ISSN: 0067-2904

Up Up-regulation of Soluble Programmed Death Ligand 1 (PD L1) Level as a Potential COVID-19 Severity-Associated Bio-parameter

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Received: 6/1/2025 Accepted: 4/5/2025 Published: 30/4/2026

Abstract

Programmed cell death protein 1 (PD-1) may be in a position to interact hydrophobically with the spike glycoprotein or its epitopes of SARS-CoV-2, which, in addition to the neuroinvasive capability it possesses, triggers hyperactivation of the Program Death Ligand1. A case-control study (67 COVID-19 patients and 23 healthy controls) was conducted to detect the role of soluble Program Death-Ligand1 (sPDL-1). The sPD-L1 serum concentrations were measured with an ELISA kit, while the SARS-CoV-2 infection was determined earlier via a real-time PCR evaluation kit. The findings demonstrated that median sPD-L1 levels in patients were substantially more significant compared to healthy controls (1349 *vs.* 737.8 pg/ml; $p < 0.0001$). Most patients were considered high producers of sPD-L1 (> median=990.8 pg/ml) compared to controls (65.7%; p=0.001). Receiver operating characteristic curve analysis indicated that an sPDL-1 level of more than 887.6 pg/ml distinguishes patients from control (area under the curve = 0.831; $p < 0.0001$) and is considered an excellent indicator that distinguishes–patients with different severity at risk (area under the curve = 0.91; $p < 0.0001$). The sPD-L1 levels showed a significant relationship with the severity of infection by COVID-19 ($p < 0.0001$), which were significantly increased with C-reactive protein (CRP) ($p = 0.021$) and Ferritin levels, making both of them a promising prognostic marker for infection progress. The sPD-L1 had a crucial role in the progression of infection along with SARS-CoV-2 wild type and their variants, and future studies are required to inhibit sPD-L1 based-interferons expression.

Keywords: sPDL-1 receptor, SARS-CoV-2, Threshold cycle, Biomarker, ELISA.

ارتفاع مستوى الربيطة الذائبة المحفزة لموت الخلية (sPDL-1) في مرضى كوفيد-19 المرتبط بالحالات الشديدة

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الخلاصة

يسمح مكان وجود بروتين موت الخلية المبرمج 1 (PD-1) بالتفاعل مائيًا مع البروتين السكري الشوكي لفيروس سارس-2-، والذي يؤدي إلى فرط تنشيط ربيطة بروتين موت البرنامج-1 (sPD-Ligand1) بالإضافة إلى قدرته على التوغل العصبي. أجريت دراسة مقارنةحالة المرضى مع الاصحاء (67 مريضًا بفيروس كوفيد-19 و23 من الاشخاص الاصحاء) للكشف عن دور ربيطة بروتين Program

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Death–Ligand1الذائبة (sPD–L1) في شدة الاصابة وتقدمها بين متغيرات فيروس سارس–2–. تم قياس تراكيز sPD–L1 في المصل باستخدام اختبار ELISA ، في حين تم تحديد اصابات سارس–2 بشكل اولي عن طريق تفاعل الوقت الحقيقي لسلة البلمرة . أظهرت النتائج أن متوسط مستويات sPD–L1 لدى المرضى كانت أكبر بكثير مقارنة بالضوابط الصحية 1349) مقابل 737.8 بيكوغرام/مل؛ .(p < 0.0001 اعتُبر معظم المرضى منتجين لمستويات عالية من sPD–L1 (> المتوسط = 990.8 بيكوغرام/مل) مقارنة بالضوابط65.7%) ؛ .(p=0.001 أشار تحليل المنحنى المميز للتشغيل المتلقي إلى أن مستوى sPDL–1 الذي يزيد عن 887.6 بيكوغرام/مل يميز المرضى عن المرضى المتحكم بهم) المنطقة تحت المنحنى = 0.831؛ (p <0.0001 ويعتبر مؤشرًا ممتازًا يميز المرضى ذوي الشدة المختلفة في الخطر) المنطقة تحت المنحنى = 0. 91؛ .(p <0.0001 أظهرت مستويات sPD–L1 علاقة كبيرة مع شدة الإصابة بفيروس كوفيد–19(p <0.0001) ، والتي زادت بشكل كبير مع مستويات البروتين الحاد في البروتين الدهني = p) (0.021ومستويات الفيريتين، مما يجعل كلاهما علامات تنبؤية جيدة لتقدم الإصابة. كان لـ sPD–L1 دور حاسم في تطور العدوى إلى جانب النوع البري من فيروس كورونا المستجد – كوفيد–19 ومتغيراته، وهناك حاجة إلى دراسات مستقبلية لتثبيط تعبير sPD–L1 بالاعتماد على الانتفيرون.

1. Introduction

The programmed cell death protein 1 (PD-1, also known as PDCD1 and CD279) was first identified in apoptotic mouse T-cell tumors. It is expressed on activated T cells, B cells, monocytes, and immune cells. It regulates human immune response through binding with PD-L1 and PD-L2, which belong to the B7 family of T cell co-inhibitory molecules [1]. PD-L1, a conserved and widely expressed variant of PD-1, is crucial in tumor cell immune evasion. Its ligands, including 1191PD-1, dampen T cell responses primarily through cytokine production, affecting IFN-γ, TNF-α, and IL-2 production [2]. PD-1 interacts with PD-L1 or PD-L2 to inhibit T cell signaling, mediate tolerance, and maintain immune homeostasis, suppressing autoimmunity and preventing autoimmune diseases. Cancer cells express PD-L1 or PD-L2 on T cell surfaces, forming an immunosuppressive tumor microenvironment [3].

Viral infections represent a significant challenge to the immune system, with numerous mechanisms evolved to evade or suppress the host defense to promote viral replication and spread. Type I IFNs comprise one of the first lines of cytokine defense against viruses, functioning in an autocrine and paracrine manner to induce a broad antiviral state in neighboring cells [4]. Type I `IFNs are also important for the maturation and activation of natural killer, dendritic, and adaptive immune cells. Some viruses have recently been shown to modulate PD-L1 levels in infected cells through IFN-dependent and -independent mechanisms. Upon activating the PD-L1/PD-1 pathway, cells expressing PD-L1 bind to the T cells expressing PD-1, inhibiting the immune responses of activated T cells and, thus, blocking the “killing” of the cells expressing PD-L1. The PD-L1/PD-1 system is well-known to be involved in immune evasion by tumors and is now also known to be involved in immune evasion by various viruses in both the acute and chronic phases [5-6].

Severe COVID-19 patients exhibit dysregulated PD-1/PD-L1 axis, affecting dendritic and monocyte cells and reducing T cell response and stimulatory capacity. Disease severity is also associated with PD-L1 expression in eosinophils and basophils [7]. Platelets from COVID-19 patients display increased PD-L1 expression, affecting CD4+ T cell IFN-γ production. Soluble PD-L1 (sPD-L1) has been detected in membrane PD-L1-positive cells related to matrix metalloproteinase enzymes [8]. sPD-L1, a protein found in tumor cell lines, has a binding domain to the PD-1 receptor and is found in high levels in patients with various cancers. Its expression negatively impacts cancer prognosis, with high levels increasing mortality risk and low levels promoting better prognosis [9].

The potential viral load significantly increased with the sPD-L1 level. Specifically, the level increased with the HBeAg-negative status, an HBV DNA level of >105 IU/mL, and a higher HBcrAg level [10]. As identified, there was an increased level of sPD-L1 during HAV and astroviruses, where its increase led to an undesirable silencing of immune responses during acute infection. The concept was similar to that underpinning HBV infection, where specific suppressive factors, PD-1, and its ligand system, promote immune tolerance, leading to chronic infection with no immunity and insufficient resistance from sPD-L1 [11].
The current study aimed to detect the role of progressive infection among variants of SARS-CoV-2 and identifies sPD-L1 as an inflammatory biomarker in progressive infection among variants of SARS-CoV-2.

2. Material and Methods

2.1 Study population

In this study, a total of 90 individuals were included. These participants comprised 67 patients who had previously been diagnosed in cooperation with the Iraqi Ministry of Health/National Central Health Laboratory. Patients were distributed, 45 falling into the mild-moderate category and 22 falling into the severe-critical category (including 4 case deaths), all of whom had contracted SARS-CoV-2. Both the patients and healthy controls were further divided into age groups (22-35, 36-50, and 51-<65 years) and sex (male and female) to ensure a balanced representation. Patients were from Iraq in Baghdad Teaching Hospital between August 2022 and December 2022, characterized positive in reverse transcriptase real-time PCR (rRT-PCR) assay, and healthy controls had the COVID-19 antibodies (IgM and IgG), and rRT-PCR results were negative.

Remarkably, every participant took part in providing the consent forms, guaranteeing that they were all aware and completely included in the study. With a medium oxygen saturation (SpO_2) of 90% in room air, COVID-19 patients with mild-to-moderate symptoms and no indicators of hypoxia or pneumonia exhibit moderate clinical signs of pneumonia, such as fever, cough, dyspnea, and respiratory distress, but not severe pneumonia. Pneumonia with one of the following conditions, along with clinically significant indications of severe COVID-19 patients: respiratory rate >30 breaths/minute, severe breathing trouble, and SpO_2 <90% in room air. Acute respiratory distress syndrome (ARDS), sepsis or septic shock, acute pulmonary embolism, and acute coronary syndrome are among the numerous serious problems that COVID-19 patients have developed.

2.2 Sample collection

Data collection for this study involved various steps. Firstly, demographic information and medical history were collected. In the second step, the nasal swabs and blood samples were taken from the study groups to diagnose SARS-CoV-2 with variants and to measure sPD-L1 along with other biomarkers, ensuring detailed information about each participant's sPD-L1 levels for scientific excellence. A total of 90 nasal swabs and blood samples (5 mL) were taken from patients (67) and healthy controls (HC) (23). EDTA-blood tube (2 ml) was used for complete blood count and D.dimer. Serum was collected by evenly dividing blood (3 ml) into gel tubes for ferritin and LDH and freezing the remaining serum and swabs at -70 °C until the sPD-L1 testing and reverse transcriptase real-time PCR (rRT-PCR) assay.

2.3 Biomarker Detection

The blood samples were separated by three tubes: sodium citrate, gel, and ethylene-diamine-tetra-acetic acid (EDTA). After centrifuging the first section of the blood samples (sodium citrate tubes) for 20 minutes at 3500 rpm, an automated protein analyzer was used to perform plasma D.dimer analysis. Both plain and EDTA tubes were filled with five milliliters of venous blood (3 and 2 mL, respectively). After allowing the plain tube to clot, the serum

was collected by centrifuging it for 15 minutes at 4 °C. By using an electro-chemiluminescence immunoassay system (Roche Cobas Integra 400 plus, Switzerland), the serum was examined for CRP. White blood cells (WBC: total, neutrophils, and lymphocytes) were counted using EDTA blood and an automated hematology analyzer (ABX Micros ES 60, Horiba, USA). The absolute neutrophil count was divided by the absolute lymphocyte count to determine the neutrophil to lymphocyte ratio (NLR).

The gel tube containing the second part of the blood samples was spun for 10 minutes at 6000 rpm to extract serum for measuring ferritin and lactate dehydrogenase (LDH). The serum concentrations of LDH were assessed using the Roche Cobas Integra 400 plus electro-chemiluminescence immunoassay. Ferritin was also evaluated using a miniVIDAS analyzer (ELFA) from BioMerieux, following the manufacturer's instructions.

2.4 Immunoassay of sPDL-1

The sPDL-1 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit from SunLong Biotech, China (Catalogue Number: SL2269Hu). Serum levels were measured following the manufacturer's directive. The assay has a sensitivity of 26 pg/mL and an assay range of 35-2000 pg/ml.

2.5 Statistical analysis

The collected data were subjected to thorough statistical analysis to determine the role of the PD-1 polymorphism gene in the severity of infection and the role of soluble ligand (sPD-L1) in COVID-19 infection or variant of SARS. Descriptive statistics were utilized to summarize the participants' demographic characteristics and clinical variables. Pearson's correlation coefficient or Spearman's rank correlation coefficient was employed based on the distribution of variables to examine the correlation between sPD-L1 levels and severity of SARS-COV-2 infections. Additionally, appropriate multivariate statistical models, such as regression analysis, were used to assess the association while controlling for confounding factors. An examination of statistics Two-sided Numbers and percentages representing categorical variables were analyzed using Fisher's exact test. The Mann-Whitney U test was used to check the normality of continuous data. The Mann-Whitney *U* test compared non-parametric (not regularly distributed) variables reported as the median and interquartile range (IQR). A receiver operating characteristic (ROC) curve analysis was conducted to assess the area under the curve (AUC), 95% confidence interval (CI), cut-of-value, Youden index, sensitivity, and specificity. The odds ratio (OR) and 95% CI were calculated using logistic regression analysis. Patients and HC were separated into high- and low-production groups in this study based on the PDL-1 median in HC (and median, respectively), with the high-production group acting as the reference category. The relationship between the variables was investigated using Spearman's rank-order correlation. A probability (p) value ≤ 0.05 was considered statistically significant. The statistical software used for the analysis was IBM SPSS Statistics 27.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 10.0 (San Diego, California, USA). G*Power version 3.1.9.2 software was used to calculate the sample size power using the compromise analysis approach.

3. Results

The patients in the current investigation were divided into three age groups, as shown in Table 1: 22-35, 36-50, and 51-<65 years old. According to the statistical analysis, which showed substantial (p=0.001) differences between the age groups of COVID-19 patients, age is a significant risk factor. Regarding the sex observed, males had more infections than females, and there was no significant difference in sex in patients (p=0.805). Significant differences were observed in CRP, ferritin levels, D. dimer, LDH, diabetes mellitus, and

hypertension (p<0.001) between studied groups. SARS-COV-2 variants had significant differences between studied groups but were not significant in severe-critical individuals (p = 0.483). Since the Ct value is low, the viral load is high in the opposite relationship, so we notice the high levels of sPDL-1, but this has no significant difference among Ct values in severe-critical patients.

Table 1: Baseline characteristics of COVID-19 patients stratified to infection severity.

Characteristic	*COVID-19 Patients*		*p-value*
	Mild-Moderate (no.45)	severe-critical (no.22)	
Age: year	43 (39-48)	53 (43.8 - 60.5)	**p=0.002**
Age group	22-35	5 (11.1)	1 (4.6)	**p=0.001**
	36-50	31 (68.9)	7 (31.8)	
	51-< 65	9 (20)	14 (63.6)	
	p-value	**p <0.001**	**p=0.003**	
Sex	Male	28 (62.2)	13 (59.1)	p=0.805
	Female	17 (37.8)	9 (40.9)	
	p-value	p=0.101	p=0.349	
Diabetes mellitus	Yes	13 (28.9)	18 (81.8)	**p<0.001**
	No	32 (71.1)	4 (18.2)	
	p-value	**p=0.005**	**p=0.003**	
Hypertension	Yes	11 (24.4)	21 (95.5)	**p<0.001**
	No	34 (75.6)	1 (4.5)	
	p-value	**p=0.001**	**p<0.001**	
Ct value	14-20	18 (41.9)	9 (45.0)	p=0.481
	21-30	22 (51.2)	11 (55.0)	
	31-36	3 (7.0)	0 (0.0)	
	p-value	**p=0.001**	p=0.655	
CRP mg/L	9.5 (4.8-16.2)	29.6 (26.8-44.2)	**p<0.001**
Ferritin mg/ml	326 (260-412)	1012 (780-1410)	**p<0.001**
D.dimer mg/L	328 (230-491)	1029 (810-2106)	**p<0.001**
LDH IU/L	182 (160-223)	280 (263-395)	**p<0.001**

Age values can be expressed as a number and percentage (categorical variables) or as a median with interquartile range (continuous variables); p: the probability of Mann-Whitney U test (to compare continuous variables), two-tailed Fisher exact test, or Pearson Chi-square test (to compare categorical variables).

The results of sPD-L1 showed the median level was highly significant in patients compared to healthy controls (HC) (1349 pg/mL *vs* 737.8 pg/mL; p<0.0001), as shown in Figure 1. Most patients were classified as high producers of sPDL-1, with a significant difference from HC. The cases of death were 4, and most of its levels were higher than the median [1400 (2400-3300) pg/ml]

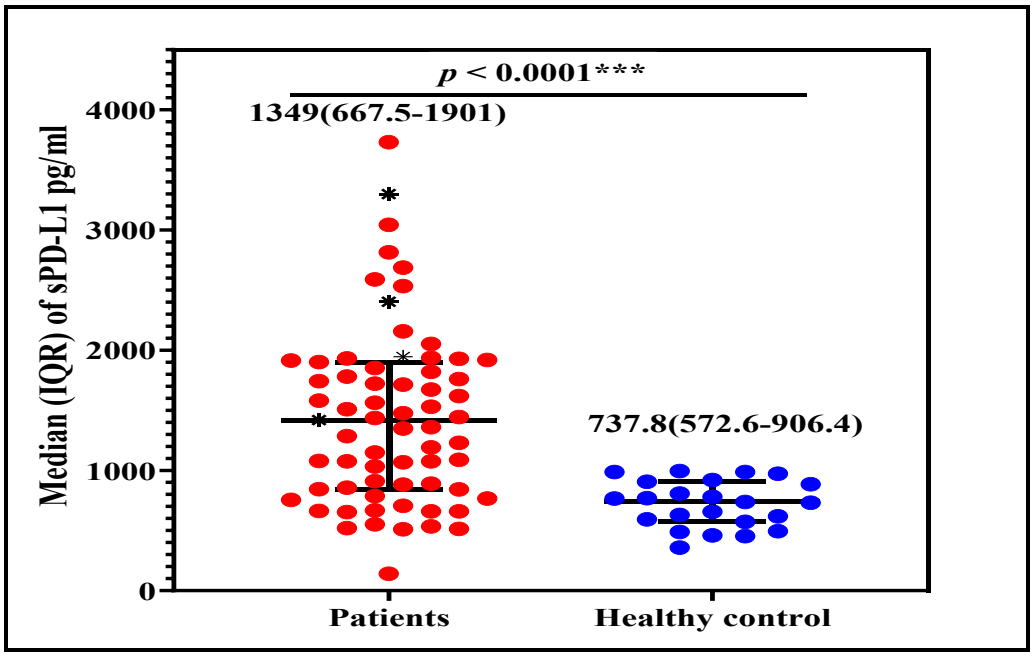

Figure 1: Levels of sPD-L1 in COVID-19 patients and healthy controls are shown as a scatter dot. Horizontal lines represent the median, while vertical lines represent the interquartile range (IQR). *p*: the probability of the *Mann-Whitney* test between continuous variables; the symbol*indicates four death cases among patients.

Participants with high production had an age, severity, and variation of SARS-CoV-2-adjusted (OR = 3.02, 95% CI = 2.84–5.73; p = 0.001), according to logistic regression analysis (Figure 2). There was a significant difference in the level of sPDL-1 between participants, < median 990.8 pg/ml (low producers) in 87% of healthy control than 3% in patients. In contrast, 86% of patients with severe-critical are high producers (> median 990.8 pg/ml).

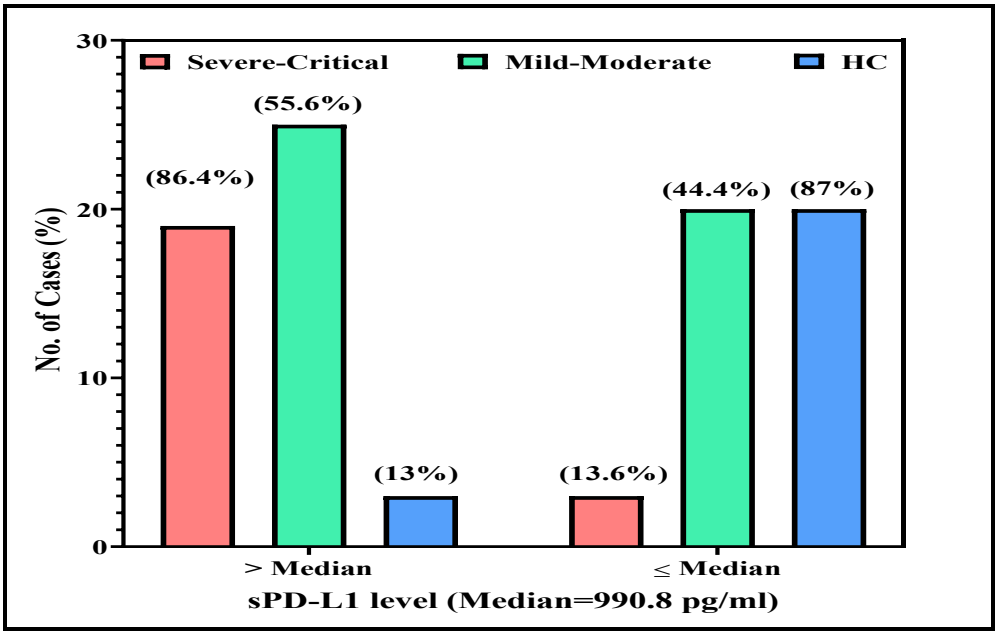

Figure 2: Frequencies of sPD-L1 in patients and healthy controls (HC) that are in high- and low-production categories (> and ≤median, respectively). After controlling for age, severity, and SARS-CoV-2 variations, multinomial logistic regression analysis revealed that individuals with sPD-L1 levels greater than the median were more likely to have severe infections (patient *vs* HC; OR = 3.02, 95 CI = 2.84-5.73, p =0.001).

The results obtained from rRT-PCR showed a significant difference between sPD-L1 levels among the three Ct value (viral load) groups (p =0.043) (Figure 3). This finding suggests that high viral load (low Ct value) induces sPD-L1 to rise and contributes to inhibiting immune reactions.

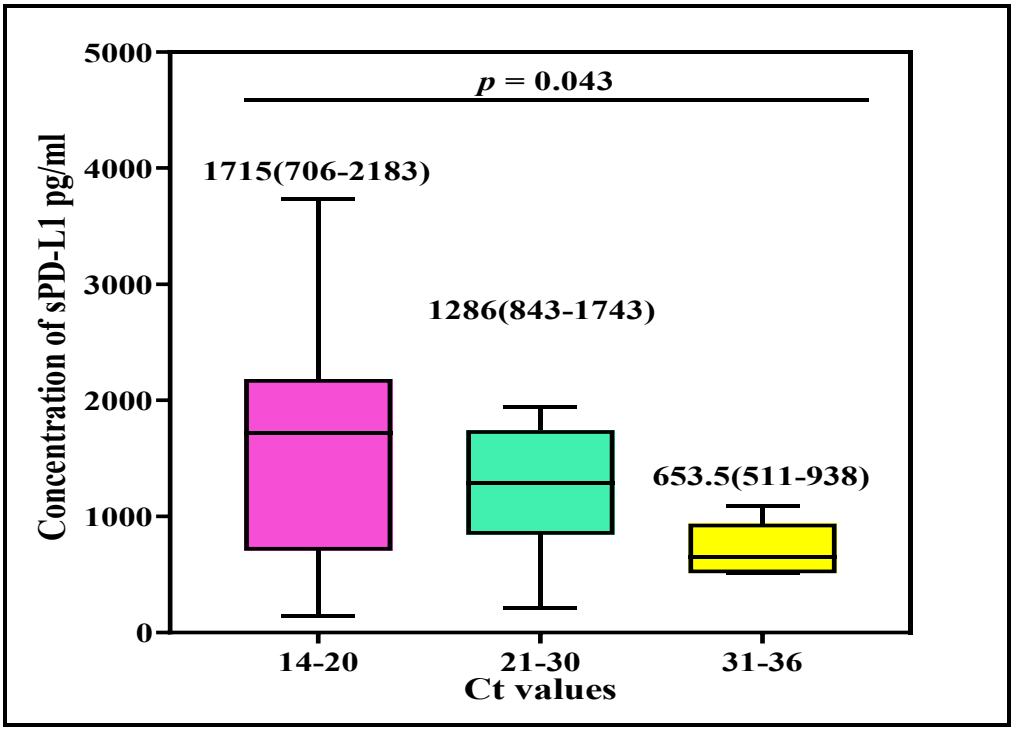

Figure 3: Box plot of sPD-L1 concentration among patients stratified by Ct values (Threshold of real-time RT-PCR assay); *p*: probability of *Kruskal-Wallis* test (to compare continuous variables).

High sPDL-1 levels were found to be very good predictors of COVID-19 patients using ROC analysis. The ideal cut-off value for distinguishing between patients and HC was 887.6 pg/mL (AUC = 0.831; 95% CI = 0.749–0.913; p < 0.0001), with a sensitivity of 77.6% and a specificity of 69.6%, respectively (Figure 4 A); as well as it is an excellent indicator for distinguishing patients progressive to risk from recovered (Figure 4 B).

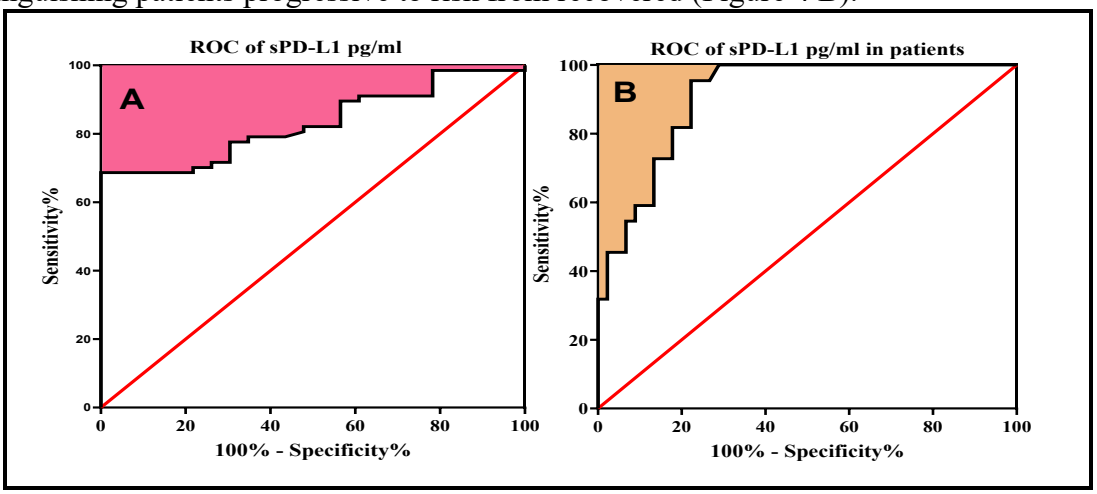

Figure 4: A ROC curve analysis of sPD-L1 level in COVID-19 patients *versus* healthy control. A sPD-L1 level cut-off value of more than 887.6 pg/ml can distinguish patients with viral infection at risk from healthy individuals (Area under the curve = 0.831; 95%

Confidence interval = 0.749 − 0.913; $p < 0.0001$; Sensitivity = 77.6%; Specificity = 69.6%). **B:** ROC curve analysis of sPD-L1 level among COVID-19 patients with different severity. A sPD-L1 level cut-off value of more than 1317 pg/ml can distinguish patients progressive to risk from recovered (Area under the curve = 0.91; 95% Confidence interval = 0.844 − 0.976; $p < 0.0001$; Sensitivity = 81.8%; Specificity = 80.1%).

According to the patient and health control, the median sPDL-1 showed no significant difference in age group, sex, hypertension pressure (HIN), and diabetes mellitus (DM), while DM was only significant in patient individuals (p= 0.023), as shown in Table 2.

Table 2: sPD-L1 median results were stratified based on the characteristics of healthy controls and COVID-19 patients.

Characters		*sPDL-1 median (IQR); pg/ml*	
		patient (no.67)	Control (no.23)
Age group	22-35	860.7 (442.6-1552)	906.4 (808.1-979.7)
	36-50	1324.5 (628.9-1921)	693.9 (514.7-859.1)
	51-<65	1359.6 (857.2-1914.8)	----
	p-value	*p*=0.246	*p*=0.075
Sex	Male	1285.8 (686.8-1907.8)	693.9 (493.6-933.7)
	Female	1428.2 (625.3-1848)	779.9 (595.5-895.9)
	p-value	*p*=0.847	*p*=0.488
HTN	Positive	1456.3 (771.2-1938.5)	629.1 (417.6-802.8)
	Negative	1075.0 (660.5-1781.2)	751.9 (572.1-905.3)
	p-value	*p*=0.151	*p*=0.106
DM	Positive	1581.1 (1075.0-1935.8)	439.5 (392.3-716.6)
	Negative	1071.5 (523.5-1766.3)	526.9 (458.9-687.4)
	p-value	***p*=0.023**	*p*=0.781

IQR: Interquartile range; DM: Diabetes mellitus; HTN: hypertension pressure; *p*: Mann-Whitney *U* test and *Kruskal-Wallis* test probability.

Results in Figure 5 explain the correlation of sPDL-1 with ferritin, LDH, CRP, and D-dimer, with values only for patients. The increase in ferritin, CRP, and D-dimer levels is associated with an increase in sPDL-1 levels (rs= 0.235, rs= 0.347, rs= 0.215, respectively). LDH with sPDL-1 levels has a weak negative inverse relationship (r_s= 0.138).

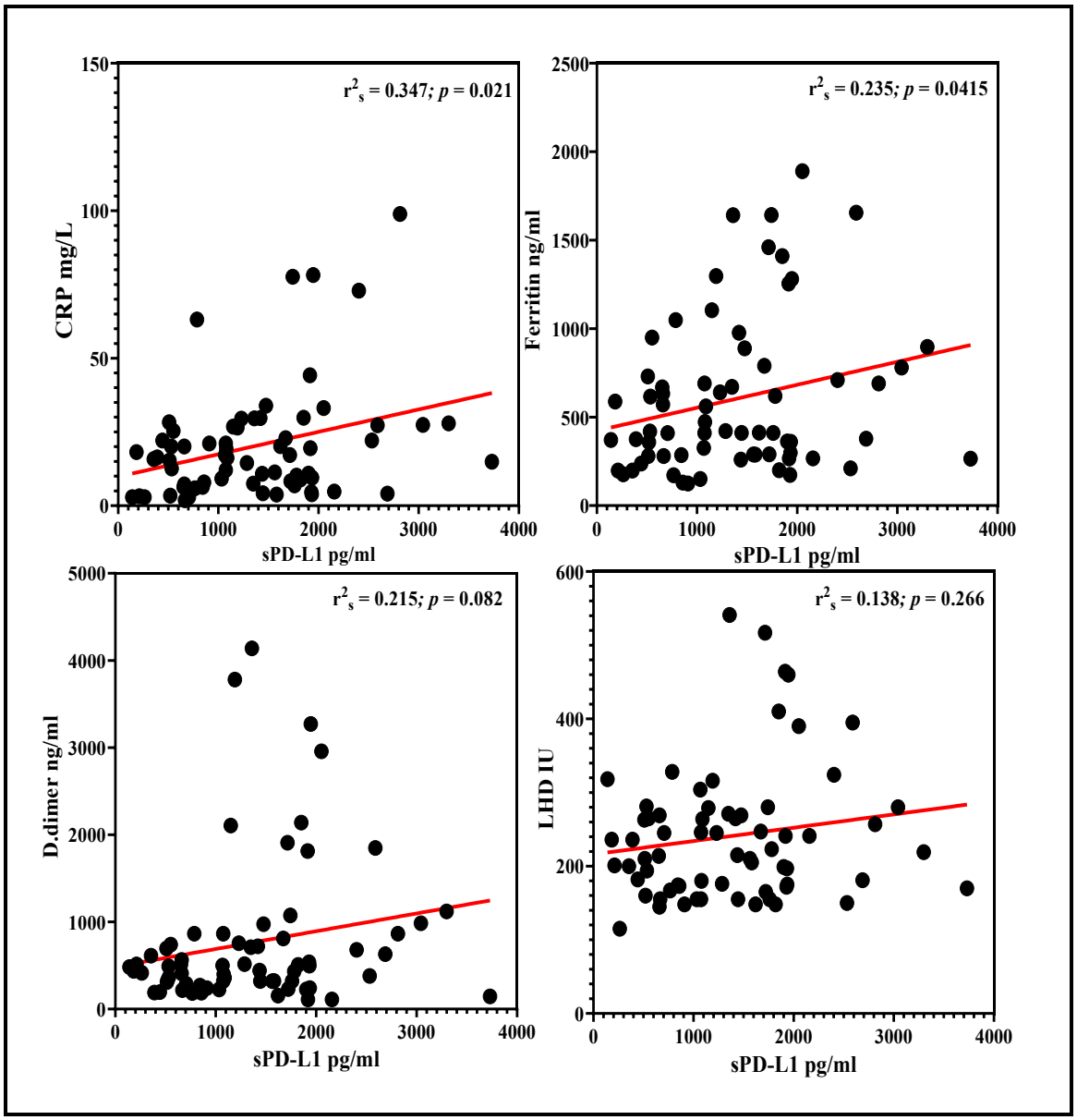

Figure 5: Scatter plot Spearman rank-order correlation coefficient (rs) for analysis between sPD-L1 level with CRP, ferritin, D.dimer, and LDH levels in COVID-19 patients.

4. Discussion

According to our knowledge, there are several local studies about SARS-CoV-2 infection, but no research has evaluated the role of sPDL-1 levels in progressive disease. The patients who participated in the current investigation were divided into age groups 22-35, 36-50, and 51-65. According to the statistical analysis, which showed significant (p = 0.001) differences between the age groups of COVID-19 patients, the older the age, the more the risk in severe-critical while in mild, it is less dangerous in ages below a year. This result aligns with another study demonstrating a causal connection between aging and COVID-19 infection [12]. Male and advanced ages were identified as risk factors for severe illness and mortality in COVID-19 patients in another survey conducted by Terpos *et al.,* [13]. Co-morbid conditions and the greater likelihood of a weakened immune system might be the causes of this since older adults are more vulnerable to a number of diseases that have the poorest prognoses and the most severe development. The development of the illness may also be impacted by comorbid conditions such as diabetes, hypertension, and heart disease, which are unevenly distributed among men and women of various ages [14]. COVID-19 patients often have hypertension,

Diabetes Mellitus, and other comorbidities, which can significantly predict disease severity. Studies show that SARS-CoV-2 can increase the risk of microvascular and macrovascular problems in diabetes mellitus patients, and older age and comorbidities correlate with increased severity and death [15].

Epidemiological findings show a higher incidence of COVID-19 infection in males than females, with higher morbidity and fatality rates. This is due to higher expression of angiotensin-converting enzyme-2, sex hormones, and X-linked genes. The virus is more likely to infect men who have COVID-19, and men who smoke and drink more are more likely to have weakened immune systems and be more vulnerable to other viral illnesses. Our study showed the incidence of COVID-19 infection in males was 13(59.1%), while in females, it was 18(81.8%). Previous local studies of COVID patients have shown that the infection rate is higher in males 65.2% than in females 34.8% [16]; other studies showed Infants sex males 67 (54.9%), females 55 (45.1%) [17], male 166 (70.94) female 68 (29.06) [18], male 172 (69%) female 75(30.4%) [19].

There was a significant correlation (p <0.001) between the median CRP and the selected infection severity. Age, gender, weight, blood pressure, cholesterol, smoking status, and heredity all have an impact on baseline CRP readings. Phosphocholine, which is abundant on the surface of injured cells, is the molecule that the CRP prefers to bind to. These bindings activate the immune system's classical complement pathway, which alters phagocytic activity to remove pathogens and damaged cells from the body. CRP is a useful biomarker for monitoring the severity of the disease since its concentration drops when inflammation or tissue damage heals [20].

Ferritin is a crucial inflammatory marker in COVID-19 infection, with increased levels linked to severe conditions, increased intensive care unit exposure, and higher mortality. It mediates immune dysregulation, contributing to the cytokine storm in COVID-19 patients. In COVID-19 patients, iron oxides interact with serum coagulation cascade proteins to cause regular cell death (RCD). Iron overload can result from the virus's destruction of hemoglobin, which releases iron from porphyrins and releases it into the bloodstream. Coagulation problems sometimes accompany severe instances. For the SARS-CoV helicases to function, ATP must be hydrolyzed, which requires iron [21].

According to the current study, LDH and D-dimer levels elevated significantly with illness severity, peaking in critical and severe COVID-19 patients. Dysregulated thrombin production, fibrinolysis, coagulation activation, and poor natural anticoagulants are all associated with elevated D-dimer values [22]. Patients in critical condition may experience increased thrombotic events, coagulopathy, and antiphospholipid antibodies. Elevated LDH levels may result from lung tissue degradation, as previous research shows high levels in COVID-19 patients in the ICU [23].

Systemic sPD-L1 levels can be detected in the serum. Most, but not all, studies observed extremely low levels of serum sPD-L1 in healthy subjects. Results showed that sPD-L1 levels were revealed higher levels in patients with severe and dead cases than in healthy controls, indicating a decrease in immunity and encouraging virus replication, thus explaining the patient's high producer of sPD-L1 accompanied by a rising high viral load (low Ct value) as reported in this study. Another study demonstrated that the potential viral load significantly increased with sPD-L1 level; our finding is consistent with two studies by other researchers who documented an upregulation of sPD-L1 levels with omicron SARS-COV-2. Beserra *et al*., register elevated sPD-L1 levels in individuals with acute pancreatitis, rheumatoid arthritis, chronic hepatitis C, and HIV, and are linked to disease severity and progression [24]. While Hsiang *et al.,* observed higher PD-L1 expression in Omicron-infected cells than in SARS-CoV-2-infected cells. Blocking PD-L1 at an early stage of virally-infected AAV-

hACE2 mice significantly recovered lymphocyte counts. It lowered inflammatory cytokine levels, indicating that targeting the SARS-CoV-2-mediated NF-κB and IRF1-PD-L1 axis may represent an alternative strategy to reduce COVID-19 severity [25].

This study found significant differences in the level of soluble mediator sPDL-1 between participants, with 13% of cases being high producers due to other infections. In mild cases, more than 50% are high producers, and 86% of patients with severe-critical symptoms are high producers. Patients with HIV, rheumatoid arthritis, acute pancreatitis, and chronic hepatitis C have been found to have elevated sPD-L1 levels, which are linked to the severity and advancement of these conditions [26]. This study raises the possibility that sPD-L1 and the severity of COVID-19 are connected. Identifying specific soluble mediator patterns may serve as potential biomarkers for disease severity, prognosis, and response to vaccination, aiding in the timely implementation of appropriate interventions and monitoring treatment outcomes.
Results of this study pointed out that although the level of sPDL-1 increases with older age and females in patients, it is statistically non-significant. Still, it showed a significant increase in chronic disease among the group of people with diabetes and high blood pressure. On the other hand, this study suggests that the increased sPD-L1 level in the elderly may be induced by aging rather than age-driven diseases. However, we found that age-related increases in sPD-L1 were associated with sex and chronic disease group. Compared with healthy young females and males, SARS-CoV-2 in males has a high risk of developing disease as they age with more increase of sPD-L1 levels, and the elevated severe is present within the elderly male and female group, whether it is with or without chronic diseases. In general, the number and the percentage of elderly individuals with sPD-L1 levels between healthy elderly males and females were close to the younger level.

This is similar to other results observed; PD-L1/TC or PDL-1/TILS expression was not associated with age, gender, clinical stage, histological grade, or smoking and alcohol habits [27]. Moreover, the association of expression of PD-L1 /TILS and PDL-1/TC with ages older than 65 years, and we explain this by weak host immunity in the elderly, which may be affected by chronic diseases, with the presence of chronic infections, which may activate immunosuppressive signals, most notably PD-L1[28]; in another study, the expression of PDL-1/TC and the expression of PDL-1/TILS were not associated with gender, and this is consistent with the study of Admaski [29]. In contrast, some studies, such as the Hanna study, found a correlation between PDL-1 expression and females [30]. Satgunaseelan also found an association of PD L1 expression with females; these studies explained their results to higher estrogen levels after menopause [31]. In contrast, Li found an association of PD-L1 expression in males and smokers [32].

The current study showed that sPDL-1 levels increased with increased CRP levels and ferritin, making them useful prognostic indicators for infection development. Nonetheless, D. dimer and LHD were shown to be weakly significantly correlated with sPDL-1 levels. An elevated level of this inflammatory biomarker implicitly indicates the body's stress level, which in turn indicates the severity of the disease. This is in agreement with retrospective studies that found hospitalized COVID-19 patients with high ferritin levels had a higher risk of death. They confirmed the positive correlation between sPD-L1 levels and ferritin or CRP in COVID-19 patients, reinforcing the significance of these markers in the relation of soluble PD-L1. D-dimers and lactate dehydrogenase may contribute partially to systemic impairment [33-35].

5. Conclusions

The sPD-L1 had a crucial role in the progression of infection along with SARS-CoV-2 among variants, and future studies are required to inhibit sPD-L1 expression because chronic viral infections should consider immunological therapies that target PD-L1 (or IFN-like therapies that inhibit PD-L1 upregulation), but it has limitations. It only evaluated sPD-L1 in COVID patients' serum, not on cell surface or plaque tissues, and did not assess changes in sPD-L1 levels post-treatment or its association with prognosis. A larger sample size is needed for further research.

Acknowledgements

Many thanks and appreciation to all the colleagues who support this study in the practical part.

Ethical responsibilities of authors

Before the study was conducted, ethical approval was obtained from the Ethics Committee of Public Health and the Environment and the Department of Biology at the College of Science, University of Baghdad (CSEC/0923/0059).

Disclosure and conflict of interest

The authors declare that they have no conflicts of interest.

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