innate immune system .It is released by necrotic cells and monocytes/macrophages in response to cytokine and bacterial antigen stimulation and shown to be inflammasome (caspase-1-activating), multiprotein complexes containing a sensory protein dependent in bacterial infections .HMGB1 then interacts with other molecules (e.g. bacterial LPS), and bind to receptors including the Receptor for Advanced Glycation End products RAGE, exists in both cellular 'transmembrane' form, and a 'soluble', decoy receptor form and the Toll-like Receptors, to activate a multitude of pro-inflammatory genes In addition to liberation of inflammatory cytokines (e.g. TNF-α), HMGB1 will activate immune cell functions and induce their maturation (e.g. monocytes, myeloid /plasmacytoid dendritic cells. significant elevation of the pro-inflammatory cytokines IL-8/CXCL8 and IL-6 in patients with active PTB; IL-18 and sTNFR-1 (indicating TNF-α release) were also increased (Lui *et al.*, 2016).

.2.1.7.1.4 Latent pulmonary tuberculosis

Recent studies have shown that differences exist in the immunological response mounted by different individuals that lead to the formation of physiologically distinct

granulomatous lesions in individuals exposed to *M. tuberculosis*. Some of these lesions suppress while others promote the persistence of viable *M. tuberculosis* in the microenvironment. Low-dose infection of cynomolgus macaques that reproduce the clinical characteristics of human latent TB leads to the formation of at least two types of tuberculous granuloma. Histopathological studies have shown that the classic caseous granuloma are composed of epithelial macrophages, neutrophils, and other immune cells surrounded by fibroblasts. The central caseous necrotic region in this type of granuloma consists of dead macrophages/other cells and is hypoxic with *M. tuberculosis* residing inside macrophages in the hypoxic center. The other kind of granulomas seen in latent tuberculosis in both humans and cynomolgus macaques are fibrotic lesions, composed almost exclusively of fibroblasts that contain very few macrophages. However, it is not known at present whether *M. tuberculosis* is located inside macrophages or in the fibrotic area in these lesions. The latent infection in a person without overt signs of the disease is indicated by the delayed-type hypersensitivity (DTH) response to purified protein derivative (PPD) prepared from culture filtrates of *M. tuberculosis* tuberculin skin test (Ahmed,2011).

2.1.7.1.5 Clinical feature

Pulmonary TB is defined as tuberculosis of the lung parenchyma and the tracheobronchial tree only. The classic clinical features of pulmonary TB include chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, and hemoptysis**.** Primary pulmonary TB should be distinguished from post primary pulmonary TB, which is the most frequent TB manifestation in adults (70%–80% cases). It is characterized by local granulomatous inflammation, usually in the periphery of the lung and may be accompanied by ipsilateral lymph node involvement. The infection is usually asymptomatic but can present as an acute lower respiratory tract infection (Loddenkemper *et al.*, 2015). Common presenting symptoms include low-grade fever, night sweats, fatigue, anorexia (loss of appetite), and weight loss. A patient who presents with pulmonary tuberculosis usually has a productive cough, along with low-grade fever, chills, myalgia (aches), and sweating; however, these signs and symptoms are similar for influenza, acute bronchitis, and pneumonia (Patricia, 2017).

Post primary pulmonary TB may follow primary TB. In the generally immunocompetent, there is a lifetime chance of reactivation of the dormant primary complex of 5%–10%. These estimates were developed before the availability of

molecular techniques that can distinguish reactivation from reinfection with another strain of *M. tuberculosis*, and it may be that the overall risk is rather less in most people with latent TB infection not exposed again to *M. tuberculosis*. The first two years following primary infection are the period of maximal risk of progression. This can be reduced significantly by treating LTBI, which is indicated particularly in high-risk groups. It is not known why only ~10% of individuals infected with *M. tuberculosis* develop active disease. Apart from diverse risk factors such as diabetes, smoking, and chronic renal failure several genes have been found to be associated with increased susceptibility to, or resistance against, *M. tuberculosis*. The most frequent symptoms of active disease are fever, anorexia or reduced appetite, weight loss, night sweats, anemia, and persistent cough, usually productive of purulent and/or blood-stained sputum. Occasionally, patients complain of localized thoracic pain attributable to accompanying pleural inflammation. In extensive and long-lasting pulmonary disease, patients may report breathlessness. Hemoptysis is usually the result of cavitating lung disease causing erosion of pulmonary blood vessels (Loddenkemper *et al.*, 2015).

2.1.7.2 Extra pulmonary TB

2.1.7.2.1 Tuberculous Lymphadenitis

TB lymphadenitis is seen in nearly 35 % of extra pulmonary TB which constituted about 15 to 20 % of all cases of TB. Tuberculous lymphadenitis most frequently involves the cervical lymph nodes followed in frequency by mediastinal, axillary, mesenteric, hepatic portal, perihepatic and inguinal lymph nodes. It may present as a unilateral single or multiple painless slow growing mass or masses developing over weeks to months, mostly located in the posterior cervical and less commonly in supraclavicular region (Mohapatra and Janmeja, 2009).

2.1.7.2.2 Central nervous system TB

Central nervous system disease is the most serious form of tuberculosis, and is associated with high mortality and severe neurological sequelae (Nicholas *et al.*, 2012). Definitive diagnosis of tuberculous meningitis (TBM) depends upon the detection of the tubercle bacilli in the CSF. Every patient with TBM (Cherian and Thomas, 2011).

2.1.7.2.3 Miliary TB

Miliary tuberculosis (TB) is a potentially lethal disease if not diagnosed and treated early. Diagnosing miliary TB can be a challenge that can perplex even the most

experienced clinicians. Clinical manifestations are nonspecific, typical chest radiograph findings may not be evident till late in the disease, high resolution computed tomography (HRCT) shows randomly distributed miliary nodules and is relatively more sensitive (Sharma *et al*., 2012).

2.1.7.2.4 Pleural TB

Globally, pleural TB remains one of the most frequent causes of pleural exudates, particularly in TB-endemic areas and in the HIV positive population. Most TB pleural effusions are exudates with high adenosine deaminase (ADA), lymphocyte-rich, straw-coloured and free flowing, with a low yield on mycobacterial culture. TB pleurisy can also present as loculated neutrophil-predominant effusions which mimic para pneumonic effusions. Rarely, they can present as frank TB empyema, containing an abundance of *M. Tuberculosis* Up to 80% of patients have parenchymal involvement on chest imaging. The diagnosis is simple if *M. tuberculosis* is detected in sputum, pleural fluid or biopsy specimens, and the recent advent of liquid medium culture techniques has increased the microbiological yield dramatically (Shaw *et al* .,2018).

2.1.7.2.5 Skeletal TB

Skeletal tuberculosis (STB) is one of the most common forms of extra pulmonary tuberculosis; however, limited epidemiological data are available on this public health concern worldwide, especially in developing countries (Fan, *et al*.,2020). The diagnosis of tuberculosis (TB) in osteoarcheological series relies on the identification of osseous lesions caused by the disease. The study of identified skeletal collections provides the opportunity to investigate the distribution of skeletal lesions in relation to this disease. The vertebrae of individuals who have died of TB may have internal cavities in the absence of external lesions (Mariotti *et al*.,2015).

2.1.8 Diagnosis of tuberculosis

The World Health Organization (WHO) has provided several key guidance documents for national TB control and laboratory programs for the use of light-emitting diode (LED) microscopes to improve the sensitivity of and turnaround time for the front-line assay, sputum smear microscopy, and for improving the accuracy of TB detection and drug susceptibility testing through the use of liquid culture and molecular line probe assays (LPAs). International and national laboratory partners and donors are currently involved in evaluations of new diagnostics that will allow the more rapid and accurate diagnosis of TB at point-of-care (POC) settings and also

evaluations of alternative algorithms using new molecular tools for TB reference laboratories. Progress in the implementation of these initiatives is dependent on key partnerships in the international laboratory community and ensuring that quality assurance programs are inherent in each country's national laboratory network (Parsons *et al.*, 2011).

2.1.8.1 Microscopic examination of tuberculosis

Sputum smear microscopy has been the primary method for diagnosis of pulmonary tuberculosis in low and middle income countries which is where nearly 95 % of TB cases and 98 % of deaths due to TB occur. It is a simple, rapid and inexpensive technique which is highly specific in areas with a very high prevalence of tuberculosis. It also identifies the most infectious patients and is widely applicable in various populations with different socioeconomic levels However, sputum smear microscopy has significant limitations in its performance. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organism's /ml sputum sample. It also has a poor track record in extra pulmonary tuberculosis, pediatric tuberculosis and in patients coinfected with HIV and tuberculosis. Due to the requirement of serial sputum examinations, some patients who do not come back for repeated sputum examinations become diagnostic defaulters. Some do not come back for results, and are lost to treatment and follow up. Fluorescence microscopy was introduced in the 1930, in an attempt to improve outcomes of smear microscopy. Fluorochrome dyes are used to stain the smear. A halogen or high-pressure mercury vapour lamp is traditionally used to excite the dye, and make it flouresce. A meta-analysis of studies comparing fluorescent and conventional microscopy found that the sensitivity of fluorescent microscopy was 10 % higher than that of conventional microscopy (Desikan, 2013).

2.1.8.2 Culture of MTB

2.1.8.2 .1 Solid media

Following decontamination, specimens may be cultured for the growth of *M. tuberculosis*. Culture is traditionally performed on solid egg-based media, such as Lowenstein-Jensen (L-J) media, which is composed of egg proteins, potato flour, salts, and glycerol. L-J media supports good growth of *M. tuberculosis*, but is not as reliable for all mycobacterial species including *M. bovis* and *M. genavense*. While some laboratories still use L-J media, many have transitioned to using more chemically defined agar-based media optimized for faster mycobacterial growth. The

use of Middlebrook 7H10 or 7H11 agars, for example, allows for visible *M. tuberculosis* colony growth in 10–12 days as compared to 18–24 days with L-J media. However, agar-based media is less stable and more prone to deterioration. For example, exposure to excessive heat or light may lead to the release of formaldehyde which is toxic to *M. tuberculosis* and may inhibit growth. Most *Mycobacterium* species, including *M. tuberculosis*, grow best at a temperature of 35–37 °C. Select species such as *M. haemophilum*, *M. marinum*, *M. paratuberculosis*, and *M. ulcerans* have an optimum growth temperature of 30 °C and may be cultured in a separate incubator. Like many, *M. tuberculosis* growth is stimulated by incubation in air containing 5–10% CO_2. Unlike other bacteria, *M. tuberculosis* have a growth rate with 12–24 hours for each generation of cell division. Due to this slow growth rate, it may take several weeks for colonies to become visible on culture plates. Cultures are typically held for 6–8 weeks before being discarded and reported as negative (Caulfield and Wengenack, 2016).

.2.1.8.2 .2 Liquid media

Optimal recovery of *M. tuberculosis* from clinical specimens is achieved through the use of a combination of solid and liquid media. In general, *M. tuberculosis grow* faster in broth than on solid media plates, which allows for improved patient management and clinical outcomes. Growth of *M. tuberculosis* from clinical specimens takes an average of 10 days by automated broth systems versus 20–25 days on solid media. (Caulfield and Wengenack 2016)**.** There are three FDA-cleared commercial platforms for the semi-automated broth-based culture of *M. tuberculosis:* the BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems), the VersaTREK system (Trek Diagnostic Systems), and the MB/BacT Alert 3D (bioMérieux). The MGIT system is named for its use of *Mycobacterial* Growth Indicator Tubes .Each tube contains a modified Middlebrook 7H9 broth and a fluorescent indicator that is quenched by the presence of oxygen within the tube. Growth of *M. tuberculosis* in the medium consumes oxygen over time and allows the fluorescent indicator to signal as positive once a certain growth threshold has been met. The instrument continuously monitors tube fluorescence allowing lab staff to quickly identify positive tubes and begin the task of identifying any *M. tuberculosis* present. The MGIT system is an improvement over past BACTEC platforms that utilized radiometric assessment of growth and required manual intervention to place bottles on the machine once or twice per day for reading. In

addition to faster growth, many studies have documented the improved sensitivity of the MGIT broth system as compared to solid culture (Caulfield and Wengenack 2016).

2.1.8.3 Molecular Approaches for TB diagnosis

2.1.8.3.1 Nucleic acid amplification test (NAAT)

Diagnosis of TB has entered an era of molecular detection that provides faster and more cost-effective methods to diagnose and confirm drug resistance in TB cases, meanwhile, diagnosis by conventional culture systems requires several weeks. New advances in the molecular detection of TB, including the faster and simpler nucleic acid amplification test (NAAT) and whole-genome sequencing (WGS), have resulted in a shorter time for diagnosis and, therefore, faster TB treatments **(**Rodrigues, 2012**).** Increased use of NAATs in diagnosis of pulmonary TB could decrease the time-to treatment initiation and consequently decrease transmission **(**Peralta *et al.*, 2016). The *mycobacterial* insertion sequence IS*6110* proved crucial in deciphering tuberculosis (TB) transmission dynamics. This sequence was also shown to play an important role in the pathogenicity (transmission ability and/or virulence) of *M. tuberculosis*, the main causative agent of TB in humans (Thabet and Souissi, 2017**).**

2.1.8.3.2 XPERT MTB/RIF assay:

Xpert® MTB/RIF (Xpert) is a World Health Organization (WHO) - recommended, rapid, automated, nucleic acid amplification assay that is used widely for simultaneous detection of *M. tuberculosis* and rifampicin resistance in sputum specimens (Kohli *et al.*, 2018).

2.1.8.3.3 Whole-genome sequencing (WGS)

Whole genome sequencing (WGS) has gained increasing recognition as the new standard approach for epidemiological typing of M. *Tuberculosis* It has the highest resolution and an additional advantage in allowing for simultaneous identification of the *M. tuberculosis* (sub) species and genotype families, as well as detection of resistance to anti-tuberculous drugs in a reliable way. Multiple studies regarding the epidemiology of TB have pointed out that the resolution of WGS is superior to that of MIRU-VNTR typing and that epidemiological links can be traced more accurately (Jajou, *et al.*,2019). Due to the highly conserved genome of *M. tuberculosis* strains, it is possible to analyse WGS data from any *M. tuberculosis* strain by comparison to a common reference genome. The *M. tuberculosis* H37Rv genome has been widely used as a reference genome and mutations compared to the H37Rv genome are

reported as single nucleotide polymorphisms (SNPs) or insertions and deletions (Jajou *et al.*, 2019).

2.1.9. Tuberculin Sensitivity and Testing

Screening tests provide indirect information on the presence of latent tuberculosis infection (LTBI). Historically, the diagnosis of LTBI has relied on the use of the tuberculin skin test (TST). Recently, interferon gamma release assays (IGRAs) have been developed. These may overcome some of the limitations of TST (e.g., cross-reactivity in Bacilli Calmette-Guerin vaccinated people, error in measuring the size of induration of the skin reaction) and can be used as a replacement or adjunct to the TST (Auguste *et al.*, 2017)**.**

2.2Cytokines

2.2.1 Roles and Nomenclature

The history of the cytokines starts in the second half of the 1970's when many groups realized that activated lymphocytes produced secreted proteins that had dramatic effects on other leukocytes. The soluble mediators were given names of the assays that detected their activities like "macrophage activation factor" or "macrophage inhibitory factor." Several teams started to apply biochemical efforts to distinguish or molecularly characterize the mediators of these activities and this led to the realization that two of the earliest cytokines exhibited specific biochemical characteristics. This led to the identification of the first two interleukins, interleukin 1 and interleukin 2, which were named at the Second International Lymphokine Conference. Another dramatic step forward was the development of molecular biology tools which led to the initial efforts to "clone" the genes encoding important cytokines. One of the first to be cloned was interferon gamma (by Genentech). Conversely, there are many interleukins that are related evolutionarily to each other but this is not apparent from their names (IL-4 and IL-13, IL-2, IL-15 and IL-21, IL-10, and IL-22, etc.). In retrospect, the term "interleukin" had a significant advantage: it is a "neutral" designation, one that does not describe a specific characteristic or biological activity. In contrast, consider cytokines like interferon gamma (IFNγ); which is a major immunoregulatory cytokine, and this is what it is known for (not its "interferon" bioactivity). It is a major macrophage activator including induction of antigen presenting activity. Thus, this is an example of a cytokine that received a name based on one of the first biological activities detected, even though it is not one of the most relevant (Zlotnik, 2020).

2.2.2 Properties of cytokines

Cytokines are soluble factors which are mostly generated by immune cells and in turn play crucial roles in the differentiation, maturation, and activation of various immune cells. These cytokines may exert either proinflammatory or anti-inflammatory effects, or both, depending on specific local microenvironments. They are also critical mediators that bridge innate and adaptive immune systems, constituting a rather complex immune response network. The abnormalities of various cytokines may reflect the imbalance among different immune cell subsets, such as Th1/Th2 (Shen *et al.*, 2018).

2.2.3 Cells produce cytokines

T cell subset differentiation. Mature CD4 and CD8 T cells leave the thymus with a naive phenotype and produce a variety of cytokines. In the periphery, these T cells encounter antigen presenting cells (APCs) displaying either major histocompatibility complex (MHC) class I molecules (present peptides generated in the cytosol to CD8 T cells) or MHC class II molecules (present peptides degraded in intracellular vesicles to CD4 T cells). Following activation, characteristic cytokine and chemokine secretion profiles allow the classification of CD4 T helper (Th) cells into two major subpopulations in mice and humans. Th1 cells secrete mainly IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β), whereas Th2 cells secrete mainly IL-4, IL-5, IL-6, IL-10 and IL-13. Th1 cells support cell-mediated immunity and as a consequence promote inflammation, cytotoxicity and delayed-type hypersensitivity (DTH). Th2 cells support humoral immunity and serve to downregulate the inflammatory actions of Th1 cells (Cameron and Kelvin, 2013).

2.2.4 Types of cytokines according to T helper

2.2.4.1 Interleukins

The term 'interleukin' (IL) has been used to describe a group of cytokines with complex immunomodulatory functions -- including cell proliferation, maturation, migration and adhesion. These cytokines also play an important role in immune cell differentiation and activation. Interleukins initiate a response by binding to high-affinity receptors located on the surface of cells; ILs act in a paracrine or autocrine fashion, rather than as an endocrine signal, which is more common with steroidal and amino acid-derived hormones. The response of a particular cell to these cytokines depends on the ligands involved, specific receptors expressed on the cell surface and the particular signalling cascades that are activated (Brocker *et al.*, 2010).

2.2.4.2 Interferons (IFN)

Type I IFNs have been considered indispensable and unique antiviral mediators for the activation of rapid innate antiviral protection. However, the recent discovery of type III IFNs is challenging this paradigm. Since their identification in 2002/2003 by two independent groups, type III IFNs or IFN-λs, also known as IL-28/29, have been the subject of increased study with consequent recognition of their importance in virology and immunology. Initial reports suggested that IFN-λs functionally resemble type I IFNs. Although IFN-λs and classical type I IFNs (IFN-α/β) utilize distinct receptor complexes for signaling, both types of IFNs activate similar intracellular signaling pathways and biological activities, including the ability to induce antiviral state in cells, and both type I and type III IFNs are induced by viral infection (Kotenko, 2011).

2.2.4.3 Tumor necrosis factor (TNF)

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that has important roles in mammalian immunity and cellular homeostasis. Deregulation of TNF receptor (TNFR) signalling is associated with many inflammatory disorders, including various types of arthritis and inflammatory bowel disease, and targeting TNF has been an effective therapeutic strategy in these diseases (Brenner *et al*., 2015).

2.2.5 TNF alpha

Tumor necrosis factor (TNF)-alpha was originally identified in the 1970s as the serum mediator of innate immunity capable of inducing hemorrhagic necrosis in tumors. Today, a wide spectrum of biological activities has been attributed to this molecule, and clinical translation has mainly occurred not in using it to treat cancer, but rather to inhibit its effects to treat autoimmunity (Josephs *et al*., 2018).

Although activity that induced tumor regression was observed and termed tumor necrosis factor (TNF) as early as the 1960s, the true identity of TNF was not clear until 1984, when Aggarwal and coworkers reported, for the first time, the isolation of 2 cytotoxic factors: one, derived from macrophages (molecular mass 17 kDa), was named TNF, and the second, derived from lymphocytes (20 kDa), was named lymphotoxin. Because the 2 cytotoxic factors exhibited 50% amino acid sequence homology and bound to the same receptor, they came to be called TNF-α and TNF-β. Identification of the protein sequences led to cloning of their cDNA. Based on sequence homology to TNF-α, now a total of 19 members of the TNF superfamily have been identified, along with 29 interacting receptors, and several molecules that

interact with the cytoplasmic domain of these receptors. The roles of the TNF superfamily in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, and morphogenesis have been documented. Their roles in immunologic, cardiovascular, neurologic, pulmonary, and metabolic diseases are becoming apparent. TNF superfamily members are active targets for drug development, as indicated by the recent approval and expanding market of TNF blockers used to treat rheumatoid arthritis, psoriasis, Crohns disease, and osteoporosis (Aggarwal *et al.*, 2012).

2.2.5.1The cellular source of TNF α

Monocytes and macrophages have been suggested as a major cellular source of TNF-α in human .1n TB, TNF-α is produced primarily by myeloid cells and also by lymphocytes and its availability is regulated by cytokines, enzymes, lipid mediators and miRNAs (Dorhoi, 2014).

2.2.5.2TNF α and tuberculosis

Host immune response against *M. 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 *M. tuberculosis*, TNF-alpha seems to have a primordial function. This cytokine acts in synergy with IFN-gamma, stimulating the production of reactive nitrogen intermediates (RNIs), thus mediating the tuberculostatic function of macrophages, and also stimulating the migration of immune cells to the infection site, contributing to granuloma formation, which controls the disease progression (Cavalcanti *et al.*,2012). TNF-α is one of the earliest cytokines implicated in pathogenesis of lung fibrosis. Furthermore, the TNF-α gene polymorphism has been found to be significantly associated with an increased risk for pulmonary fibrosis. Given that genetic variation may potentially alter inflammation and fibrosis in lung (Fan *et al.*,2010). TNF-α had been found to be associated with TB when it was originally identified as cachexin and until today TB research continues to unveil novel roles of this cytokine of highest relevance for the disease process and for novel intervention strategies. The essentiality of TNF-α for containment of active TB is reflected by redundancy of cellular sources of this cytokine, by complexity of mechanisms regulating TNF-α abundance and by substantial polyfunctionality of this mediator. The propensity of TNF-α to modulate granuloma biogenesis and integrity in TB represents the quintessential process in infection outcome (Dorhoi and Kaufmann, 2014).

2.2.6 C-reactive protein and tuberculosis

C-reactive protein (CRP) was discovered by Tillett and Francis in 1930. The name CRP arose because it was first identified as a substance in the serum of patients with acute inflammation that reacted with the "c" carbohydrate antibody of the capsule of pneumococcus. CRP is a pentameric protein synthesized by the liver, whose level rises in response to inflammation. CRP is an acute-phase reactant protein that is primarily induced by the IL-6 action on the gene responsible for transcription of CRP during the acute phase of an inflammatory/infectious process. CRP has both proinflammatory and anti-inflammatory properties. It plays a role in the recognition and clearance of foreign pathogens and damaged cells by binding to the phosphocholine, phospholipids, histone, chromatin, and fibronectin. It can activate the classic complement pathway and also activates phagocytic cells via Fc receptors to expedite the removal of cellular debris and damaged or apoptotic cells and foreign pathogens (Nehring *et al.*, 2020).

Systematic screening for active pulmonary tuberculosis (TB) is recommended for high-risk populations however the lack of an accurate, simple, and low-cost screening test that can be used in high burden areas is a major obstacle to its implementation. C-reactive protein (CRP) possesses the necessary test characteristics to screen individuals for active pulmonary TB (Yoon *et al.*, 2017).

2.3. Multi drug resistance tuberculosis (MDR-TB)

Multidrug-resistant tuberculosis (MDR-TB) is defined as disease due to *M. tuberculosis* that is resistant to isoniazid (H) and rifampicin (R) with or without resistance to other drugs. Rifampicin-resistant TB (RR-TB) defined as resistance to rifampicin detected using genotypic or phenotypic methods with or without resistance to other first-line anti-TB drugs. MDR-TB/RR-TB has been an area of growing concern to human health worldwide and posing a threat to the control of TB. The Global TB Report 2016 estimated that of 3.9% newly diagnosed and 21% of previously treated TB cases had MDR-TB. It has been estimated that 580,000 cases of TB resistant to at least rifampicin (RR-TB) globally in 2015, of whom, 480,000 were having resistant to both rifampicin and isoniazid (MDR-TB), and 250,000 deaths occurred due to MDR-TB/RR-TB in 2015 globally. Out of estimated 580,000 MDR-TB/RR-TB cases, only 132,120 (23%) were detected, and even fewer 124,990 (20%) started treatment, and only 52% of them were treated successfully. MDR-TB/RR-TB

is treatable but is very expensive and requires long duration of treatment and contains potentially toxic drugs (Prasad *et al*., 2018).

2.3.1. Extensively drug-resistant TB (XDR-TB)

Extensively drug-resistant TB (XDR-TB) is defined as TB caused by a multidrug-resistant (MDR) strain that resistant to at least rifampicin and isoniazid) that is also resistant to any fluoroquinolone (FQ) and any of the second-line injectable drugs, such as capreomycin, kanamycin, or amikacin. From 2006, when the first report on XDR-TB was published, until the end of 2012, 92 countries had reported the presence of at least one case of XDR-TB (Matteelli *et al*., 2014).

2.4. Prevention and control of tuberculosis

At present, the BCG vaccine is the only vaccine available against tuberculosis. The effectiveness of this live vaccine is controversial, because studies have demonstrated ineffectiveness to 80% protection. The greatest potential value for this vaccine is in developing countries with high prevalence rates for tuberculosis. At this time, at least four types of anti-tuberculosis vaccines are currently being evaluated in experimental studies in animals (Patricia,2017).

2.5. Treatment

Therapy directed against *M. tuberculosis* depends on the susceptibility of the isolate to various antimicrobial agents. To prevent the selection of resistant mutants, treatment of tuberculosis requires four drugs: isoniazid, rifampin, ethambutol, and pyrazinamide. Initial therapy includes all four drugs for 8 weeks. However, if drug susceptibility is determined for isoniazid, rifampin, and pyrazinamide, ethambutol may be discontinued. This is the preferred therapy for initial treatment, followed by isoniazid and rifampin for an additional 18 weeks. The most common two-drug regimen is isoniazid (INH, also known as isonicotinoylhydrazine) and rifampin. The combination is administered for 9 months in cases of uncomplicated tuberculosis; if pyrazinamide is added to this regimen during the first 2 months, the total duration of therapy can be shortened to 6 months. Ethambutol may also be added to the regimen. INH prophylaxis is recommended for individuals with a recent skin test conversion who are disease free (Patricia , 2017).

2.5.1. Second-line anti-TB drugs

To effectively treat patients diagnosed with drugs- resistant tuberculosis and protect the population from further transmission of this infectious disease, an uninterrupted

supply of quality assured (QA), second- line anti-TB drugs(SLDs) is necessary (Claiborne *et al.*, 2013).

2.6 Previous studies

In study conducted by Mirzaei, and Mahmoudi (2018) In Iran aimed to evaluate the level of TNF alpha in patients with tuberculosis infection, found that the concentration of TNF-α in patients with TB was significantly higher than in the control group ($P< 0.05$). However, the difference was only significant in the age groups 20–30 and 50–60 years; in the age groups 30–40, 40–50 and 50–70 years, the difference was not significant, although certain trends were apparent.

Other study by Shameem *et al* (2015) in India aimed to evaluate the levels of TNF-α in new, under treatment (UT), and multidrug-resistant (MDR) pulmonary and extra pulmonary cases. They found That TNF-α levels were elevated in new cases ($P< 0.05$) and MDR cases ($P< 0.05$) but not significantly for UT cases ($P< 0.05$).

Other study done by Andrade *et al* (2008) in Brazil to evaluate the level of TNF- α alpha in patients with tuberculosis infection, found that the levels of TNF-alpha had significant differences between the tuberculosis and control groups ($p<0.05$).

Other study done by Amanda *et al* (2020) in Uganda aimed to evaluate the level of CRP in active pulmonary tuberculosis patients. They found that among those who were *M. tuberculosis* culture positive, 36 had CRP levels of ≥10 mg/liter, providing a sensitivity of 78% (95% CI, 64 to 89%). Among those with *M. tuberculosis*-negative cultures, 38 had CRP levels of <10 mg/liter, also CRP levels were strongly associated with performance status, with (95%) of those in the moderately or severely impaired category having elevated CRP, compared with (52%) of those in the unaffected or mildly impaired category (risk ratio, 13.5; 95% CI, 1.9 to 97.4; $P < 0.00$).

Other study conducted by Brown *et al* (2016) In UK aimed to report the relationship between baseline serum CRP prior to treatment and disease characteristics in a metropolitan population with TB resident in a low TB incidence region. They found that CRP results were significantly higher in culture positive cases compared to culture negative cases: median 49 mg/L (16-103 mg/L) vs 19 mg/L (IQR 5-72 mg/L), p <0.001. In those with pulmonary disease, smear positive cases had a higher CRP than smear negative cases: 67 mg/L (31-122 mg/L) vs 24 mg/L (7-72 mg/L), p < 0.001.

CHAPTER III

MATERIAL AND METHOD

3.1 Study design

This study was an analytical case-control study

3.2 Study area and duration

This study was conducted in Abo Anga Hospital in Khartoum State during the period from March 2021to March 2022.

3.3 Study population.

Study population consisted of 88 Sudanese individuals of age between 17-65 years, divided into two groups as follows: 44 Active pulmonary TB patients and 44 apparently healthy control group.

3.3.1. Inclusion criteria

Patients with active pulmonary TB infection positive with gene Xpert, untreated, and had symptoms of TB less than three months, control group was apparently healthy subjects.

3.3.2 Exclusion criteria

Active pulmonary TB infected patients with underlining disease that affected cytokine level including physiological factors such as (pregnancy, smoking and alcohol consumption) and others diseases such as (autoimmune diseases, infectious diseases, allergy, hypersensitivity, cancer, heart failure and Parkinson diseases), patient under treatment and other types of tuberculosis were excluded.

For control group, any person suffering from any infectious disease or any disease that affected cytokine level including physiological factors such as (pregnancy, smoking and alcohol consumption) and others diseases such as (autoimmune diseases, infectious diseases, allergy, hypersensitivity, cancer, heart failure and Parkinson diseases) were excluded.

3.4 Ethical consideration

The study was approved by Research Board of college of Medical Laboratory Science, Sudan University and Technology, and verbal consent was obtained from participants before collection of the blood samples.

3.5. Sampling technique

Non-probability convenience sampling technique was used in this study.

3.6. Sample size

A total of 88 subjects were enrolled in this study n= 44 samples were collected from active tuberculosis patients and n= 44 samples were collected from healthy volunteer.

3.7. Data collection

A structured questionnaire was used to collect demographic and clinical data.

3.8. Specimen collection

Under sterile condition five ml of venous blood sample was withdrawn from each participant, the serum was separated by centrifugation at 5000 rpm for five minutes, serum was separated into plain container then stored at -20C˚ until used.

3.9. Laboratory investigation

TNF level was measured by – Enzyme linked immunosorbent assay Kit. (biolegend, USA).

CRP level was measured by latex turbidimetric kit using DIRUI Auto Chemistry-analyser CS-T180 (spinreact, Spain).

3.9.1 ELISA Procedure

In day I, a 100 µL of diluted capture antibody solution was added to each well, the plate was sealed and incubated overnight between 2˚C and 8˚C

In Day II, the plate was washed 4 times with at least 300µL of wash buffer per well and blotted any residual buffer by firmly tapping the plate upside down on clean absorbent paper. To block the plate by adding 200 µL 1X assay diluents A to each well, the plate was sealed and incubated at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking were performed similarly. The plate washed 4 times; A 100 µL of diluted standareds and samples were added to the appropriate wells. The plate was sealed and incubated at room temperature for 2 hours with shaking. Then the plate was washed 4 times. A 100µL of diluted detection antibody solution was added to each well, the plate was sealed and incubated at room temperature for 1 hour with shaking. The plate was washed 4 times; 100 µL of diluted Avidin-HRP solution was added to 28 each well, the plate was sealed and incubated at room temperature for 30 minutes with shaking. The plate was washed 5 times; soaked for 30 seconds to 1 minute per wash. A 100µL of freshly mixed TMB substrate solution was added to each well and incubated in the dark for 15 minutes. A 100µL of stop solution was added to each well. The optical density was read at 450 nm and 570 nm within 15 minutes by ELISA reader. To determine the concentration of each sample, first find

the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration.

3.9.2. ELISA Washer principle

First the wash solution is pump from the wash bottle, the solution is dispense to the cuvette by short pins, and then the wash liquid is aspirate from the cuvette by long pins , at the end the waste liquid was pumped into the waste bottle by the vacuum pump.(www.diasource.be 2022).

3.9 3. ELISA reader principle

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted. It is filtered by interference filters and focused onto the photodiodes. The photodiode converts the received light into an electrical signal which is transformed into a digital form, from which the microprocessor calculates the absorbance, taking in account of the blank and dichromatic selection (www.diasource.be 2022).

3.9.4 C-reactive protein procedure

Was measured by DIRUI auto-chemistry analyzer CS-T180 using fully automated procedure.

The samples were putted in the sample position by using the original blood tube also the reagent was putted in the reagent position using the original reagent container and turn on the instrument. Mixer mix immediately after adding reagent and it go to the photoelectron road where the light produced by the halogen lamps was passed through the sample and the optical density was written by using 340nm~800nm Wavelengths. Then the result was read in the software display using windows 10. (Analysis control software Graphical operating software English version)(www.healthcare, 2022).

3.10. Statistical analysis

Data was analyzed using statistical package for social science (SPSS version 20) Frequencies and mean ± SD were calculated in this study. Independent T test was used for testing significant difference and correlation test to find out correlation. Probability value ≤ 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

Active pulmonary TB patients (n=44) and apparently healthy control (n=44) were enrolled in this study their ages varied from 17-70 years, 44 subjects were active pulmonary tuberculosis patients, 33 of them (75%) were males and 11 of them (25%) were females, with mean age of cases (41±18.3 std) years. The other44 subjects are apparently healthy control, 33(75%) of them were males and 11 (25 %) were females with mean age (41±18.3 std) years as show in table (4-1).

Table (4-1): Distribution of sex among the cases and control groups

Gender	Cases (No %)	Controls (No %)
Males	33 (75%)	33 (75%)
Females	11(25%)	11(25%)
Total	44 (100%)	44 (100%)

Thrifty two (50%) of active pulmonary tuberculosis patients were in age group (31-50) years and 19 (34.2%) of them were in age group (11-30) years and 3 (6.8%) of them were in age group (51-70) years and 32 (50%) of healthy control group were in age group (31-50) years and 19 (34.2%) of them were in age group (11-30) years and 3 (6.8%) of them were in age group (51-70) years as shown in table (4-2)

Table (4-2): Distribution of age groups the cases and control groups

Age groups /years	Cases (No %)	Control (No %)
11-30	19(34.2%)	19 (34.2%)
31-50	32(50%)	32 (50%)
51-70	3 (6.8%)	3 (6.8%)
Total	44 (100%)	44 (100%)

The mean level of TNF-α in active pulmonary tuberculosis patient (44.4 pg/ml), in control group (9.7 pg/ml) with statistical significant difference between case and control (*p*-value 0.000)

Table (4-3): Association of TNF-α Level among cases and controls groups

Subjects	Mean	*P.* value
Cases (N=44)	44.4 pg/ml	0.000
Control (=44)	9.7 pg/ml	

**P*. value ≤ 0.05 = significant.

The mean level of TNF-α was higher in males (46pg/ml) than females (39 pg/ml) and showed no statistical significant difference (*P*-value 0.5).

Table (4-4): Association of TNF-α and gender

Sex	Mean ±STD	*P.* value
Male (N=33)	46 ± 40 pg/ml	0.564
Female (N=11)	39 ± 16 pg/ml	

**p*-value ≤ 0.05 = significant.

The results also showed that the TNF-α level were not affected by age in the case subject and *p*.value showed an insignificant correlation (*P*-value = 0.902).

Table (4-5): Association of TNF-α and age Group.

Age groups	Mean ±STD	*P.* value
11-30 (N=19)	42 ± 20 pg/ml	0.902
31-50 (N=32)	50 ± 45 pg/ml	
51-70 (N=3)	16 ± 1 pg/ml	

The mean level of CRP in active pulmonary tuberculosis patient (53.9mg/l), in control group (3.5mg/l) with statistical significant difference between case and control (*p*-value 0.000)

Table (4-6): Association of CRP Level among cases and control groups

Subjects	Mean	*P.* value
Cases (N=44)	53.9mg/l	0.000
Control (N=44)	3.5mg/l	

*p-value ≤ 0.05 = significant.

The mean level of CRP was higher in males (55mg/l) than females (48 mg/l) and showed no statistical significant difference (*P*-value 0.7).

Table (4-7): Association of CRP and gender

Sex	Mean ±STD	*P.* value
Male (N=33)	55 ± 36 mg/l	0.769
Female (N=11)	48 ± 37 mg/l	

*p-value ≤ 0.05 = significant

The results also showed that the CRP level were not affected by age in the case subject and *p*. value showed an insignificant different (*P*.value 0.4).

Table (4-8): Association of CRP and age Group.

Age groups	Mean ±STD	*P.* value
11-30 (N=19)	51± 31 mg/L	0.406
31-50 (N=32)	50 ± 39 mg/L	
51-70 (N=3)	97 ± 9 mg/L	

*p-value ≤ 0.05 = significant.

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

Tumor necrosis factor (TNF) is a proinflammatory cytokine that has important roles in mammalian immunity and cellular homeostasis. Deregulation of TNF receptor (TNFR) signaling is associated with many inflammatory disorders. (Brenner, 2015). The roles of the TNF superfamily in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, and morphogenesis have been documented. Their roles in immunologic, cardiovascular, neurologic, pulmonary, and metabolic diseases are becoming apparent. (Aggarwal *et al.*, 2012).

In the present study serum level of TNF-α (pg/ml) was significantly higher in active pulmonary tuberculosis patients compared to healthy control group (*p*.value 0.000), This finding was supported by Mirzaei and Mahmoudi (2018) in Iran, they showed that the concentration of TNF-α in patients with TB was significantly higher than in the control group (P<0.05). Furthermore, Majewski *et al* (2018) in Poland showed that, the Mean serum concentration of TNF-α was significantly higher in the TB group (8.51±1.92) compared with healthy controls (2.69±0.19) *(p* < than 0.001). Similar results were observed by Joshi *et al* (2015) in India, they showed that the median values of TNF-α cytokine were significantly high among active pulmonary tuberculosis patients compared to healthy control group (*P*< 0.0001). TNF-α is involved in the protective response against *M. tuberculosis*, its role is complex and it was accepted that it had a role in the formation of intermediate reactive to nitrogen and oxygen, mediating the anti-TB activity of macrophages so the level was elevated in serum of infected patient

This study also demonstrates that there is no association between TNF-α and gender and age among cases (*P*.value = 0.5, 0.9 respectively). This finding was supported by Vinhaes *et al* (2019) in Brazil who demonstrated that there is no statistical difference of TNF-α in gender and age in case and control group (*P*.value = 0.6, 0.3), respectively.

Serum-CRP is one of the most common acute phase reactant proteins used as an indicator of inflammation. In the present study also the serum level of CRP was significantly higher in active pulmonary tuberculosis patients compared to healthy control group (*p*.value 0.000), This finding was supported by Mohamed (2012) in India who demonstrate that there are significant increases of CRP in active pulmonary

tuberculosis patients compared with healthy control group (*P*.value < 0.0001). also this finding was supported by Rao *et al* (2009) in India they found that C-reactive protein levels were found to be significantly higher in smear- positive group as compared with the smear-negative group, the values being 37.598 ± 23.195 and 5.40 ± 188 respectively ($p < 0.0005$). Also Opolot *et al* (2015) in South Africa were found that active TB patients had a higher CRP serum levels compared with control group also this finding was reported by Shaikh *et al* (2012) in Pakistan, they found that C-reactive protein turned to be negative in those who had inactive lesions (99 patients) and also in 32 cases that were in the process of improvement, it showed weak reaction, but in 26 cases with active lesions and resistant mycobacterium, C-reactive protein was strongly positive.

This study also demonstrates that there is no statistical correlation between CRP and gender and age among cases (*P*. value: 0.7, 0.4 respectively). This finding was supported by Shaikh *et al* (2012) in Pakistan they found that: All patients with pulmonary tuberculosis > 12 years of age, of either gender were evaluated for their serum CRP level The overall mean CRP in patients with TB was 9.87±4.83 where as it was 11.21±3.32 and 13.82±4.63 in male and female subjects respectively. The mean±SD of normal and raised serum CRP was 2.76±1.34 and 13.26±4.42 ($p < 0.01$). On the contrary, Brown *et al* (2016) in London, they found that men had higher baseline CRPs than women (51 mg/L vs 32 mg/L $p < 0.001$) and adults had higher CRPs than children, may be this difference due to small sample size in my study.

5.2 Conclusion

This study concluded that: -

TNF alpha and CRP serum levels were significantly high among active pulmonary tuberculosis patients comparing to control group.

There was no association between TNF alpha and CRP serum levels in Active pulmonary TB patients with age and gender.

TNF alpha serum level can be considered useful as diagnostic tool for active pulmonary tuberculosis infection in combination with CRP serum level.

5.3 Recommendations

-Further studies may be conducted considering: -

-Regular measurement of TNF alpha and CRP serum level in Sudanese active pulmonary TB patients.

-Studying the role of TNF alpha and CRP in the progressing of extra pulmonary tuberculosis.

-Using of TNF alpha and CRP serum levels in follow up of active pulmonary TB treatments.

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APPENDICIES

APPENDIX I

Sudan University of Sciences and Technology

College of Graduate Studies

Questionnaire

Estimation of Tumor Necrosis Factor alpha (TNF-α) and C - Reactive Protein (CRP) serum Levels in Sudanese Active Pulmonary Tuberculosis Patients Attending Aboanga Hospital in Khartoum State, Sudan

1. ID Number: ……

2. Gender: Male ( ) Female ( )

3. Age……. year

4. Age: 20-30 ( ) 31-60 ( ) More than 60 years ( )

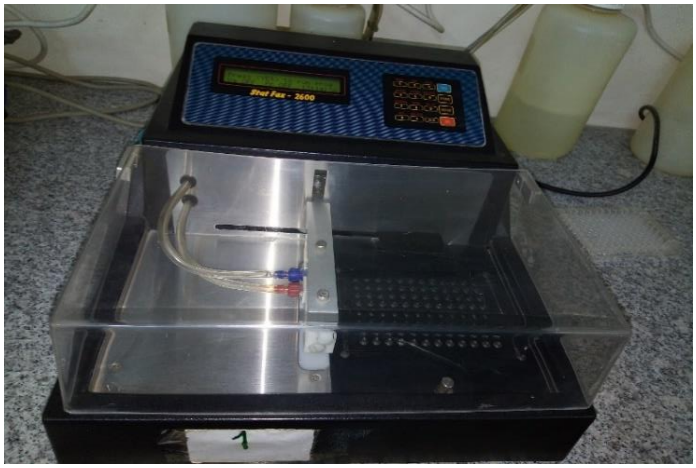
5. Duration of the disease: More than two months ( ) less than two months ( )

Result:

1. TNF-α level…… pg/ml

2. CRP level……mg/L

APPENDIX II



Color plate (1): ELISA reader

Color plate (2): Rotator

APPENDIX III

Color plate (3): ELISA kit

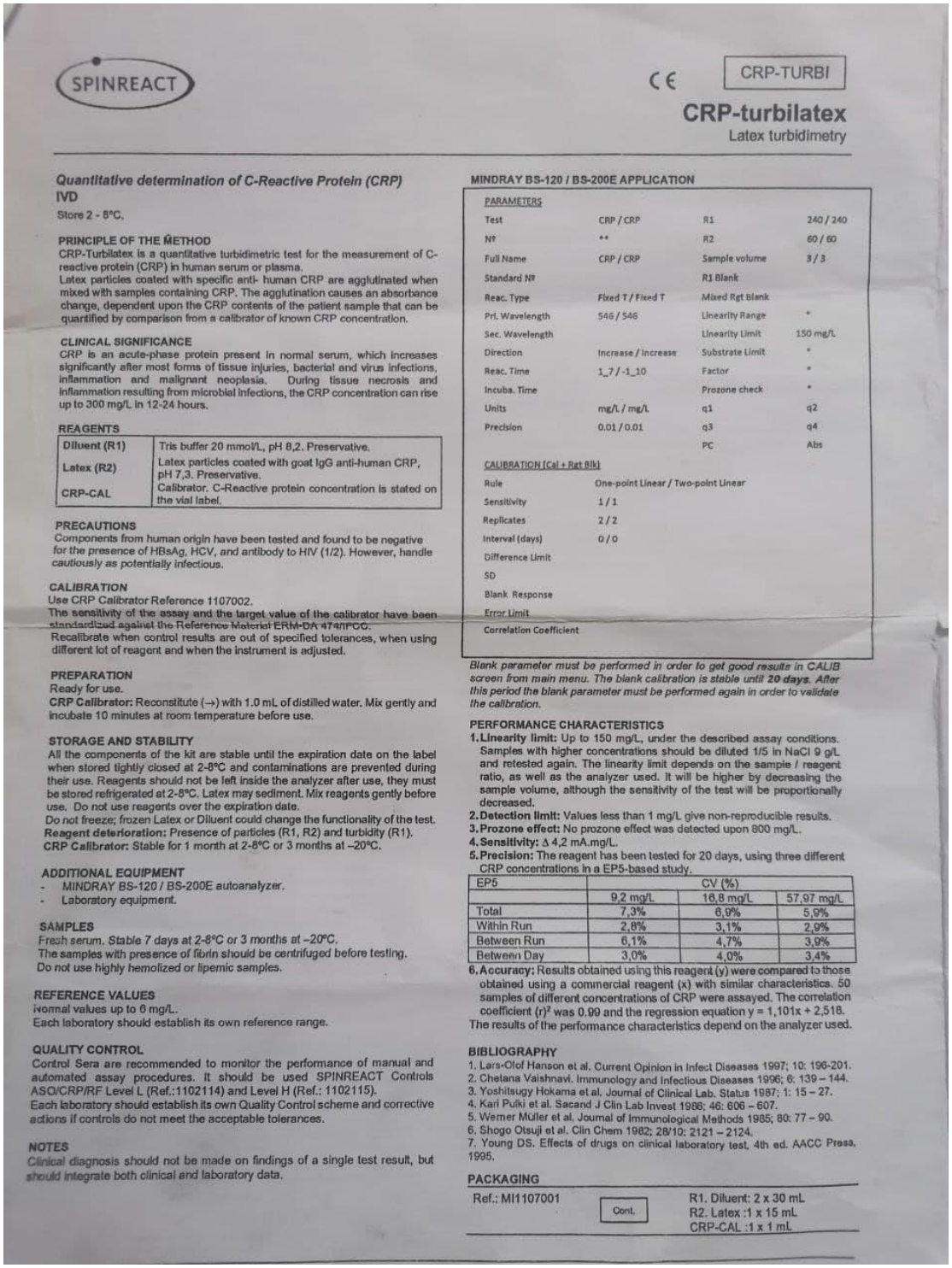
Color plate (4): CRP kit

APPENDIX IV

Color plate (5): ELISA micro plate

Color plate (6): Auto-chemistry analyzer

APPENDIX VI



SPINREACT

CE CRP-TURBI

CRP-turbilatex

Latex turbidimetry

Quantitative determination of C-Reactive Protein (CRP)

IVD

Store 2 - 8°C.

PRINCIPLE OF THE METHOD

CRP-Turbilatex is a quantitative turbidimetric test for the measurement of C-reactive protein (CRP) in human serum or plasma.

Latex particles coated with specific anti- human CRP are agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change, dependent upon the CRP contents of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration.

CLINICAL SIGNIFICANCE

CRP is an acute-phase protein present in normal serum, which increases significantly after most forms of tissue injuries, bacterial and virus infections, inflammation and malignant neoplasia. During tissue necrosis and inflammation resulting from microbial infections, the CRP concentration can rise up to 300 mg/L in 12-24 hours.

REAGENTS

Diluent (R1)	Tris buffer 20 mmol/L, pH 8,2. Preservative.
Latex (R2)	Latex particles coated with goat IgG anti-human CRP, pH 7,3. Preservative.
CRP-CAL	Calibrator. C-Reactive protein concentration is stated on the vial label.

PRECAUTIONS

Components from human origin have been tested and found to be negative for the presence of HBsAg, HCV, and antibody to HIV (1/2). However, handle cautiously as potentially infectious.

CALIBRATION

Use CRP Calibrator Reference 1107002.

The sensitivity of the assay and the target value of the calibrator have been standardized against the Reference Material ERM-DA 474/IFCC.

Recalibrate when control results are out of specified tolerances, when using different lot of reagent and when the instrument is adjusted.

PREPARATION

Ready for use.

CRP Calibrator: Reconstitute (→) with 1.0 mL of distilled water. Mix gently and incubate 10 minutes at room temperature before use.

STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C and contaminations are prevented during their use. Reagents should not be left inside the analyzer after use, they must be stored refrigerated at 2-8°C. Latex may sediment. Mix reagents gently before use. Do not use reagents over the expiration date.

Do not freeze; frozen Latex or Diluent could change the functionality of the test.

Reagent deterioration: Presence of particles (R1, R2) and turbidity (R1).

CRP Calibrator: Stable for 1 month at 2-8°C or 3 months at –20°C.

ADDITIONAL EQUIPMENT

- MINDRAY BS-120 / BS-200E autoanalyzer.
- Laboratory equipment.

SAMPLES

Fresh serum. Stable 7 days at 2-8°C or 3 months at –20°C.

The samples with presence of fibrin should be centrifuged before testing.

Do not use highly hemolized or lipemic samples.

REFERENCE VALUES

Normal values up to 6 mg/L.

Each laboratory should establish its own reference range.

QUALITY CONTROL

Control Sera are recommended to monitor the performance of manual and automated assay procedures. It should be used SPINREACT Controls ASO/CRP/RF Level L (Ref.:1102114) and Level H (Ref.: 1102115).

Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

NOTES

Clinical diagnosis should not be made on findings of a single test result, but should integrate both clinical and laboratory data.

MINDRAY BS-120 / BS-200E APPLICATION

PARAMETERS			
Test	CRP / CRP	R1	240 / 240
Nº	**	R2	60 / 60
Full Name	CRP / CRP	Sample volume	3 / 3
Standard Nº		R1 Blank	
Reac. Type	Fixed T / Fixed T	Mixed Rgt Blank	
Pri. Wavelength	546 / 546	Linearity Range	*
Sec. Wavelength		Linearity Limit	150 mg/L
Direction	Increase / Increase	Substrate Limit	*
Reac. Time	1_7 / -1_10	Factor	*
Incuba. Time		Prozone check	*
Units	mg/L / mg/L	q1	q2
Precision	0.01 / 0.01	q3	q4
		PC	Abs

CALIBRATION [Cal + Rgt Blk]	
Rule	One-point Linear / Two-point Linear
Sensitivity	1 / 1
Replicates	2 / 2
Interval (days)	0 / 0
Difference Limit	
SD	
Blank Response	
Error Limit	
Correlation Coefficient	

*Blank parameter must be performed in order to get good results in CALIB screen from main menu. The blank calibration is stable until **20 days**. After this period the blank parameter must be performed again in order to validate the calibration.*

PERFORMANCE CHARACTERISTICS

1. **Linearity limit:** Up to 150 mg/L, under the described assay conditions. Samples with higher concentrations should be diluted 1/5 in NaCl 9 g/L and retested again. The linearity limit depends on the sample / reagent ratio, as well as the analyzer used. It will be higher by decreasing the sample volume, although the sensitivity of the test will be proportionally decreased.
2. **Detection limit:** Values less than 1 mg/L give non-reproducible results.
3. **Prozone effect:** No prozone effect was detected upon 800 mg/L.
4. **Sensitivity:** Δ 4,2 mA.mg/L.
5. **Precision:** The reagent has been tested for 20 days, using three different CRP concentrations in a EP5-based study.

EP5	CV (%)		
	9,2 mg/L	16,8 mg/L	57,97 mg/L
Total	7,3%	6,9%	5,9%
Within Run	2,8%	3,1%	2,9%
Between Run	6,1%	4,7%	3,9%
Between Day	3,0%	4,0%	3,4%

6. **Accuracy:** Results obtained using this reagent (y) were compared to those obtained using a commercial reagent (x) with similar characteristics. 50 samples of different concentrations of CRP were assayed. The correlation coefficient $(r)^2$ was 0,99 and the regression equation y = 1,101x + 2,518.

The results of the performance characteristics depend on the analyzer used.

BIBLIOGRAPHY

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2. Chetana Vaishnavi. Immunology and Infectious Diseases 1996; 6: 139 – 144.
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4. Kari Pulki et al. Sacand J Clin Lab Invest 1986; 46: 606 – 607.
5. Werner Müller et al. Journal of Immunological Methods 1985; 80: 77 – 90.
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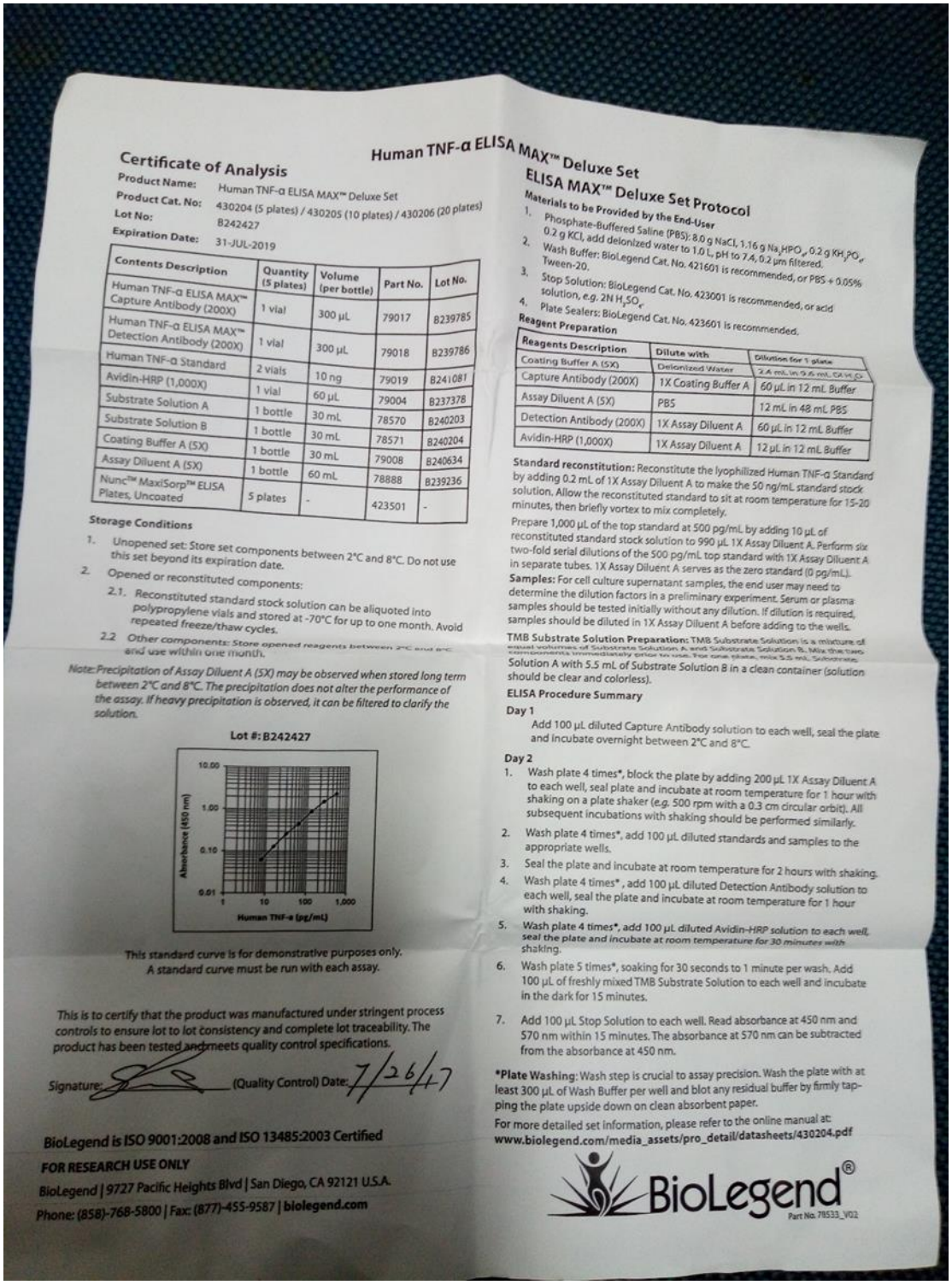
PACKAGING

Ref.: MI1107001	Cont.	R1. Diluent: 2 x 30 mL R2. Latex :1 x 15 mL CRP-CAL :1 x 1 mL

Color plate (7): CRP leaflet

APPENDIX VII



Certificate of Analysis

Product Name: Human TNF-α ELISA MAX™ Deluxe Set
Product Cat. No: 430204 (5 plates) / 430205 (10 plates) / 430206 (20 plates)
Lot No: B242427
Expiration Date: 31-JUL-2019

Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human TNF-α ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79017	B239785
Human TNF-α ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79018	B239786
Human TNF-α Standard	2 vials	10 ng	79019	B241081
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B237378
Substrate Solution A	1 bottle	30 mL	78570	B240203
Substrate Solution B	1 bottle	30 mL	78571	B240204
Coating Buffer A (5X)	1 bottle	30 mL	79008	B240634
Assay Diluent A (5X)	1 bottle	60 mL	78888	B239236
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #: B242427

This standard curve is for demonstrative purposes only.
A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 7/26/17

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Phone: (858)-768-5800 | Fax: (877)-455-9587 | biolegend.com

Human TNF-α ELISA MAX™ Deluxe Set

ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL CaH_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 12 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution: Reconstitute the lyophilized Human TNF-α Standard by adding 0.2 mL of 1X Assay Diluent A to make the 50 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 500 pg/mL by adding 10 µL of reconstituted standard stock solution to 990 µL 1X Assay Diluent A. Perform six two-fold serial dilutions of the 500 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution Preparation: TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal plate and incubate at room temperature for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times* , add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 15 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/430204.pdf

BioLegend®
Part No. 78533_V02

Color plate (8): TNF- α leaflet