

## ARTICLE / INVESTIGACIÓN

**Soluble (PD-1) and Autophagy protein (ATG-5) in patients with tuberculosis**

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**Abstract:** Immune checkpoints such as programmed cell death protein (PD-1) and autophagy-related protein 5 (ATG5) can directly affect the immune response and thus, interfere with infection with *Mycobacterium tuberculosis* (Mtb). This study aimed to determine the role of soluble ATG5 and soluble PD-1 proteins in tuberculosis (TB) patients. The study included a collection of 90 blood samples (47 samples from patients with confirmed tuberculosis and 43 samples from age and gender-matched healthy subjects to estimate the concentration of soluble autophagy (ATG-5) and programmed cell death protein (PD-1). Enzyme-linked immunosorbent assay was used to estimate the serum levels of these markers. The median value of ATG5 level in patients was 2.08 µg/ml, which is higher than the level of the control group (median = 0.93 µg/ml) with a highly significant difference. Similarly, the median value of PD-1 level in patients was 259.4 ng/ml (range 107.76-924.25 ng/ml), which was higher than that of the control (median = 146.4 ng/ml, range = 50.32-1117.1 ng/ml) and with a highly significant difference. It can be concluded that both soluble ATG5 and PD-1 increased significantly in patients with TB compared with controls.

**Key words:** Tuberculosis, *M. tuberculosis*, Autophagy, Programmed cell death protein-1.

## Introduction

Tuberculosis (TB) is considered one of the essential epidemic diseases, and it is caused by *Mycobacterium tuberculosis* (Mtb) which commonly attacks the lungs and other organs such as bones, lymph and joints<sup>1</sup>. Patients often present with pulmonary symptoms such as a bloody cough (hemoptysis), chest pain and shortness of breath (SOB), and general clinical symptoms such as fever, poor appetite, weight loss and night sweats<sup>2</sup>.

According to World Health Organization (WHO) statistics in 2018, one-third of the world's population is infected with MTB, 90% of whom have latent infection. With most severe cases are in developing countries. The prevalence is associated with poor treatment, drug resistance and limited vaccines<sup>3</sup>.

Understanding the host response to Mtb infection is a critical factor in efforts to eradicate TB by developing effective vaccines and immune therapeutics. The ability of Mtb to survive and replicate in host macrophages is an essential step for its pathogenesis, with intracellular growth being often associated with virulence<sup>4,5</sup>.

Autophagy is an intracellular self-digestion process whereby cytoplasmic constituents are delivered to and degraded by lysosomes<sup>6</sup>. Besides being crucial for quality control and energy supply, autophagy plays critical roles in immune defenses against invading bacterial and viral pathogens, such as regulation of inflammation, antigen presentation and microorganism capture and degradation<sup>7-9</sup>.

Programmed cell death protein 1 (PD-1) is a 288 amino acid cell surface molecule that has been designated as a membrane protein of the immunoglobulin superfamily in humans. This protein is expressed on T cells, pro-B cells, and

myeloid-derived dendritic cells, leading to negative regulation of the proliferation and activity of these cells<sup>10</sup>.

Increasing evidence suggests PD-1 expression and some autophagy proteins such as ATG5 are upregulated in patients with active TB<sup>11-13</sup>. This study aimed to measure the serum levels of soluble PD-1 and ATG5 in patients with TB.

## Materials and methods

### The Study Population

The study involved 47 patients with TB attending the TB control center/ Baghdad during the period from 1<sup>st</sup> January 2019 to 31<sup>st</sup> October 2020. The TB diagnosis was made first with acid-fast stain (figure 1), then confirmed with culturing on Löwenstein–Jensen (LJ) culture and biochemical test specific for Mtb.

Other age- and gender-matched 43 apparently healthy subjects were selected to represent controls. Blood samples were obtained from patients and controls. The sera were separated and stored at -20°C for immunological studies. Demographic data (age, gender, socioeconomic status, smoking habit) were collected from each participant through direct interview. The ethical committee approved the study of the College of Science, Tikrit University.

### Serum Level of Soluble PD-1 and ATG5

Ready commercial kits (SunLong Biotech Co., LTD/ China) were used to measure serum levels of soluble ATG5 and PD-1 through enzyme-linked immunosorbent assay (ELISA). This assay is based on the presence of antibodies

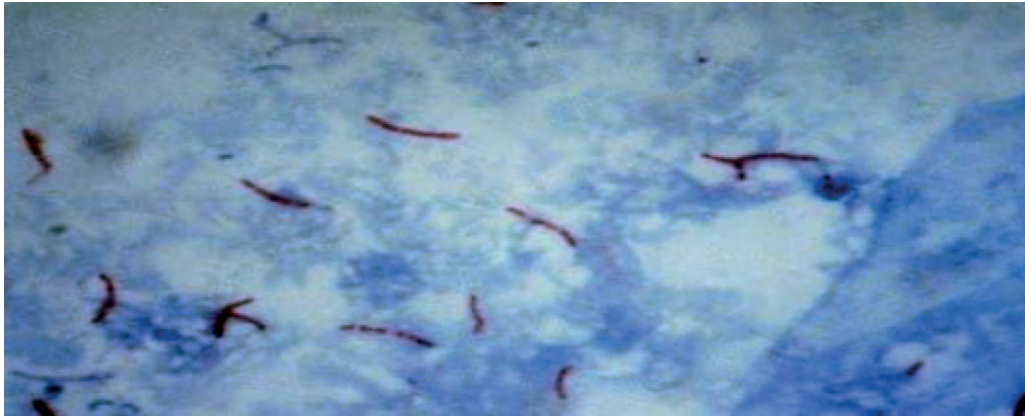
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**Figure 1.** Acid fast bacilli of *M. tuberculosis* 100X.

(for PD-1 or ATG-5) attached to microliter wells. Soluble PD-1 or ATG-5 reacts with these antibodies. An enzyme-conjugated anti- PD-1 or ATG-5 (horseradish peroxidase (HRP) is then added to the reaction. The enzyme acts as an amplifier of the detection signal by converting a substrate that results in color changes in the wells. The density of color is proportional to the serum concentration of PD-1 or ATG-5. The manufacturer's instructions were followed precisely. A fully automated apparatus read the results. These assays were performed at the Medical Research Unit, College of Medicine, Al-Nahrain University/ Baghdad.

### Statistical Analysis

Statistical analyses were performed using SPSS software version 25.0 (SPSS, Chicago). Continuous data were subjected to a normality test (Shapiro Wilk test); Data with normal distribution were presented as mean and standard deviation and analyzed with a Student t-test. Data with non-normal distribution were presented as median and range and analyzed with the Mann Whitney U test. Categorical variables were expressed as numbers and percentages and analyzed with the Chi-square test. The receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of PD-1 and ATG5 in the discrimination TB patients and controls. A p-value less than 0.05 was considered to indicate a statistically significant difference.

## Results

### Demographic characteristics of the study population

The mean age of the patients was  $40.94 \pm 16.79$  years, which did not differ significantly from the control group ( $39.0 \pm 16.21$  years). Similarly, the distribution of females and males, the socioeconomic status and the percentage of smokers were similar between the two groups, with no significant differences. On the other hand, 25.53% of patients without comorbidities compared to 58.14% of the control group with a significant difference. In particular, the incidence of diabetes was higher in patients versus the control group (29.79% versus 13.95%)(Table 1).

### Serum level of soluble ATG5 and PD-1

Data regarding soluble ATG5 and PD-1 levels were found to be non-normally distributed. Accordingly, they expressed as median and range and analyzed with Mann Whitney U test. The median value of ATG5 level in patients

was 2.08  $\mu\text{g/ml}$  (range 1.1-4.06  $\mu\text{g/ml}$ ), which was higher than that of the control group (median = 0.93  $\mu\text{g/ml}$ , range = 0.45-5.26  $\mu\text{g/ml}$ ) with a highly significant difference (Figure 2).

Similarly, the median value of PD-1 level in patients was 259.4 ng/ml (range 107.76-924.25 ng/ml) which is higher than that of the control (median = 146.4 ng/ml, range = 50.32-1117.1 ng/ml) and with a highly significant difference (Figure 3).

### Diagnostic value of ATG5 and PD-1

The receiver operating characteristic curve was used to determine the diagnostic value of ATG5 and PD-1 in discriminating between patients and control. For ATG5, the area under the curve was 0.921, 95% CI = 0.984-0.857,  $p < 0.001$ . The sensitivity and specificity of the test at the cut-off value for ATG5 1.32  $\mu\text{g/mL}$  were 89% and 84%, respectively. For PD-1, the area under the curve was 0.797, 95% confidence interval = 0.898-0.695,  $P < 0.001$  The sensitivity and specificity of the test at the cut-off value of PD-1 = 205.4 ng/ml were 80% and 74 % respectively (Figure 4).

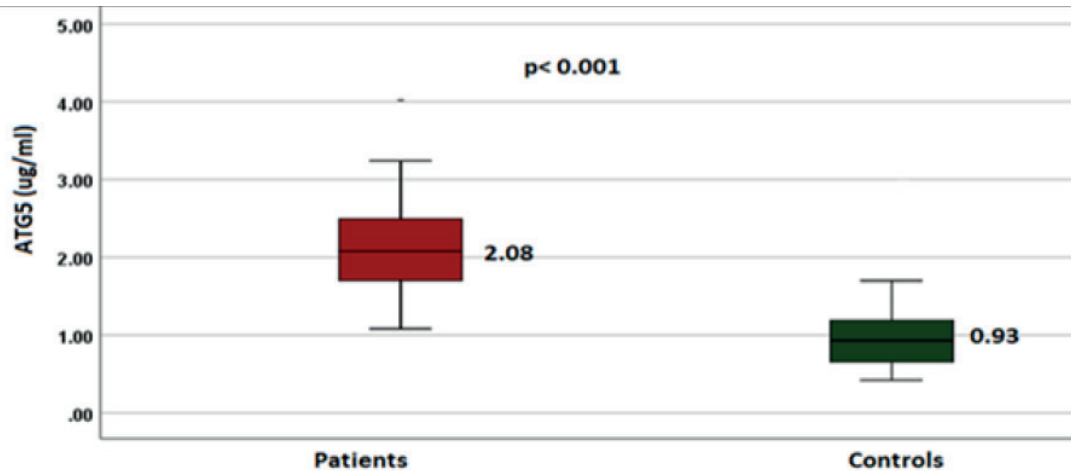
## Discussion

When the host exposes to Mtb, and autophagy mechanism is induced. This results in fusion of lysosomal compartments with mycobacterial phagosome and triggers the generation of neo-antimicrobial peptides, essential for mycobacterial killing<sup>14</sup>. Moreover, autophagy may play an indirect role in Mtb phagocytosis through the regulation of scavenger receptors expression<sup>15</sup>. *In vivo* experiments have documented that autophagy protein ATG5 in macrophages is required to restrain Mtb infection in laboratory animals<sup>16,17</sup>. Moreover, using autophagy inhibitors enhances mycobacterial load in zebrafish embryo model<sup>18</sup>. This reflects the importance of autophagy proteins, particularly ATG5, in host defense against TB. Thus, it is reasonable to elevate serum levels of ATG5 protein in TB patients because this protein may reflect the gene expression of membranous ATG5.

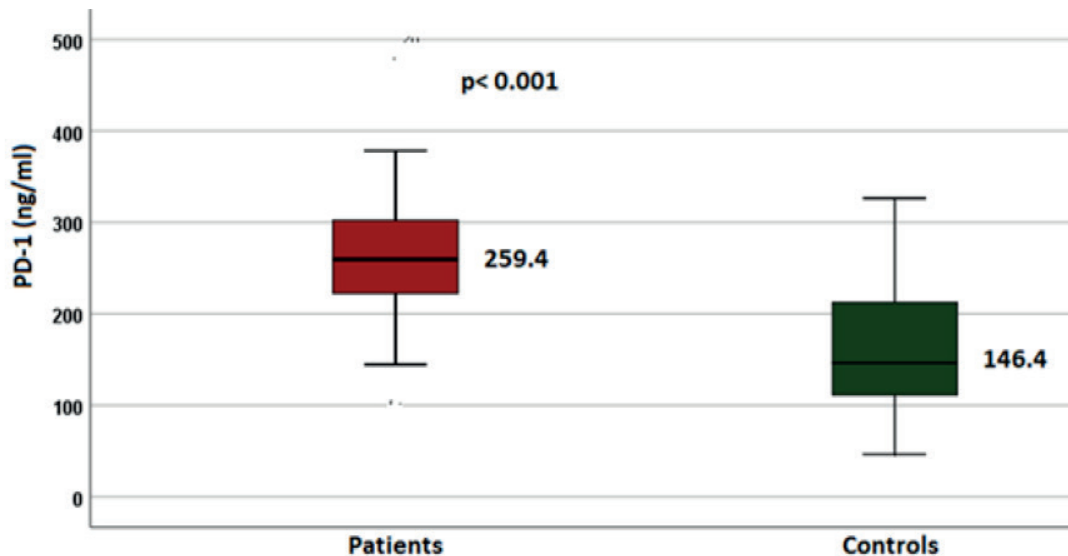
In laboratory animals, PD-1 expression was found to be upregulated in severe TB disease infection<sup>19</sup>. Mtb infection of PD-1<sup>-/-</sup> mice leads to enhanced susceptibility to TB disease characterized by increased bacterial loads, increased inflammatory and necrotic responses in the lungs, and reduced survival<sup>20,21</sup>. These studies in PD-1-deficient mice demonstrate an essential role for PD-1 in limiting excessive IFN- $\gamma$  production by CD4 T cells, which has been asso-

Variables	control group (43)	patients group (47)	P-value
<b>Age (year)</b>			
Mean $\pm$ SD	39.0 $\pm$ 16.21	40.94 $\pm$ 16.79	0.580
Range	12-65	12-66	
<b>Gender</b>			
Male	24(55.81%)	26(55.32%)	0.962
Female	19(44.19%)	21(44.68%)	
<b>Socioeconomic status</b>			
low	9(20.93%)	19(40.43%)	0.057
Middle	27(62.79%)	18(38.3%)	
High	7(16.8%)	10(21.28%)	
<b>comorbidities</b>			
None	25(58.14%)	12(25.53%)	<b>0.011</b>
Diabetes	6(13.95%)	14(29.79%)	
Hypertension	5(11.63%)	11(23.4%)	
kidney stones	5(11.63%)	3(6.38%)	
Other	2(4.65%)	4(8.51%)	
<b>Smoking</b>			
Never	26(60.47%)	26(55.32%)	0.622
Ex/current	17(39.53%)	21(44.69%)	

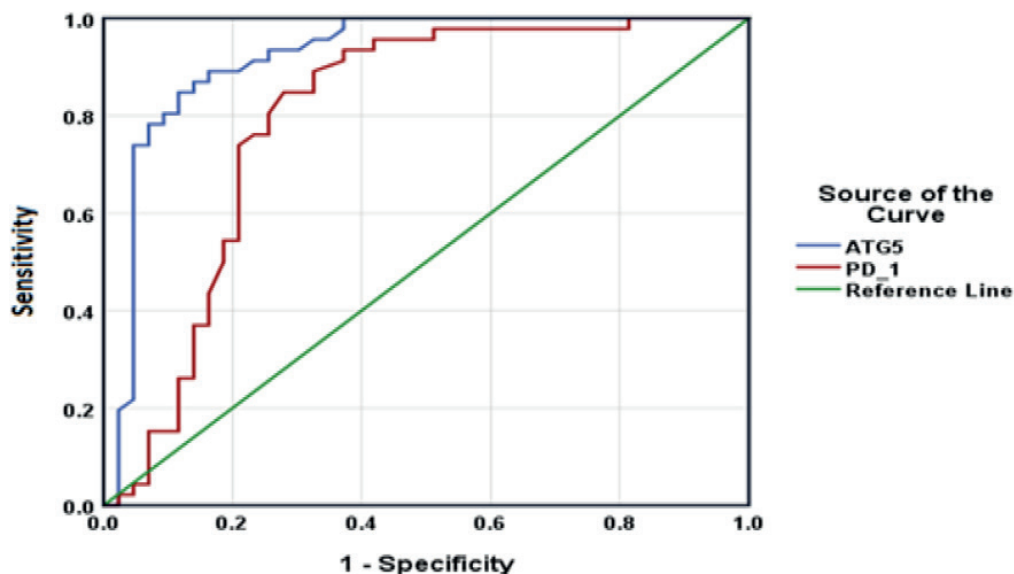
**Table 1.** Demographic characteristics of the study population.



**Figure 2.** The Median serum level of soluble ATG5 in patients and controls.



**Figure 3.** The Median serum level of soluble ATG5 in patients and controls.



**Figure 4.** Receptor operating characteristic curve for ATG5 and PD-1 to distinguish between TB patients and controls.

ciated with exacerbated disease in murine models of TB<sup>22</sup>. Increasing evidence suggests PD-1 expression is upregulated on innate and adaptive immune cells in the setting of Mtb infection and disease in humans<sup>23</sup>. Although all these studies focused on membranous PD-1, soluble PD-1 may also reflect the host's immune status.

In a Chinese study, Li *et al.*<sup>24</sup> showed that the concentration of soluble PD-1 in the serum of patients with cystic echinococcosis was elevated compared with the healthy control subjects or patients that received treatment. The authors explained this elevation in terms of an increase in soluble PD-1 expression inhibits the PD-1/PD-L1 signaling pathway in T cells through negative feedback, which reduces the inhibition of T cell activation and increases the activity of the immune system in managing the infection. This supports the findings of Wang *et al.*, which suggest that sPD-1 blocks the membrane PD-1 binding site on activated T-cells, thereby attenuating the PD-1 signaling pathway and increasing the immune response<sup>25</sup>.

## Conclusions

Serum levels of soluble PD-1 and ATG5 protein are increased in tuberculosis patients compared with controls. The soluble ATG5 protein has an outstanding diagnostic value in discrimination between patients with TB and controls and could be used as additional diagnostic tools in suspicious cases of TB.

## Author Contributions

Suhaib Waad Oklah Musa: sample and data collection; manuscript writing; Reyam F. Saleha: statistical analysis and manuscript editing.

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