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Original Article

# Prognostic importance of PD-L1 expression in bladder cancer patients


## ABSTRACT

**Background:** PD-L1/PD-1 molecules are known as important mediators in immune-escape mechanisms of tumors. PD-L1 is highly expressed in various malignancies, including bladder cancer. However, the prognostic value of PD-L1 in bladder cancer patients remains controversial.

**Aim:** To investigate the prognostic significance of PD-L1 expression in tumor tissues of bladder cancer patients.

**Subjects and Methods:** RNA was isolated from FFPE tumor tissues of 48 bladder cancer patients using the monophasic phenol and guanidine isothiocyanate method. Total RNA was converted to cDNA and gene expression levels were analyzed by qRT-PCR. The differential expression levels of the PD-L1 gene between tumor grade and cancer stage groups were analyzed by independent student's t-test and one-way ANOVA.

**Results:** Statistically significantly increased PD-L1 expression was observed in the high-grade tumor group ($p < 0.05$). No significant difference in PD-L1 expression was found among pTa, pT1, and pT2 groups. In addition, the difference in overall survival was not significantly different between groups.

**Conclusion:** The results showed that high PD-L1 expression in bladder cancer was associated with tumor aggressiveness and grade. Despite the inability of the qRT-PCR to show the PD-L1 expression at different locations of tumor tissue, evaluation of PD-L1 mRNA expression by qRT-PCR, which is a highly sensitive and specific assay, appears to be a robust approach. Furthermore, these findings may contribute to a rationale for recommending anti-PD-L1 immunotherapy as an alternative to standard therapy for bladder cancer patients who are most likely to benefit from it.


**KEY WORDS:** Bladder cancer, cancer immunotherapy, gene expression, PD-L1, prognosis


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## INTRODUCTION

Bladder cancer, one of the most common cancers worldwide, is a frequently recurring type of cancer associated with a high risk of morbidity and mortality.[1,2] Bladder cancer has been shown to be a heterogeneous tumor group with the presence of many subgroups with diverse histopathological, molecular, and clinicopathological features. Treatment of bladder cancer includes chemotherapy, radiotherapy, surgical applications, and innovative immunotherapeutic methods. Anti-PD-L1/PD-1 immunotherapies called immune checkpoint inhibitors (ICIs) are recommended for patients who are not suitable for platinum-based chemotherapy.

Immune checkpoint molecules PD-L1 and PD-1 are expressed in many cancer types and have important roles in the immune-suppression and immune-escape mechanisms of cancer cells.[3,4] In recent years, immune checkpoints have been identified as potential therapeutic targets for many types of cancer, and new treatment strategies have been developed with ICIs, which aim to block the connection between PD-1 and its ligand PD-L1. Various studies have shown in the literature that PD-L1 is generally expressed at high levels in several malignancies, including bladder cancer, and bladder cancer patients with high PD-L1 expression have a poor prognosis. However, a few studies have failed to show a clear relationship between PD-L1 and PD-1 expression and prognosis. Discussions continue about the relationship between the expression level of PD-L1/PD-1 and the aggressiveness of the cancer types and prognosis of the patients.

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**Cite this article as:** Durur S, Uzun H, Bedir R, Nalkiran I, Nalkiran HS. Prognostic importance of PD-L1 expression in bladder cancer patients. J Can Res Ther 0;0:0.

**Submitted:** 22-Jun-2022
**Revised:** 17-Jul-2022
**Accepted:** 18-Jul-2022
**Published:** 17-Mar-2023

Access this article online
Website: www.cancerjournal.net
DOI: 10.4103/jcrt.jcrt_1297_22
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In the present study, we aimed to investigate PD-L1 and PD-1 gene expression levels in bladder cancer patients' tissues grouped by pathological stage and tumor grade. In addition, the role of PD-L1 as a prognostic factor for bladder cancer was evaluated.

## MATERIAL AND METHODS

### Patients and tissue specimens

FFPE tumor tissue samples of 62 patients diagnosed with bladder cancer and five tonsils used as a positive control of PD-L1 expression were obtained from Recep Tayyip Erdogan University Hospital (Rize, Turkey). Ethics approval (approval no: 2019/167) was obtained from Recep Tayyip Erdogan University Clinical Research Ethics Committee. The tissue sample was excluded from the study if the sample was degraded, or the sample size was smaller than 5 $mm^3$. Thus, 14 of the FFPE tumor tissue samples of the 62 patients provided did not meet the criteria for inclusion in the study group, the analysis was continued with 48 patient samples.

### Isolation of RNA by monophasic phenol and guanidine isothiocyanate method

RNA isolation from FFPE tissues was performed using the monophasic phenol and guanidine isothiocyanate method. The RNA isolation protocol briefly covers the following steps: 1 mL of xylene (100%) was added to the tissue sections in the tubes to remove paraffin, vortexed, and incubated at 50°C for 3 minutes in a heat block. The samples were centrifuged at maximum speed then xylene was removed. 1 mL of ethanol (100%) was added to the samples, vortexed, and centrifuged at room temperature for 2 min at maximum speed. 1 mL of ethanol was added to the samples, vortexed, and centrifuged at room temperature for 2 min at maximum speed. Ethanol was removed and the lysis solution was added to the samples [20 mM Tris, 1 mM $CaCl^2$, 10% SDS, 10 µl Proteinase K (20 mg/mL)] and mixed by vortexing. The samples were incubated at 55°C for 3 hours in a heat block. Then monophasic phenol and guanidine isothiocyanate was added and incubated for 5 minutes at room temperature. Chloroform was added to the samples, and the tubes were vortexed and incubated for 10 minutes at room temperature. Then the tube was centrifuged at 12000 xg for 15 min at + 4°C. The clear aqueous phase was transferred to the tubes containing 0.5 mL isopropanol. The samples were centrifuged at 12000 x g for 10 min at +4°C. The samples were washed with 75% ethanol and centrifuged at 7500 x g for 5 min at +4°C. The pellet was reconstituted in 30 µl of RNase-free water and stored in a -80°C freezer.

### Control of RNA quality by agarose gel electrophoresis

Quality control of RNAs isolated from control and tumor tissue samples was performed by 1.2% agarose gel electrophoresis runs in 0.5 X TBE buffer at 70 V for 45 min and visualized using a gel documentation system (DNR Bio-Imaging Systems, Israel). In addition, RNA concentration was measured using µDrop plates on Thermo Multiskan Go Microplate Reader (Thermo Fisher Scientific, USA).

### cDNA synthesis

The synthesis of cDNA was performed as directed by the manufacturer (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Briefly, a 2X Reverse transcription master mix was prepared. After adding RNA to reverse transcription reaction, it was performed in a thermal cycler (Applied BiosystemsVeriti™ 96 Well Thermal Cycler, USA)

### Quantitative real-time-PCR (qRT-PCR)

After the conversion of cDNA, expression levels of the PD-L1 and PD-1 genes in bladder tumor and control tissues were examined using Light Cycler 480 Probes Master commercial kit (hydrolysis probe/TaqMan probe) using Light Cycler 480 II Roche (LightCycler® 480 Instrument II, Switzerland. PD-L1 (Hs01125301_m1) and PD-1 (Hs01550088) probes (Thermo Fisher Scientific, USA) were used in the study. Expression levels of the GAPDH gene were used as an internal reference to normalize the mRNA expression of genes of interest. The forward primer of GAPDH reference gene: 5′-GAAGGTGAAGGTCGGAGTC-3′ reverse primer: 5′-GAAGATGGTGATGGGATTTC-3′ and GAPDH YAK: YAK-CAAGCTTCCCGTTCTCAGCCT-BBQ sequence information was obtained from the database of the manufacturer (TIB MOLBIOL, Berlin, Germany). The results were evaluated with the Basic Relative Quantification for All Samples analysis method in the LightCycler 480 II SW 1.5.1 program. The total amount of RNA was normalized to GAPDH as a reference control gene, and the expression was calculated using the $2^{-\Delta\Delta}$Ct formula.

### Statistical analyses

Statistical analysis of the data was performed using the IBM SPSS Statistics Version 25 program. The tumor tissue samples were divided into two groups; low-grade and high-grade tumors. Independent students t-test was used to compare the expression levels for both target genes (PD-L1/PD-1). Then, the expression levels of the PD-L1/PD-1 genes in tumor tissue samples were examined in terms of tumor stages with a One-Way ANOVA test, and Post-Hoc tests were performed. The association of PD-L1 expression, tumor grade, and cancer stage with overall survival was calculated by Kaplan-Meier analysis. $P < 0.05$ values were considered statistically significant.

## RESULTS

PD-L1 and PD-1 expressions in FFPE tissues of 48 patients diagnosed with bladder cancer were analyzed by qRT-PCR. PD-L1 expression was detected in 48 tissues of patients with bladder cancer by the qRT-PCR method, PD-1 expression could only be detected in 12 tumor samples. Therefore, the results showing the relationship of the PD-L1 gene expression levels with tumor grade, cancer stage, and overall survival were stated.

### Patients and tissue specimens

Patients' age and sex, and pathological features such as pathological grade and stage of the patient samples included in the study are shown in Table 1.

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**Table 1: Clinopathological features**

| Characteristics | Number of patients (%) |
|---|---|
| Female/Male | 2/46 |
| Pathological Grade | |
| Low grade | 20 (41.7) |
| High grade | 28 (58.3) |
| Pathological Stage | |
| pTa | 32 (66.7) |
| pT1 | 5 (10.4) |
| pT2 | 11 (22.9) |
| Alive/Dead | 41/7 |
| Age (year, median) | 65 [29-85] |
| Follow-up Period (month) | 56 |

### The relationship of PD-L1 expression with cancer stage and tumor grade

There were 20 low-grade and 28 high-grade bladder cancer tissue samples in the study group. The difference in PD-L1 expression levels between the high-grade bladder tumor group ($n = 28$) and low-grade bladder tumor group ($n = 20$) was examined and a statistically significantly increased PD-L1 expression was observed in the high-grade tumor group ($P = 0.036$). The boxplots representing the fold-change of PD-L1 expression in tumor grades is shown in Figure 1a.

Of the 48 patients included in our study, 32 had pTa, 5 had pT1, and 11 had pT2 cancer stage. Examining the relationship between cancer stages and PD-L1 expression showed that there was no statistically significant difference in PD-L1 expression levels among groups (p = 0.089) [Figure 1b]. In addition, no significant results were obtained between groups (posthoc testing).

### Association of PD-L1 expression, cancer stage, tumor grade, and overall survival

The relationship of PD-L1 expression, tumor grade, and cancer stage with overall survival in bladder cancer patients was calculated by Kaplan-Meier analysis, and a comparison of survival curves was performed with Log Rank (Mantel-Cox). While 41 of the patients in the study group were alive, seven of them were dead. The overall survival of the patients is at least 5 months and a maximum of 56 months. Of the 7 patients who died; 2 had low-grade and 5 had high-grade bladder cancer. In addition, 3 of the 7 deceased patients had Ta, and 4 had T2 pathological tumor stage.

The relationship between tumor grade and survival was examined. Although survival times of 33 months in low-grade and 35 months in high-grade patient groups were found, the difference result was not statistically significant ($p = 0.573$) [Figure 2a].

When the overall survival rates were analyzed according to cancer stages: It was observed that the patients in the Ta stage had a survival time of 38.5 months, those in the T1 stage had a survival time of 35 months, and the patients in the T2 stage had a survival time of 40 months. However, in

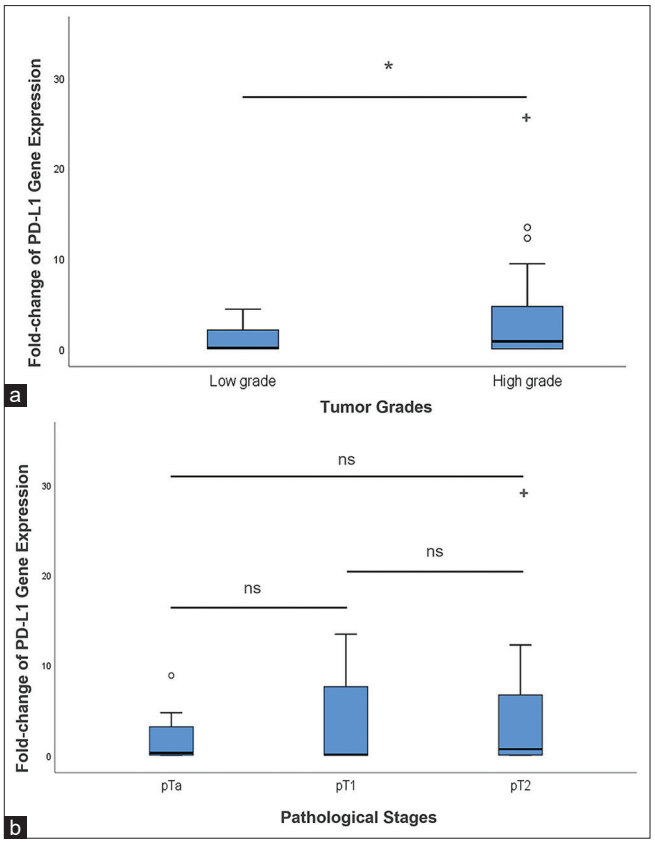



**Figure 1:** PD-L1 gene expression in bladder cancer tumors. (a) Fold-change of PD-L1 expression according to cancer stages,(b) PD-L1 expression fold-change according to tumor grades.Gene expression analysis of PD-L1 was normalized to GAPDH levels. *;$P < 0.05$, ns; not significant. The results were represented as fold-change and expressed as means ± SD in comparison to tonsil tissue samples. Circle indicates outlier and plus indicates extreme outlier in the dataset

the statistical evaluation, the difference between the overall survival times and the cancer stage groups was not found significant (p = 0.334) [Figure 2b].

It was found that the patients with high expression of PD-L1 had an overall survival of 43 months, and patients with low expression of PD-L1 had an overall survival of 35 months. However, there was no statistically significant difference between the survival data of the low PD-L1 expression group and the high PD-L1 expression group (p = 0.064) [Figure 2c].

## DISCUSSION

Nowadays, molecules with prognostic and therapeutic importance are increasingly being investigated by focusing on the immunogenic properties of bladder cancer.[5] In recent years, the PD-L1 molecule involved in the immune checkpoint is utilized as a potential therapeutic target in some cancer types, including bladder cancer. The presence of detectable PD-L1 expression in the vast majority of tumor tissues of bladder cancer patients may provide insight into the recommendation of anti-PD-L1 immunotherapies as an

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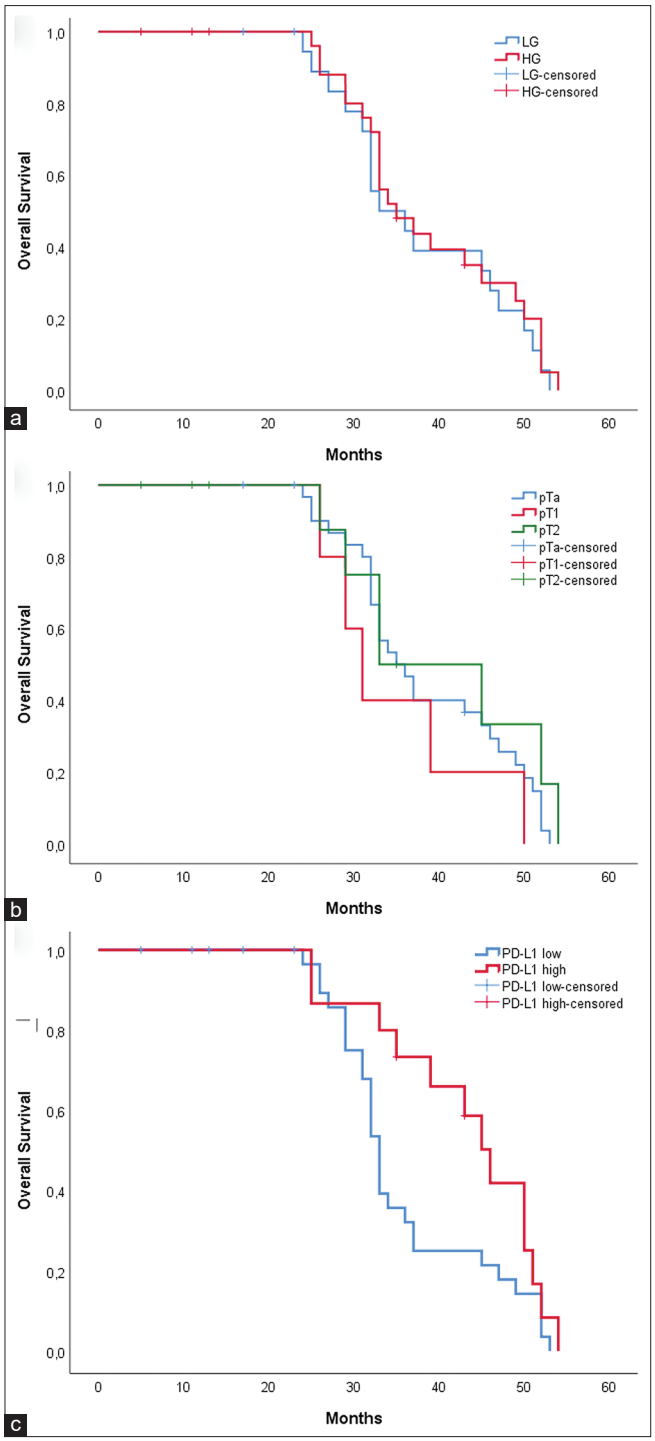



**Figure 2:** Kaplan Meier survival curves for overall survival according to tumor grade, cancer stage and PD-L1 expression (a) Kaplan-Meier curve showing the relationship between tumor grades and overall survival. (b) Kaplan-Meier curve showing the relationship between tumor stages and overall survival (c) Kaplan-Meier curve showing the relationship between PD-L1 expression and overall survival. PD-L1 expression values were grouped into low and high PD-L1 expression based on the mean value. The *P* values were determined via log-rank test. LG; low grade, HG; high grade

alternative treatment option for patients who do not respond to standard therapy. Today, it is of great importance to study predictive biomarkers that facilitate and guide the selection of sequential treatment strategies and the correct treatment for patients with advanced or metastatic bladder cancer.

In this study, the hypothesis that high PD-L1 expression in bladder cancer samples could be associated with poor prognosis of patients, high tumor stage, and cancer grade was tested. Here, we examined the prognostic potential of PD-L1 and PD-1 expression levels, in a cohort of bladder cancer patients grouped according to tumor grade, cancer stage, and its association with overall survival. While PD-L1 expression could be detected in 48 tissues of patients with bladder cancer by the qRT-PCR method, PD-1 expression could only be detected in 12 tumor samples. Consistent with our result of low PD-1 expression in tumor tissues, Li *et al*. (2020)[5] analyzed 260 bladder cancer tumor samples with tissue microarray and found PD-1 expression positivity in tumor tissue to be low since PD-1 expression is associated with immune cells infiltrating the tumor. Normal cells use the PD-1/PD-L1 interaction to prevent tissue inflammation and contribute to homeostasis maintenance.[6] The PD-L1 can typically be expressed by various hematopoietic cells such as macrophages, dendritic cells, mast cells, and non-hematopoietic cell types including endothelial cells, whereas T-cell, B-cell, macrophage, and dendritic cell subsets can express PD-1.[7] Mo *et al*. (2016)[8] examined the expression of PD-1, PD-L1, and PD-L2 in endometrial cancers immunohistochemically in 35 normal endometrial tissue samples and 75 endometrial cancer tissue samples. Approximately 61% of endometrial cancers have positive PD-1 staining on the tumor-infiltrating immune cell surface, but it has been reported that PD-1 is not expressed in tumor cells or normal endometrial tissues. Moreover, in a study, PD-1 expression was analyzed in cancerous and non-cancerous bladder tissue samples. Interestingly, the presence of PD-1 expression in the non-cancerous bladder tissue was reported. However, the results of the study showed no significant differences between the two groups in the expression of the PD-1 gene.[9]

In our study, significantly increased PD-L1 expression was found in the high-grade bladder cancer patient group. The difference in PD-L1 expression levels between cancer stage groups was not statistically significant. This could be due to an unbalanced distribution of patients in each group (n = 32; pTa, n = 5; pT1, and n = 11; pT2). In addition, overall survival data were analyzed with the Kaplan Meier method, and no significant difference was found between overall survival and tumor grade, and cancer stage. Low and high PD-L1 expression in tumor tissues did not demonstrate a statistically significant difference in overall survival time for groups. The follow-up period in the patient cohort was limited to the longest 56 months, which create a limitation in the survival data. Davick *et al*. (2018)[10] conducted a study with 165 cystectomy specimens to examine the expression of PD-L1 in tumor cells and the immunological environment of bladder cancers. These samples were investigated for

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tumor type, tumor grade, pathological stage, and overall survival. Consistent with our results, it has been reported that high-grade tumors have been found to be more PD-L1 positive than low-grade tumors, but there was no significant difference between PD-L1-positive and PD-L1-negative bladder cancers in terms of survival rates. Similarly, Kawahara *et al*. (2018)[9] examined the expressions of PD-1/PD-L1 genes in normal and bladder cancer tissue by qRT-PCR. No significant difference was found in PD-1 and PD-L1 expressions between normal and cancerous tissue, but they observed that PD-1 and PD-L1 were expressed in all bladder cancer tissues and were more expressed in high-grade bladder cancer than low-grade bladder cancer. However, it has been emphasized that PD-1 and PD-L1 could be a new biomarkers associated with the pathological grade of bladder cancer and act as a mediator of stage progression in bladder cancer. In a meta-analysis study including 11 studies and 1,697 patients, high PD-L1 expression was associated with poor overall survival rate and advanced tumor stage.[11] Boorjian *et al*.[12] associated PD-L1 and PD-1 expressions in bladder urothelial carcinoma with advanced disease. Another study, comprising 110 urothelial bladder cancer patients, investigated the relationship between PD-L1 expression (analyzed by immunohistochemistry and qRT-PCR), and clinical outcomes. PD-L1 expression was shown to be higher in cancer tissues compared to normal tissues and was positively associated with grade, stage, and survival.[13] Contrary to these studies, in another meta-analysis of the clinicopathological and prognostic value of PD-L1 expression in urothelial carcinoma of the bladder, no significant association was found between PD-L1 expression in tumor cells and both advanced cancer and higher tumor grade. However, it has been shown that PD-L1 expression on tumor cells is associated with muscle invasion in bladder cancer patients and PD-L1-positive patients respond better to anti-PD-1/PD-L1 antibody therapy.[14] The predictive and prognostic role of PD-L1 in the treatment of bladder cancer remains a matter of debate. According to current literature data, it is reported that the objective response rate is higher after being treated with anti-PD-L1 in patients with PD-L1-positive tumors, but both PD-L1-positive and negative-groups benefit from treatment in overall survival rates.

The differences in the sampled organ and collection method (such as TUR-CT, cystectomy, nephroureterectomy), age of FFPE tissues, material-related changes (formalin-fixed paraffin block and frozen tissue), methods detecting PD-L1 expression, and changes in expression positivity cut-off points make it difficult to directly compare studies in the literature. The different results in the literature reveal that there is a need for multicenter studies to reach a higher sample size. In addition, PD-L1 expression might not be uniform in the tumor and its near margin. There is a possibility that external factors such as TNF$\alpha$ and IL-1$\beta$ produced locally by juxtaposed T lymphocytes induce the PD-L1 expression.[15,16] Therefore, in the current study, it is important to note that the lack of distinguishing PD-L1 expression patterns for specific locations in tumor tissues by qRT-PCR could weaken the power of this assessment approach.

In conclusion, the data obtained in the current study showed that PD-L1 expression determined by the qRT-PCR method in bladder cancer might not be a strong prognostic biomarker alone. However, high expression of PD-L1 shows a strong correlation with tumor aggressiveness and tumor grade. Our results may contribute to the stratification of the appropriate patient group for immunotherapy with anti-PD-L1/PD-1 antibodies for patients with a low response to standard therapy.

## Acknowledgements

We would like to thank Yusuf Ekinci for his help in taking sections from FFPE tissues.

## Financial support and sponsorship

This work was supported by Recep Tayyip Erdogan University, Scientific Research Projects Unit under grant [number TYL-2020-1203].

## Conflicts of interest

There are no conflicts of interest.

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