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*Original Research Article*

# Immunohistochemistry Detection of PD-1 and PD-L1 Expression in serum and Colonic Tissues of Patients with Ulcerative Colitis

***Article History:***

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***Abstract***: Background: Programmed cell death 1or simply Protein PD-1 (PD-1) and it is ligand programmed cell death ligand 1 (PD-L1) belong to CD28 and B7 superfamily respectively, these receptors prominently expressed on activated CD4 and CD8 T lymphocytes and play a critical role in both innate and adaptive immune responses and contribute to gut immune homeostasis. Aims: To assess serum and tissue PD-1 and PD-L1 expression in UC patients and examine their relationship with UC severity and extension. Methods: The current study group consist from newly diagnosed UC (60 adults' patients) and healthy control group (30 Adults). Both serum level of sPD-1 and sPD-L1 are assessed using ELISA technique. While, Immunohistochemistry was used to study tissue expression score of PD-1 and it is ligand PD-L1 in colonic mucosal tissue biopsies that collected during colonoscopy. Results: Both serum and tissue expression score of PD-1 and itis ligand PD-L1 were significantly elevated on diseased group in compared to healthy control, both PD-1 and PD-L1 had significantly related to disuses extension and severity. Although sPD-L1 was significantly elevated in UC and control group sPD-1 was elevated in non-significant relation to control group. Conclusions: Both tissue expression of PD-1 and PD-L1 obtained during colonoscopy and sPD-L1 may be valuable in improving the diagnosis of disease severity.

**Keywords**: Ulcerative colitis, Immunohistochemistry, PD1, PD-L1, sPD-1, sPDL1.

## INTRODUCTION

Ulcerative colitis, a form of inflammatory bowel disease disorders, is featured by inflammatory ulceration of mainly rectum and less extent the colon. Furthermore, in extensive UC, backwash ileitis terminal ileum can be involve also (1).Sign and symptoms often includes bloody diarrhea, Abdominal pain, systemic manifestations in severe cases that may accomplished extra-intestinal complications like systemic arthritis or sclerosing cholangitis (2) . The exact etiology of UC is not fully understood. Still, it appears that a mixed of genetic with environmental factors, including unhealthy lifestyle, long term stress, smoking habit, processed foods, can impact the development of chronic inflammation and the immunological processes associated with UC (3). IBD affects nearly 7 million people worldwide and has a bimodal age distribution, with a peak age onset of IBD at 30–40 years, and a second peak at 60–70 years (4). In contrast to most autoimmune disorders, which demonstrate a female predominance, this disease exhibits an equal incidence among males and females (5). Programmed cell death protein ( PD-1) is a type I transmembrane glycoprotein primarily expressed may on T cells, B cell , NK cells and other inflammatory cells (6, 7). Programmed cell death protein 1 structured by 288 amino acids protein arranged in N-terminal IgV and Transmembrane domains with cytoplasmic tail (8). As an important, PD-1 player in both immune tolerance and exhaustion, PD-1 expression is tightly controlled. On naïve T-lymphocytes, PD-1 is only expressed in a low basal level, while after antigenic stimulation PD-1 expression on T lymphocytes and B lymphocytes are

 

increased (9). On the other hands, PD-1 pathway have an important role in maintaining peripheral immune tolerance and ameliorating autoimmune diseases by blocking excessive inflammatory reactions and corresponding immune tissue damage.(10, 11)

## MATERIAL AND METHODS

During the period from April 2024 to January 2025, Nighty individuals from gastroenterology Centre in Azadi Teaching Hospital in Kirkuk/Iraq had been enrolled in this prospective study. Individuals were divided into two categories UC patients and control group. A sixty-patient diagnosed with UC according to European Crohn's and Colitis Organization (ECCO) with exclusion of patients less than 18 years, pregnant women, patients received steroids or biological agents in previous 6 months, patients with GIT carcinoma. A healthy control groups were normal individuals that undergo endoscopic examination for reasons other than malignancy nor IBD. Blood was drawn from each group for serum sample collection. Then stored in (-20) $C^0$ until ELISA analysis. ELISA techniques were carried out for serum biomarkers (16). PD-1(MyBioSource-USA/MBS2023507), PD-L1(MyBioSource-USA/ MBS380-0929)(12). Colonic tissue biopsies were taken during colonoscopic investigation from both ulcerated areas in UC group, and normal apparent colonic mucosal tissue for normal control group. The PD-1 and PD-L1 protein tissue expression was examined by using immunohistochemical study. Colonic biopsies specimen was fixed in neutral buffer formalin (10%) for at least 72 hrs. Then the biopsies specimens submitted to grade of ethanol concentration (70%, 80%, 90% and 100%), followed by at least twice xylene immersion and then embedded in paraffin wax to yield paraffin embedded block. Multiple Sections of 5 µm-thickness were obtained then, used for immunohistochemistry analysis to PD-1 and PD-L1 detection. Specific Primary monoclonal antibodies against PD-1 were used (Elabscience, Catalog Number: E-AB-65810). A specific Polyclonal rabbit Anti-human PD-L1 Protein against PD-L1 were used (Elabscience , Catalog Number: E-AB-65810). The immune reaction (antibody-antigen interaction) is then imagined utilizing chromogenic detection, by using light microscope. Scoring the immunoreactivity of studied biomarkers obtained by calculated the percentage of immunoreactive positive cells per total number of other cells (immune inflammatory and epithelial cells). The score of positively stained cells was evaluated as 0, 1, 2 and 3 score by examination of wide areas within each colonic tissue section: 0, No positive cells-stained cells; 1, <20% stained cells; 2 (20%-30%) stained cells and score 3<30% stained cells, in comparation to total numbers of (13).

### 3.Statistical analysis

Data was analyzed by "Statistical Package for Social Sciences" (SPSS) version 26. While Graph Pad Prism was used for graph drawing. the data expressed as means and Standard deviation. chi-square test used to study the significant relationship between categorical variables. Effects were considered significant at $p<0.05$. Receiver-operating characteristics curve that used as a diagnostic performance curve for studied biomarkers.

## RESULTS

Immunohistochemical study PD-1 and PD-L1 in colonic tissue biopsies demonstrated in Figures (3-1,3-2). According to our PD-1 results the majority of control cases had a negative score (57%), while in score 3, PD-1 was seen only in 3 cases (10%). This expression was mainly in cytoplasmic monocytes and some other inflammatory cells. While in UC group, the IHC expression of PD-1 was negative in twenty percent of UC group while the maximal expression was seen in score 2 (18 of 60) (30%) show moderate PD-1 expression in colonic lamina propria monocytic cells. The differences between these groups were significant at p-value (<0.000). According to UC severity most mild group (69%) had a negative score, while in severe group nearly half of the cases were in score 3 with a significant difference between these groups. PD-1 levels were highest in patients with Pancolitis, followed by left-side colitis then proctitis with a significant elevation between these groups. as shown in table (3-3). Tissue Expression PD-L1 in Ulcerative Colitis was highly increased in UC patients in compared to healthy control group with p-value (<0.000). Severe UC patients had a highest PD-L1 expression in compared to other severity groups as demonstrated in table (3-5). While (52%) of proctitis patients had score 1. While, Serum levels of PD-1 and it is ligand sPD-1 was measured was measured by ELISA technique. The mean serum level of soluble PD-1 in UC patients' group was 41.6 pg/ml, while in healthy control group was 39.35±5.06 with ($P > 0.05$). According to disease severity, sPD-1 was (39.01±5.9, 41.50±7.31, and 43.12±7.38) pg/ml in mild Mayo score , moderate and severe patients' groups respectively. Although the sPD-1 in severe group was higher than moderate and mild group but the differences between these groups was non-significant. The mean serum level of sPD-L1 was (145.7±33.8 pg/ml), while in healthy control was (100±11.4 pg/ml) with a significant P-value (<0.000). The mean serum sPD-L1 concentrations increased progressively with disease severity, measuring 103.6 ± 17.9 pg/mL in the mild disease severity group, 145.9 ± 16.2 pg/mL in the moderate group, and reaching 169.1 ± 31.4 pg/mL in the severe UC group. Moreover, differences between these groups were significant with P-value (<0.000). According to Montreal classification sPD-L1 was significantly elevated in all UC disease extension groups with mean (112.8±19.1,146.7±18,185.6±23.6) to proctitis, left-side colitis and extensive colitis respectively. Soluble PD-L1 had a higher sensitivity and specificity in compared

 

to sPD-1 in assessment and monitoring UC severity index with (87%) specificity and (90%) sensitivity respectively

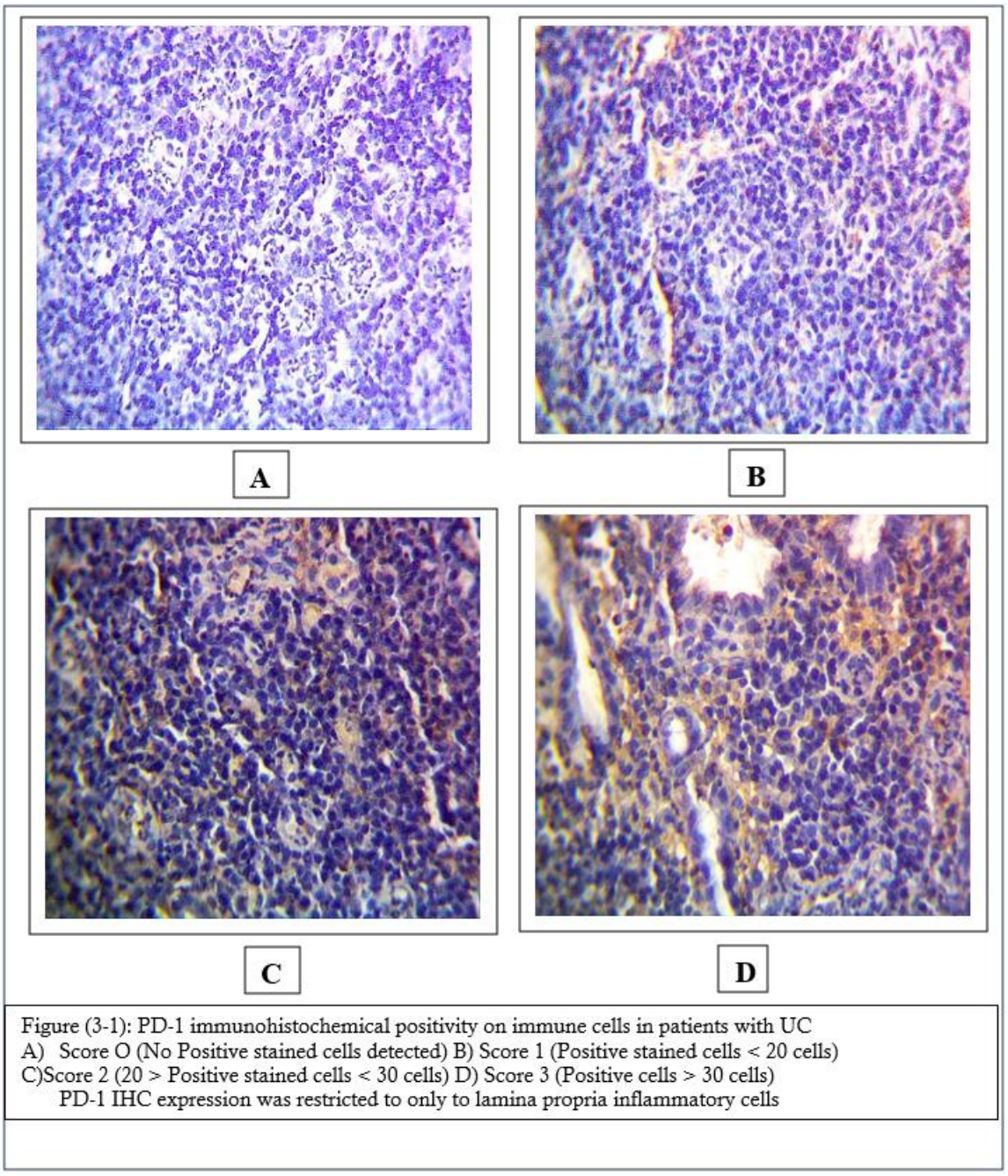


Figure (3-1): PD-1 immunohistochemical positivity on immune cells in patients with UC
A) Score O (No Positive stained cells detected) B) Score 1 (Positive stained cells < 20 cells)
C)Score 2 (20 > Positive stained cells < 30 cells) D) Score 3 (Positive cells > 30 cells)
PD-1 IHC expression was restricted to only to lamina propria inflammatory cells

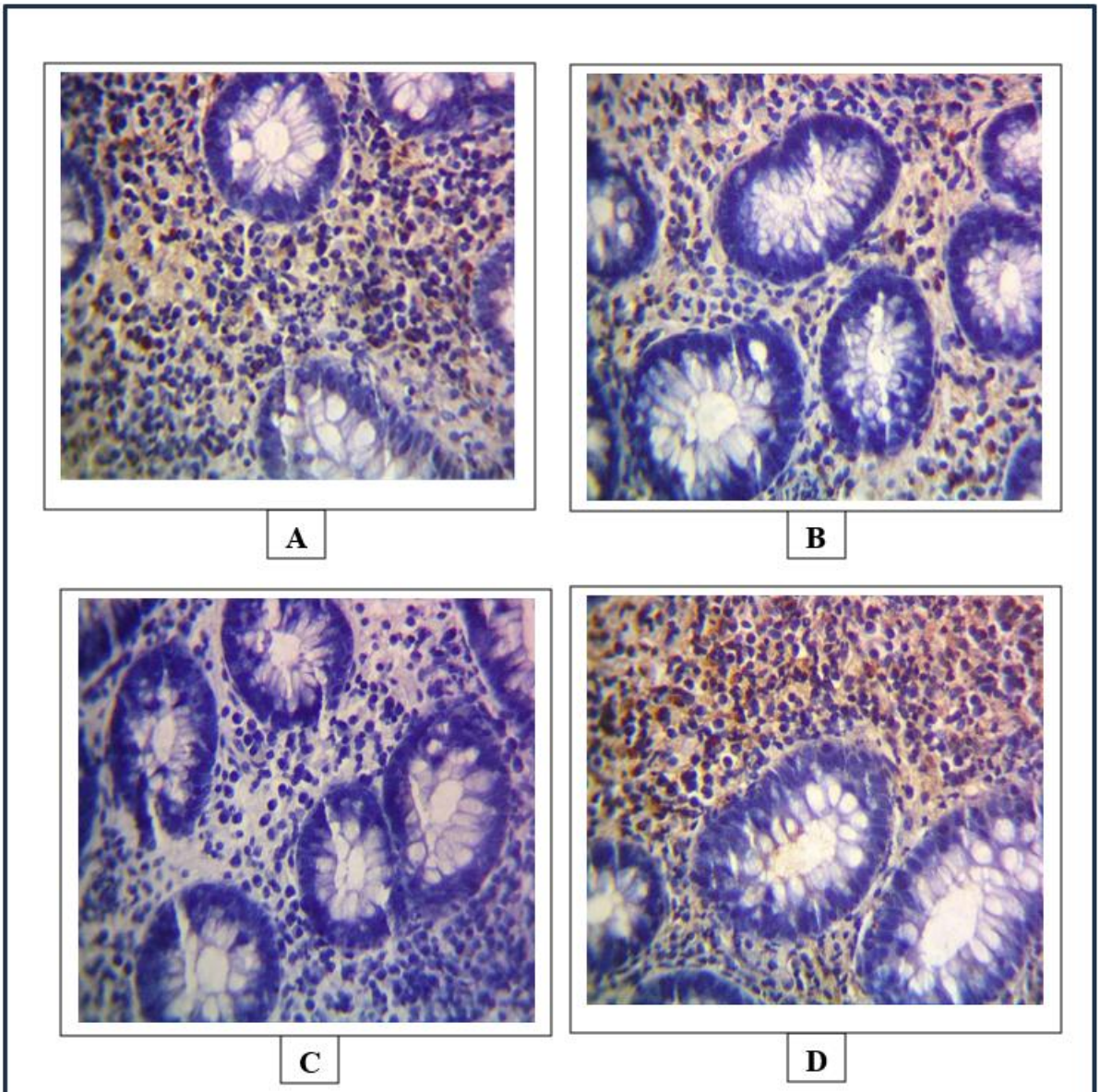


**Figure (3-2): PD-1L immunohistochemical positivity on immune and epithelial cells in patients with UC**
A) Score O (No Positive stained cells detected) B) Score 1 (Positive stained cells < 20 cells)
C)Score 2 (20 > Positive stained cells < 30 cells) D) Score 3 (Positive stained cells > 30 cells)
**PD-L1 IHC expression was found in both the inflammatory and epithelial cells**

**Table (3-1): PD-1 immunohistochemical expression in UC patients and control group**

| Studied groups PD*1 | | Score-O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Control | Count | 17 | 5 | 5 | 3 | 30 | 0.005 |
| | % | 56.6 % | 16.7 % | 16.7 % | 10 % | 100% | |
| UC | Count | 12 | 14 | 18 | 16 | 60 | |
| | % | 20 % | 23.3 % | 30 % | 26.7 % | 100% | |

**Table (3-2): PD-1 immunohistochemical expression in UC patients according to severity**

| Studied groups PD*1 | | Score-O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Mild | Count | 9 | 3 | 0 | 1 | 13 | 0.000 |
| | % | 69.2 % | 12.5 % | 0.0 % | 7.7 % | 100% | |
| Moderate | Count | 3 | 8 | 9 | 4 | 24 | |
| | % | 12.5 % | 33.3 % | 37.5 % | 16.7 % | 100% | |
| Severe | Count | 0 | 3 | 9 | 11 | 23 | |
| | % | 0.0 % | 13.0 % | 39.2 % | 47.8 % | 100% | |

**Table (3-3): PD-1 immunohistochemical expression in UC patients according to extension**

| Studied groups PD*1 | | Score-O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Proctitis | Count | 12 | 7 | 0 | 0 | 19 | 0.000 |
| | % | 63.2 % | 36.8 % | 0.0 % | 0.0 % | 100% | |
| Left-Side colitis | Count | 0 | 7 | 18 | 1 | 26 | |
| | % | 0.0 % | 26.9 % | 69.3 % | 3.8 % | 100% | |
| Pancolitis | Count | 0 | 0 | 0 | 15 | 15 | |
| | % | 0.0% | 0.0% | 0.0% | 100 % | 100% | |

**Table (3-4): PD-L1 immunohistochemical expression in UC patients and control group**

| Studied groups PD*L1 | | Score -O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Control | Count | 16 | 6 | 5 | 3 | 30 | 0.000 |
| | % | 53.3% | 20 % | 16.7 % | 10 % | 100% | |
| UC | Count | 9 | 16 | 15 | 20 | 60 | |
| | % | 15% | 26.7% | 25% | 33.3% | 100% | |

**Table (3-5): PD-L1 immunohistochemical expression in UC patients according to severity**

| Studied groups PD*L1 | | Score-O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Mild | Count | 9 | 3 | 0 | 1 | 13 | 0.000 |
| | % | 30.8% | 46.2% | 15.4% | 7.7% | 100% | |
| Moderate | Count | 0 | 10 | 10 | 4 | 24 | |
| | % | 0% | 41.7% | 41.7% | 16.7% | 100% | |
| Severe | Count | 0 | 3 | 5 | 15 | 23 | |
| | % | 0% | 13% | 21.7% | 65.2% | 100% | |

**Table (3-6): PD-L1 immunohistochemical expression in UC patients according to extension**

| Studied groups PD*L1 | | Score-O | Score-1 | Score-2 | Score-3 | Total | P-value |
|---|---|---|---|---|---|---|---|
| Proctitis | Count | 9 | 10 | 0 | 0 | 19 | 0.000 |
| | % | 47.4% | 52.6% | 0% | 0% | 100% | |
| Left-Side colitis | Count | 0 | 6 | 15 | 5 | 26 | |
| | % | 0% | 23.1% | 57.7% | 19.2% | 100% | |
| Pancolitis | Count | 0 | 0 | 0 | 15 | 15 | |
| | % | 0% | 0% | 0% | 100 | 100% | |

**Table (3.7) ROC analysis performance of sPD-1 and sPD-1L to differentiating UC from control group**

| Biomarkers | Area Under the curve | Systemic error | 95% confidence intervals | P value | Cut-off values pg/ml | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|---|---|
| **Serum sPD-1** | 0.606 | 0.6 | 0.487-0.724 | 0.104 | 39.79 pg/ml | 67% | 53% |
| **Serum sPD-L1** | 0.905 | 0.033 | 0.840-0.970 | <0.000 | 112.04 pg/ml | 87% | 90% |

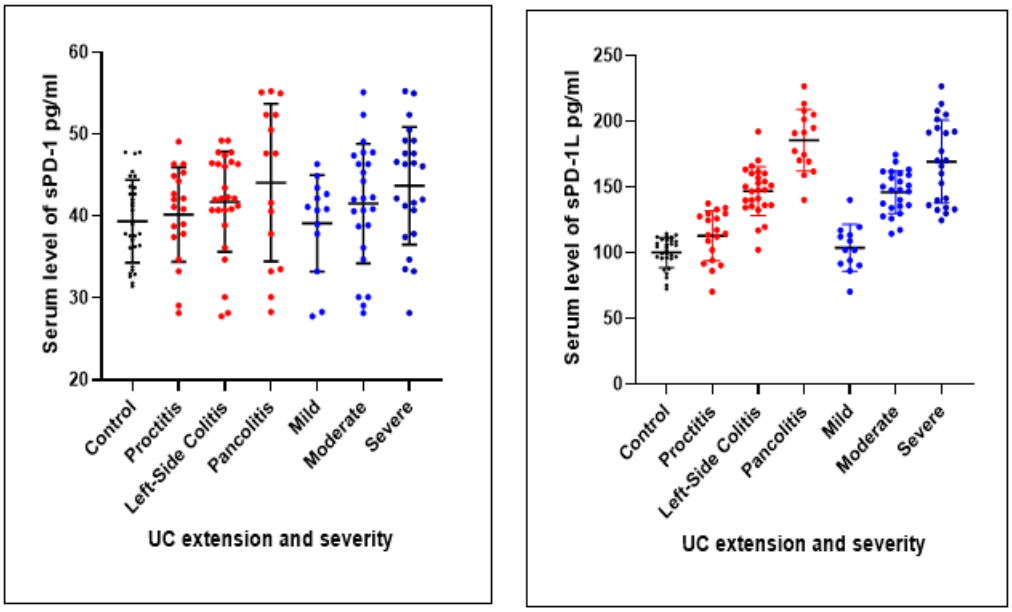


**Figure (3-4): Serum level of sPD-1 and sPD-L1 in UC patients and their distribution according to disease extension and severity group**

## DISCUSSION

Ulcerative colitis, is a chronic relapsing form of inflammatory bowel disease, featured by intestinal mucosal superficial ulceration, and even intestinal fibrosis in advanced stage (14, 15) Although UC incidence and prevalence continues to rise specially in developed countries, the exact underlying immunological mechanisms remain unclear, and treatment protocol outcomes are suboptimal.(16) The main immunopathological features of IBD is the sustained chronic inflammatory milieu in the intestine mucosa, which considers as first physical barrier against bacterial and food allergen penetration. In this study, PD-1 and it is ligand PD-L1 expression was evaluated using immunohistochemistry assay rather than real-time PCR, because post-translational modifications and protein localization may affect accurate detection, which cannot be captured by gene expression analysis alone(17). PD-1/PD-L1 expression was not detected in all UC tissue samples, regardless of disease severity (mild, moderate, or severe), suggesting the presence of three possible explanations Collectively, these findings suggest that immune heterogeneity, temporal expression, treatment effects. First, during acute flare up of UC most of the inflammatory cells in mucosal layer are neutrophiles not monocytes. Second, chronic inflammatory status seen in mucosal layer usually affects lymphocytes sensitivity to pro-inflammatory cytokines and other signaling molecules (18). Third, As PD-L1 Proteinous ligand may undergo glycosylation post-translational modifications that changes the binding affinity of PD-L1 by special antibodies that usually used in IHC (19) .

This expression was mainly in cytoplasmic monocytes and some other inflammatory cells; these results agreed with previous study that described that PD-1 immunostaining was exclusively seen cytoplasmic and membranous mucosal monocytes in normal intestinal tissue(18) .PD-1 was mainly expressed in colonic lamina propria monocytic cells.

PD-1/PD-L1 immunological pathway exerts an inhibitory signal to several aspect of immune system to maintenance the tolerance to self-antigens by controlling the induction of immune response (20) . A study by Suzuki *et al.* and his colleagues identified that PD-1 agonists which inhibit exhausted T lymphocytes in murine models with drugs induce colitis. Although this treatment strategy may induce intestinal adverse reaction, like structural and mucosal tissue changes similar to UC (21). Previous suggest that interrupted PD-1 pathway can contribute to loss of tolerance to intestinal microflora that can lead to severe autoimmune colitis in mice model (22). Other study by immunohistochemistry assay shows that increase expression of PD-1 on different cell types and it is level significantly increased in UC patients in compared to control groups and can be used as predicators to effectiveness of vedolizumab in UC patients (23) .In the current study PD-1 levels were highest in patients with Pancolitis, followed by left-side colitis then proctitis with a significant elevation between these groups. these results disagreed with previous report by Mi *et al* that found a non-significant elevation of relative expression of PD-1 gene according to UC extension (24).PD-1 expressing T regulatory cells in the inflamed gut mucosa of IBD patients specifically down-regulated anti-inflammatory cytokine production and resulted in aggravated colitis symptoms, partially explaining role of PD-1 in regulating severity of IBD (25). Immunoassay was used to evaluate serum soluble PD-1/ in blood to further study the role of sPD-1 in UC and the results was not significant in UC in compared to control groups. These results agreed with previous results that found a non-significant elevation of sPD-1 in UC group in compared to control group(18).The possible explanation to these results is sPD-1 may act as decay receptors by binding to membranous PD-1L and PD-2L(26) Although, the serum PD-L1 levels were significantly increased in patients with ulcerative colitis compared with healthy controls , In contrast, the body may not markedly increase sPD-1 production, particularly in severe cases, as elevated sPD-1 could neutralize PD-L1 and thereby exacerbate and worsen the inflammatory state observed in inflammatory bowel disease., These results agreed with previous study by Beswick *et al* observed that PD-L1 mRNA and protein levels are continuously expressed within normal colonic epithelia layer, with predominate expression on $CD90^{+}$ mesenchymal cells thus suggesting it is primary role in gut immune hemostasis and tolerance (27) .and it is tightly controlled by MyD88-dependent TLRs pathway (28). Immunohistochemical and gene expression study by Yalan *et al* in ulcerative colitis patients and DSS mouse colitis models reveals significant elevation of PD-L1 in patients in compared to control group, and linked to expression of many proinflammatory cytokines (24).in active IBD, prolonged chronic mucosal inflammation usually associated with local hypoxia and lactate accumulation. (29) Hypoxia through the action of hypoxia-inducible factor 1-alpha (HIF-1α), increase and stabilize PD-L1 gene expression (30).Microenvironment acidity increase PD-L1 expression by the activation of The acidic is another of a key microenvironment STAT3 signaling pathway (31).Membranous PD-L1 post-translational modifications by the action of Matrix metalloproteinases (ex.MMP-9) enzymes consider as major sources of soluble PD-1L.(32) .so increase metalloproteases enzymes activity could upregulte the levels of the 25 kDa sPD-L1 (33). exogenous MMP-9 and MMP-13 can removed PD-L1 from fibroblast surface, this process exacerbates tissue inflammation with imitation to them immunosuppressive capacity(34, 35).Soluble PD-L1 had a higher sensitivity and specificity in compared to sPD-1 so can be used as biomarker to disease assessments and monitoring progression, these results agreed with previous results by studies in patients with ulcerative colitis (UC) have demonstrated that serum sPD-L1 levels are significantly higher compared to healthy controls, with concentrations rising in parallel with increasing disease severity. Another important finding of our study was sPD-L1 had a higher sensitivity and specificity than sPD-1 so can be used for as an important biomarker for UC severity and monitoring disease progression, these results agreed by *Shi et al.* that found a significant relation between UC severity and sPD-L1 nor sPD-1 in monitoring UC progression(18) .

## CONCLUSION

The Assessment combined evaluation of PD-1 protein and it is ligand PD-L1 expression in colonic tissue obtained during colonoscopy and circulating sPD-L1 and sPD-1 levels may enhance the accuracy of complementary information about disease severity and extension

**Ethics Statement**

The study protocol received approval following a thorough review by the medical ethics committee at the College of Health and Medical Techniques MEC 23 in 23/3/2025. Additionally, the research obtained the requisite authorization from the research ethics division of the Ministry of Health (Iraq), specifically at the Kirkuk health directory No.13 at 7/01/2025, Azadi teaching hospital

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