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Investigating the mRNA levels of ANGPTL4 and its correlation with the expression of some immunological markers in Iraqi patients with tuberculosis

Mujahid Gati Dahdouh, Sanaz Sheikhzadehh

Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
S.sheikhzadehh@gmail.com

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Abstract

Tuberculosis (TB) is a leading cause of morbidity and mortality from a single infectious agent, despite being preventable and curable. Early and accurate diagnosis of active TB is critical to both enhance patient care, improve patient outcomes, and break Mycobacterium tuberculosis (Mtb) transmission cycles. Angiopoietin-like 4 (Angptl4) is a protein that belongs to the angiopoietin-like family. Angptl4 promotes the recruitment of immune cells and the production of inflammatory cytokines, contributing to the modulation of inflammatory processes. It can be induced by pro-inflammatory signals, such as tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), and lipopolysaccharides (LPS). Given its multifaceted roles in metabolic disorders, angiogenesis, and inflammation, Angptl4 has attracted attention as a potential therapeutic target. In this study, using western blot and RT-PCR, the expression level of ANGPTL4, IL-6, Sirt1, NF- κ B, TNF- α , JNK-MAPK and IL-1 β in patients with tuberculosis (50 people) and healthy people were measured. Expression level of ANGPTL4, IL-6, NF- κ B, TNF- α , JNK-MAPK and IL-1 β increased significantly ($P < 0.05$) in patients compared to the control (healthy group) and the amount of Sirt1 decreased. The results of our study show that the identification of specific immunological markers and their association with ANGPTL4 expression can lead to the discovery of new diagnostic biomarkers for tuberculosis. These biomarkers can be used to improve the accuracy and efficiency of TB diagnosis, especially in resource-limited settings where traditional diagnostic methods may be limited.

Keyword: Tuberculosis; Angptl4; Biomarkers; miRNA

1 Introduction

Tuberculosis (TB) is an infectious disease that usually affects the lungs and is caused by the bacterium *Mycobacterium tuberculosis*. It is estimated that about a quarter of the world's population is infected with the tuberculosis bacteria (1). In 2022, a total of 1.3 million people (including 167,000 people with HIV) will die from TB. Worldwide, TB is the second leading infectious killer after COVID-19 (beyond HIV and AIDS). Multidrug-resistant tuberculosis (MDR-TB) remains a public health crisis and a health security threat (2). 13 billion dollars is needed annually for TB prevention, diagnosis, treatment and care to meet the global goal agreed at the 2018 UN Summit to end the TB epidemic by 2030 (2). Tuberculosis is treated using a combination of 4 types of antibiotics for a period of 6 to 9 months, which leads to a high rate of treatment abandonment. This situation has increased the recurrence and emergence of multi-drug-resistant tuberculosis (MDR-TB), extremely drug-resistant tuberculosis (XDR-TB) and total drug-resistant tuberculosis (TDR-TB). Therefore, it is very necessary to find a new way of identification and treatment (3). Although identifying and diagnosing ATB in an infected individual is critical, there are challenges. Although culture and GeneXpert have high sensitivity and specificity in smear positive cases, its diagnostic accuracy is low in people with smear negative disease, children and extrapulmonary tuberculosis. In addition, culture yields results slowly and GeneXpert MTB/RIF is costly and requires significant infrastructure to implement, limiting its widespread use. These methods have additional limitations in monitoring response to treatment, which currently includes at least 4–6 months of treatment (4).

Using biomarkers is a very appropriate strategy. In the field of tuberculosis, pathogen and host biomarkers have been widely investigated. Pathogen-based biomarkers include DNA and antigen detection (5). There is a wide variety of host-based biomarkers, including blood markers, antibody response to antigen, cytokines and chemokines, RNA, other proteins, metabolites, and combinations of several markers (6). The utility of these biomarkers can be broken down by their use as diagnostic tests, including for point-of-care testing, prognostic tests for risk of progression to active TB, and markers of treatment response that may predict TB treatment outcomes. A biomarker is inexpensive and obtained from a readily available sample, such as blood or urine, and the equipment used to measure the biomarker is easy to use and at the point of care (7).

ANGPTLs are a group of proteins involved in vascular remodeling and lipid metabolism and are found in the liver, small intestine, blood plasma, and adipose tissue (8). ANGPTL-4 is a critical member of the family of special secreted proteins. ANGPTL-4 has been confirmed to be involved in angiogenesis-related disorders such as inflammation (9), and can be induced by pro-inflammatory signals such as tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β) and lipopolysaccharides (LPS) and acts as a mediator of the inflammatory response. Angptl4 promotes the recruitment of immune cells and the production of inflammatory cytokines, helping

to modulate inflammatory processes. Consequently, it is an attractive and promising biomarker for the detection or diagnosis of various inflammations (10).

The research aims to quantify the mRNA levels of ANGPTL4 in Iraqi patients diagnosed with tuberculosis. This analysis will involve collecting samples, isolating RNA, and using molecular techniques such as quantitative polymerase chain reaction (qPCR) to determine the relative expression levels of ANGPTL4 mRNA. The goal is to investigate whether there are significant differences in ANGPTL4 mRNA levels between tuberculosis patients and healthy individuals. The study also seeks to characterize the expression levels of specific immunological markers in tuberculosis patients. These markers may include cytokines, chemokines, immune cell surface markers, or other molecules associated with the immune response. The primary objective is to investigate the potential correlation between ANGPTL4 mRNA levels and the expression levels of selected immunological markers.

2 Methods

2-1 Data Collection

This research was mainly done in Tabriz Immunology Research Center. Sampling was done from 2 groups of healthy and TB patients (50 people in each group). Blood samples of more than 50 patients with tuberculosis were obtained from Imam Reza Hospital (AS) and Tabriz International Hospital in Tabriz with the explicit written consent of the participants. Specialist doctors identified patients with tuberculosis (TB).

2-2 Isolation of blood cells with ficoll

PBMCs include lymphocytes (i.e. T cells, B cells, and NK cells), monocytes, and dendritic cells, and are defined as white blood cells with round nuclei. Preparation of a PBMC fraction from whole blood is a common step prior to the isolation of specific immune cell subsets. The most common PBMC isolation method involves using a density gradient medium (e.g. Ficoll™ or Lymphoprep™) and centrifugation. This method takes advantage of the differences in density between the cells in blood and the density gradient medium. Whole blood is first diluted with phosphate buffered saline (PBS) and then carefully layered over the density gradient medium. During centrifugation, the cells with higher densities (i.e. granulocytes and erythrocytes) sediment through the density gradient medium. The peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood. Briefly, 25 ml of supernatant was carefully layered over 15 ml of Ficoll after blending with PBS and centrifuged at 3200 ×g for 20 min without brake and acceleration. After centrifugation, the interphase was collected and centrifuged again at 2500 ×g for 6 min. Finally, they seeded in RPMI medium supplemented with 10% fetal bovine serum (FBS) (11).

2-3 RT-PCR assay

Total RNA with Trizol reagent (GENEALL, Korea) was extracted from cells. Quantified using the nanodrop the RNA extracted concentrations are measured. GAPDH and U6 was used as an internal control for IL-6, Sirt1, NF- κ B, TNF- α , JNK-MAPK and IL-1 β . Each sample was analyzed in triplicate. Gene expression was presented using a modification of the $2^{-\Delta\Delta C_t}$ method. RNA extracted by Trizol, in 20 μ l of total volume, was subjected to reverse transcription with the cDNA synthesis kit (GENEALL, Korea). The enzyme was inactivated for 5 min at 95 °C. The PCR was performed as follows: 95 °C for 3 min, 30 cycles of 93 °C for 30 s, 55 °C for 40 s and 72 °C for the 60 s (Rosch, Germany) (11).

2-4 Western Blot

Cells after washing twice with PBS, the cells were placed in a lysis buffer for 30 min and suspended. The suspended cells were centrifuged at 14,000 rpm for 20 min. The supernatant was collected, and the protein content was measured through the Bradford method. Protein lysates were separated by 12% SDS gel electrophoresis and then transferred to a PVDF membrane. The membrane was then placed in a blocking solution (2% skim milk in TBS buffer) for 1 day. After that, IL-6, Sirt1, NF- κ B, TNF- α , JNK-MAPK and IL-1 β antibodies were mixed with the blocking solution, and the membrane was further incubated for 16 to 18 h. After initial staining of the membrane with secondary rabbit antibody with concentration (1: 1000) for all primary antibodies for one hour and 15 min at room temperature. The GAPDH and U6 antibody was used as a loading control (12).

2-5 Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey HSD test or by Student's t-test. Data are presented as mean \pm SEM (animal experiments) or mean \pm SD (cell culture studies). $P < 0.05$ was considered statistically significant.

3 Results

Real-time PCR to evaluate the level of mRNA and protein expression. U6 was used as the silencing reference standard. The expression level of immunological markers related to tuberculosis including (IL-6, Sirt1, NF- κ B, TNF- α , JNK-MAPK and IL-1 β) and ANGPTL4 was measured by RT-PCR in 2 groups of patients and healthy. Based on the obtained results, the expression of ANGPTL4 in patients with tuberculosis was significantly higher than in healthy individuals ($P < 0.003$) (Fig 1).

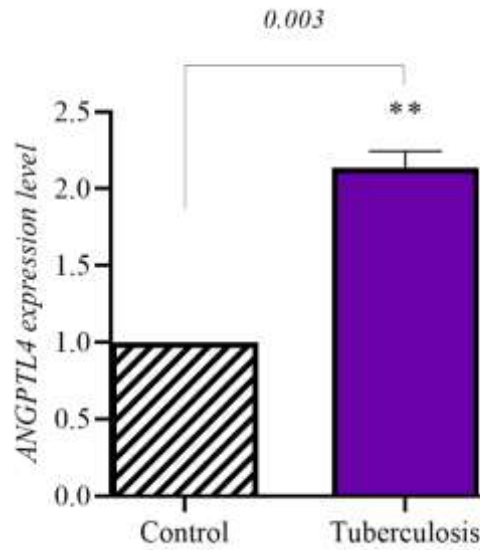


Fig 1. RT-PCR analysis of TB patients was performed for ANGPTL4 (P<0.003).

In the following, the levels of immunological markers were measured, IL-6 and TNF- α are two pro-inflammatory cytokines, and IL-6 had a slight but significant increase compared to healthy subjects (P<0.0448), the level of TNF- α expression was also had increased and this increase was more than IL-6 and has more significance (P<0.0075) (Fig 2).

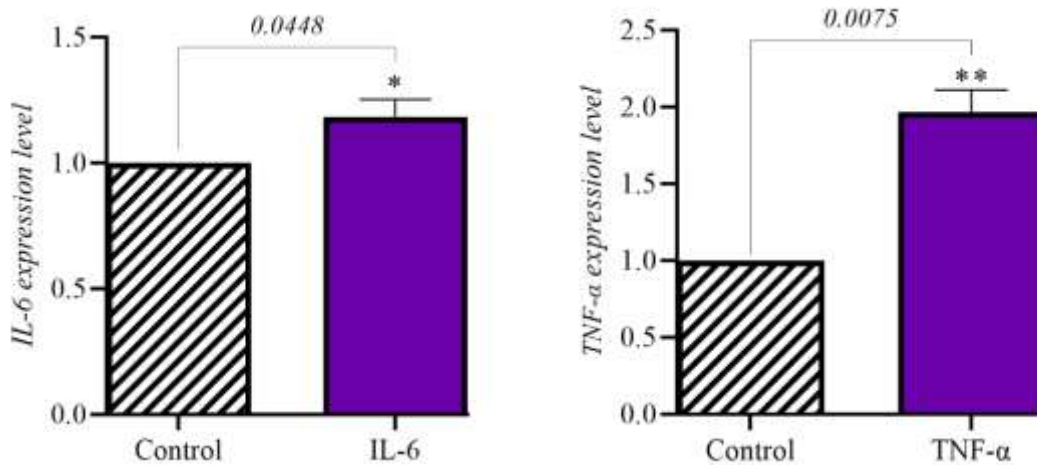


Fig 2. RT-PCR analysis of TB patients was performed for IL-6 and TNF- α

The results of the RT-PCR test showed that NF- κ B expression is classified as a family of inducible transcription factors that play an important role in mediating innate and adaptive

immunity as well as pro-inflammatory responses, significantly increased ($P < 0.0006$) and the expression of Sirt1, which is an inhibitor of NF- κ B, has decreased significantly ($P < 0.0381$) (Fig 3).

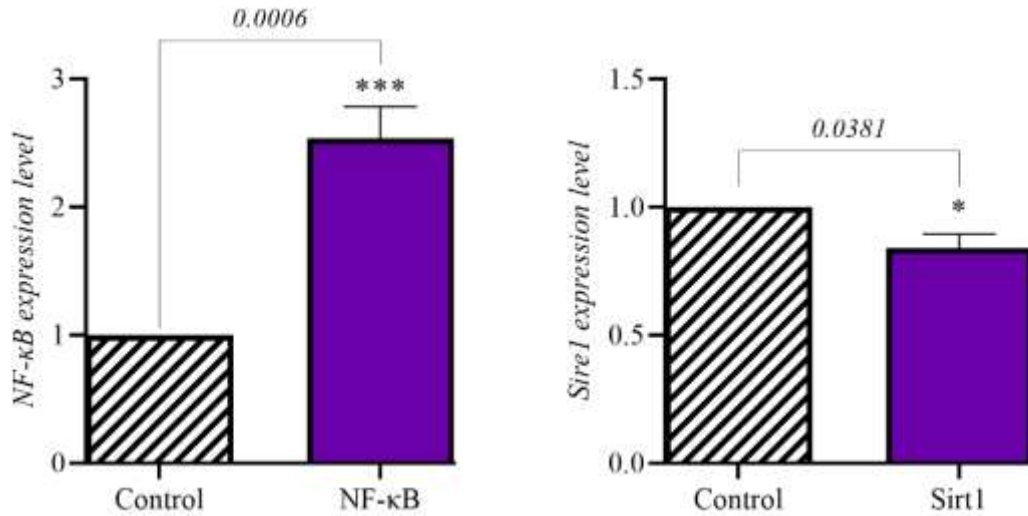


Fig 3. RT-PCR analysis of TB patients was performed for NF- κ B and Sirt1

The expression level of IL-1 β cytokine, which is a pro-inflammatory cytokine, which induces ANGPTL4 through JNK-MAPK, was also investigated. Based on the results, the expression level of both these cytokines is significantly increased in people with tuberculosis) $P < 0.006$) and ($P < 0.0055$) (Fig 4).

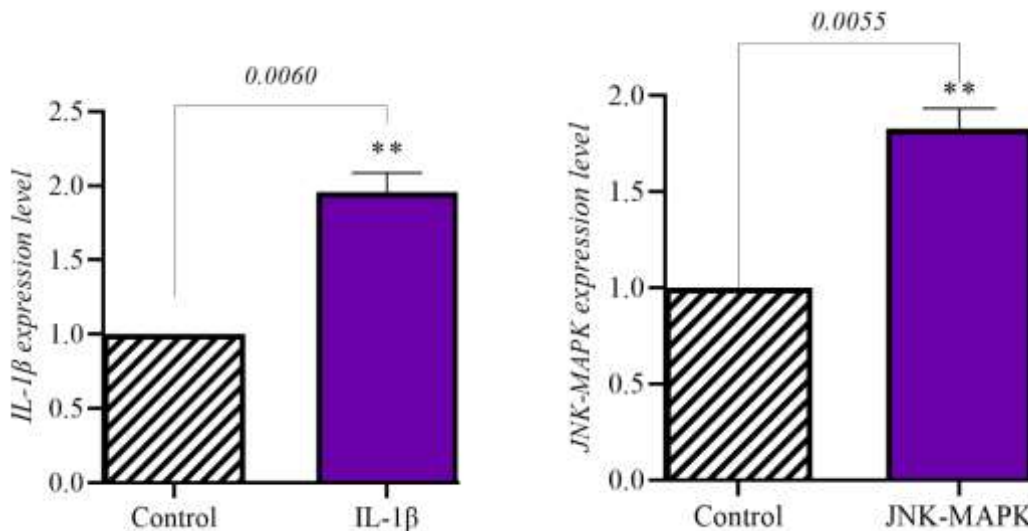


Fig 4. RT-PCR analysis of TB patients was performed for IL-1 β and JNK-MAPK

The expression level of proteins related to inflammation was measured in people with tuberculosis and healthy people. After analysis, the expression levels of ANGPTL4, IL-6, TNF- α , NF- κ B, IL-1 β and JNK-MAPK proteins were significantly increased, while Sirt1 was decreased ($P < 0.05$) (Fig 5). These data confirm the RT-PCR results.

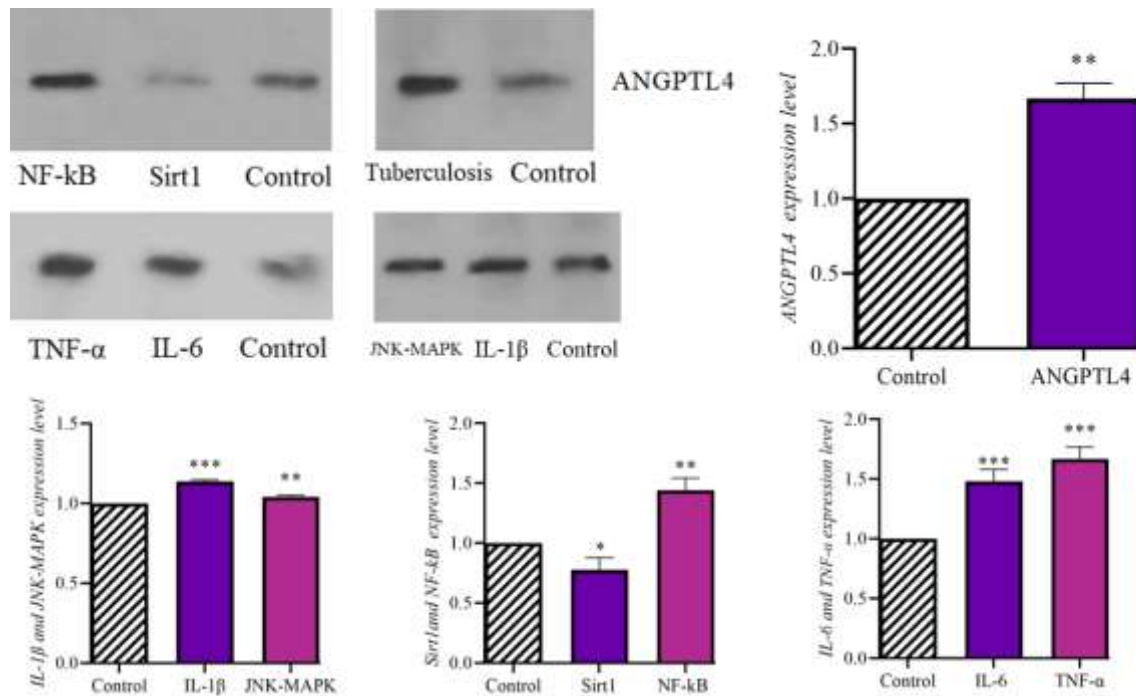


Fig 5. shown the results present the expression of TB-related proteins.

5 Discussion

Angiopoietin-like 4 (ANGPTL4) is a multifaceted secreted protein discovered by three different institutions in 2000 simultaneously. It is expressed in adipose tissues, liver, muscle, heart, kidney, skin, and other tissues. The nutritional, metabolic, and inflammatory status of the organism regulate the expression of ANGPTL4 (13).

Studies have shown that ANGPTL4 may be involved in the inflammatory response in influenza pneumonia and LPS-induced acute lung injury. Li et al. (14) identified elevated expression of ANGPTL4 in lung biopsy specimens from patients with infectious pneumonia compared to normal lung specimens. Further studies revealed that the influenza infection directly stimulated ANGPTL4 expression through the IL6-STAT3 signaling pathway (14). The concomitant increase in Furin activity cleaves fANGPTL4 to generate cANGPTL4, resulting in extensive lung injury characterized by large regions of pulmonary hemorrhage and infiltration of immune cells. ANGPTL4 deficiency improves pulmonary tissue integrity and accelerates recovery. In another recent study, Li et al. (15) showed that antibody treatment against cANGPTL4 could reduce pulmonary edema and damage in infected mice of secondary bacterial

pneumonia. And this effect was also confirmed using ANGPTL4^{-/-} mice. Based on the known involvement of ANGPTL4 in immune regulation and its potential role in angiogenesis, we hypothesized that ANGPTL4 mRNA levels would be altered in Iraqi patients with tuberculosis. Furthermore, we predicted a correlation between ANGPTL4 mRNA levels and the expression of specific immunological markers, indicating a potential crosstalk between ANGPTL4 expression and the immune response in TB infection.

As the results announced, the investigated immunomarkers in TB patients had changes compared to healthy people. Elevated expression of plasma IL-6 cytokine and expression of an IL-6 response transcriptional signature are associated with TB disease. Furthermore, elevated IL-6 responses are observed in patients with TB disease compared to healthy controls both at the site of *in vivo* standardized mycobacterial antigen challenge and *in ex vivo* stimulated monocytes. Elevated IL-6 responses in pulmonary TB are also associated with post-treatment lung impairment (16). It was shown in Miao-Miao Tian et al.'s study that the expression of ANGPTL4 is significantly increased simultaneously with the increase in the production of anti-inflammatory cytokine (peroxisome proliferator-activated receptor alpha) and pro-inflammatory cytokines including IL-6 (17). In Yuyue Zuo and et al study shown ANGPTL4 has been reported to be involved in inflammation processes of acute pulmonary diseases including influenza pneumonia and LPS-induced acute lung injury, as well as chronic pulmonary diseases such as chronic obstructive pulmonary disease. ANGPTL4 expression is directly upregulated by influenza infection through the IL6-STAT3 signaling cascade (18). In both lung tissue from an acute injury mouse model and LPS-treated human alveolar epithelial cells, ANGPTL4 expression significantly increases and is positively correlated with the inflammation in lung tissue (IL-6, and neutrophil infiltration) (19). These studies are in line with our study and confirm the possibility of increasing IL-6 in connection with ANGPTL4.

TNF- α is an essential component of the innate defense mechanism of the host against pathogenic challenge. The primary response of alveolar macrophages and dendritic cells plays a critical role in the subsequent activation of the T-cell response. Granuloma formation, representative of tuberculosis infection, is initiated by the recruitment of lymphocytes and macrophages to the infection site. Interestingly, granuloma formation is dependent on pro-inflammatory mediators of the immune response, such as tumor necrosis factor alpha (TNF- α). Unfortunately, it can also play a major role in the pathology of certain diseases, such as tuberculosis. This disease is a striking example of the role of TNF- α as a 'double-edged sword', because apart from its role in controlling the Mycobacterium tuberculosis infection, it can also cause severe tissue damage. TNF- α exhibits a very complex network of interactions and many of its activities are still not fully understood. This report aims to review the pivotal role of TNF- α in controlling the mycobacterial infection, with a particular emphasis on its influence on chemokine expression and cell movement during granuloma formation, and the issues surrounding the use of TNF- α inhibitors for therapeutic use in inflammatory diseases. In Yuyue Zuo and et al. (18) study shown in acute pancreatitis, ANGPTL4 enhances macrophage activation and leads to

hypertyrosinemia (IL-6, TNF- α). ANGPTL4 is critical for inflammation and the increase in IL-6, and TNF- α levels in the early stage of stomatitis. ANGPTL4 modulates the immune cell response to acute skin injury, wound healing, and psoriasis (TNF- α and IL-6). The increase in TNF- α gene and protein expression and its relationship with the increase in ANGPTL4 in inflammatory responses in tuberculosis can also be used.

Nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) is regarded by many as being the key mediator of the inflammatory process due to its multifaceted role in the inflammatory response. NF- κ B plays a central role in the pro-inflammatory response via the release of lysosomes in phagosomes and increasing the production of membrane transport molecules to enhance phagolysosome fusion during the invasion of foreign pathogens. There are several mechanisms by which NF- κ B affects intracellular signaling and exerts its effects on the immune response against tuberculosis. Certain mechanisms allow for an enhanced immune defense, while other pathways diminish the immune response over the course of the infection. While this pathway allows tuberculosis to prevail in its infective process, NF- κ B also supports the immune system to clear the infection (20), this article confirms the increase in NF- κ B gene and protein expression in our study patients and its relationship with IL-6 and TNF- α in tuberculosis. Recent studies have indicated that the regulation of innate immunity and energy metabolism are connected together through an antagonistic crosstalk between NF- κ B and SIRT1 signaling pathways. NF- κ B signaling has a major role in innate immunity defense while SIRT1 regulates the oxidative respiration and cellular survival. However, NF- κ B signaling can stimulate glycolytic energy flux during acute inflammation, whereas SIRT1 activation inhibits NF- κ B signaling and enhances oxidative metabolism and the resolution of inflammation. SIRT1 inhibits NF- κ B signaling directly by deacetylating the p65 subunit of NF- κ B complex. SIRT1 stimulates oxidative energy production via the activation of AMPK, PPAR α and PGC-1 α and simultaneously, these factors inhibit NF- κ B signaling and suppress inflammation. On the other hand, NF- κ B signaling down-regulates SIRT1 activity through the expression of miR-34a, IFN γ , and reactive oxygen species. The inhibition of SIRT1 disrupts oxidative energy metabolism and stimulates the NF- κ B-induced inflammatory responses present in many chronic metabolic diseases (21). In Liang Guo study the effect of ANGPTL4 on SIRT1/NF- κ B in lung cancer and its effect on inflammation was investigated. *angptl4* promoted NF- κ Bp65 expression and suppressed SIRT1 expression both in mouse lungs and A549 cells. These findings suggest that silencing *angptl4* protects against LPS-induced ALI via regulating SIRT1/NF- κ B signaling pathway (22). *Angptl4* can increase NF- κ B by inhibiting *Sirt1*. which was obtained in the results of the same process and Liang Guo's study is in line with our study. The pro-inflammatory cytokine IL-1 β is a key mediator of inflammation and plays an important role in the host resistance to tuberculosis infections. To date, most studies have examined the mechanisms of IL-1 β secretion using laboratory strains of tuberculosis and the findings may not be widely applicable to contemporary clinical strains (23). In Ryan Kolb Study shown, IL-1 β directly upregulates ANGPTL4 in adipocytes (24). And J M Noh study shown that IL-1 β increases *Angptl4* expression through a mechanism dependent on the JNK-MAPK signaling pathway in

MC3T3-E1 cells (25). Another way, Jang-Eu Cho study shown that JNK and MAPK are involved in the signal pathway responsible for tuberculosis-induced up-regulation of Lkn-1 (26). According to the studies presented and the effect of IL-1 β and JNK-MAPK in tuberculosis disease and also their regulatory effect on Angptl4 in other inflammatory diseases, along with the results of our study, the validity of these results and the relationship between the increase of two markers (IL-1 β and confirms JNK-MAPK) and Angptl4.

The results of our study show that the identification of specific immunological markers and their association with ANGPTL4 expression can lead to the discovery of new diagnostic biomarkers for tuberculosis. These biomarkers can be used to improve the accuracy and efficiency of TB diagnosis, especially in resource-limited settings where traditional diagnostic methods may be limited. By assessing ANGPTL4 mRNA levels and related immunological markers over time, clinicians can potentially track treatment efficacy and make informed decisions about treatment settings, the study found. Understanding the pathogenesis of TB and the role of ANGPTL4 can help identify subsets of patients more likely to respond to specific therapeutic approaches. By integrating the findings into clinical practice, personalized treatment strategies tailored to patients' immune profiles can be developed, potentially improving treatment outcomes and reducing the risk of drug resistance. The results of this research can clarify potential therapeutic targets related to ANGPTL4 and immunological markers. Manipulating the expression or function of these targets could potentially modulate the immune response to TB and improve treatment efficacy. This knowledge can guide the development of new therapeutic interventions, such as immunomodulatory drugs or targeted therapies, aimed at enhancing the host's immune response against TB. Research findings can help develop new drugs or treatment strategies for tuberculosis. By understanding the immunopathogenesis of TB and the role of ANGPTL4, potential drug targets or pathways involved in the immune response can be identified. This knowledge could guide the design and development of new therapies aimed at enhancing the host immune response or targeting specific immune markers associated with ANGPTL4 expression.

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