

## Gabriel Surdacki<sup>1</sup>, Bożena Sokołowska<sup>2</sup>, Aneta Gorący<sup>3</sup>, Katarzyna Chyl-Surdacka<sup>4</sup>, Marek Hus<sup>2</sup>

<sup>1</sup>Department of Urology and Urological Oncology, St. John of God Hospital, Lublin, Poland

<sup>2</sup>Department of Haematology and Bone Marrow Transplantation, Medical University of Lublin, Poland

<sup>3</sup>Department of Haematology and Bone Marrow Transplantation, Center of Oncology of the Lublin Region named after St. John of the Dukla, Poland

<sup>4</sup>Department of Dermatology, Non-Public Health Care Center Med-Laser, Lublin, Poland

# Expression of immune checkpoints on peripheral blood cells in patients with prostate cancer

### Address for correspondence:

Gabriel Surdacki, MD PhD  
 Department of Urology and Urological  
 Oncology, St. John of God Hospital,  
 Herberta 21,  
 20-468 Lublin, Poland  
 e-mail: gabriel.surdacki@wp.pl.  
 tel: 817604254

### ABSTRACT

**Introduction.** The first clinical trials of the treatment of patients with prostate cancer with checkpoint inhibitors show that only a few patients benefit from this type of treatment. This implies the need to find predictive biomarkers that would help identify patients for whom treatment with checkpoint inhibitors could be effective. Our study aimed to assess the level of PD-1, PD-L, and CTLA-4 expression on peripheral blood mononuclear cells of patients with primary prostate cancer and to demonstrate their applicability in clinical practice.

**Material and methods.** Fifty men with primary prostate cancer were enrolled in the study. The control group consisted of 20 healthy men. The material for the study was peripheral blood from which mononuclear cells were isolated by flow cytometry, and the expression of PD-1, CTLA-4, and PD-L1 on them was assessed.

**Results.** High PD-L1 expression on lymphoid dendritic cells has been demonstrated in patients with prostate cancer in comparison to the control group ( $p = 0.015$ ) and high PD-L1 expression has been demonstrated in the following groups: high risk ( $p = 0.026$ ), T2/T3 ( $p = 0.011$ ), and Gleason 7 ( $p = 0.027$ ). There was no high PD-1 expression on T lymphocytes among patients with prostate cancer in comparison to the control group, but positive correlations were found between PD-1 expression on CD3+ T cells and PSA ( $p = 0.049$ ), risk group ( $p = 0.002$ ), and TNM ( $p = 0.050$ ). Low CTLA-4 expression was found on CD3+ lymphocytes among patients with prostate cancer in comparison to the control group ( $p = 0.006$ ).

**Conclusions.** Several groups of patients with prostate cancer have been identified, showing high PD-L1 and PD-1 expression on peripheral blood mononuclear cells, and a relationship between PD-1 and PD-L1 expression and the tumor aggressiveness potential has been demonstrated. This means that the assessment of PD-1 and PD-L1 expression can be used as a prognostic and predictive biomarker in prostate cancer.

**Key words:** prostate cancer, checkpoint, PD-1, PD-L1, CTLA-4

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## Introduction

Checkpoints are part of the so-called immune synapse and are negative regulators of the immune response. Along with other receptors on the surface of

T lymphocytes, they help to maintain a proper balance between an effective immune response and tolerance to autoantigens. Their overexpression may, however, lead to the lack of activation of T lymphocytes responsible for anticancer response.

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Reports on the effectiveness of immunotherapy using checkpoint inhibitors in the treatment of various cancers have led to the initiation of studies on the use of this group of drugs in advanced prostate cancer [1–6]. The first clinical trials show that only a small proportion of prostate cancer patients benefit from this type of treatment [7, 8]. Only among patients with high microsatellite instability (MSI) with a mismatch repair deficiency (dMMR), the effectiveness of pembrolizumab treatment was confirmed, which led to the approval of this drug by the Food and Drug Administration (FDA) for the treatment of patients with prostate cancer [9, 10]. This implies the necessity to find other predictive biomarkers that would help to easily identify patients in whom treatment with checkpoint inhibitors could be effective.

The degree of PD-L1 (programmed death-ligand 1) expression on cancer and stromal immune cells is a recognized predictive biomarker that is routinely used in qualification for anti-PD-1 therapy [11] in some cancers [12]. It has been proven that the expression of PD-L1 on tumor cells is a predictive and prognostic marker in renal cancer, NSCLC (non-small-cell lung carcinoma), and melanoma [13, 14].

There are also preliminary reports on the usefulness of determining the level of PD-1 (programmed cell death protein 1), PD-L1, and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) expression on peripheral blood T lymphocytes in various cancers [11, 15, 16]. Testing venous blood samples is much less burdensome for patients and does not involve the need to perform a tumor biopsy, which is an invasive procedure and exposes the patient to possible complications. Finding

these types of biomarkers in prostate cancer could help in selecting patients for immunotherapy and avoiding unnecessary side effects associated with the treatment. Some of the biomarkers may also be of prognostic value and facilitate the assessment of the prognosis of patients with prostate cancer.

The aim of the study was to check whether the level of some prostate cancer biomarkers: PD-L1, PD1, and CTLA 4 expression on peripheral blood cells may have a similar predictive and prognostic significance as the expression of these biomarkers in tumor tissue.

## Material and methods

We examined 50 men (median age 69.1 range 51–89 years) with newly diagnosed, untreated primary prostate cancer identified on the basis of histopathological examination of transrectal biopsy sections performed under transrectal USG (TRUS) control. The control group consisted of 20 men (median age 68, range 55–82 years) with no prostate cancer. We acquired the consent of the bioethics committee to conduct the research, and we obtained consent from each patient to participate in the study. The entire population was enrolled in the PD-1 and PD-L1 expression study. Expression of CTLA-4 was tested in 22 men with prostate cancer and 15 men in the control group.

The material for the study was peripheral blood from which mononuclear cells were isolated and lymphocyte subpopulations were extracted using a flow cytometer based on the analysis of surface and intracellular antigens of cellular differentiation (CD). Then, the expression of PD-1, CTLA-4, and PD-L1 on individual cell populations of lymphocytes was assessed. The evaluation of checkpoints expression was performed on the following groups of peripheral blood mononuclear cells: CD3+, CD3+/4+, CD3+/8+, FoxP3+/25+/+/4+, CD45RO+, CD3+/69+, CD19+, NK (natural killer), BDCA1+/19+, BDCA2+/123+ (blood dendritic cells). The correlation between the expression of immune checkpoints and the tumor aggressiveness potential was also investigated. The aggressiveness of cancer was assessed on the basis of the Gleason score, TNM scale, PSA level, and the risk group of biochemical recurrence (Tab. 1).

The expression of PD-1, CTLA-4, and PD-L1 has been investigated in two ways. In the first case, it was determined what percentage of peripheral venous blood mononuclear cells positively express PD-1, PD-L1, and CTLA-4. In the second case, the number of cells expressing PD-1, PD-L1, and CTLA-4 in 1 µl of venous blood is given. Individual patients' blood counts were used to calculate the number of cells.

**Table 1. Division of patients in study group depending on the Gleason score, cTNM, PSA, and risk group**

Variable	Number of patients	
Gleason	6	19
	7	26
	≥ 8	5
cTNM	cT1N0M0	7
	cT2/3N0M0	26
	cTxN1M0/1	7
PSA (ng/mL)	< 10	18
	10–20	16
	> 20	16
Risk group	Low	5
	Intermediate	13
	High	32

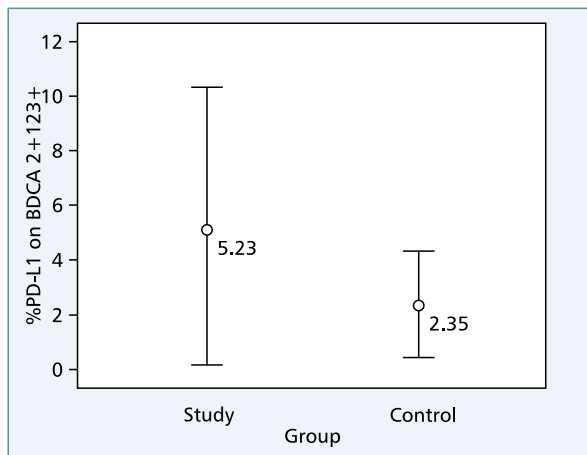
cTNM — clinical classification of malignant tumors; PSA — prostate-specific antigen

## Results

### PD-L1 expression on peripheral blood mononuclear cells

It was found that the expression of PD-L1 on lymphoid dendritic cells (BDCA2+/123+) is significantly higher in patients with prostate cancer than in men from the control group ( $p = 0.015$ ) (Fig. 1). Also, the number of BDCA2+/123+ cells expressing PD-L1 was significantly higher in patients with prostate cancer compared to the control group ( $p = 0.024$ ) (Tab. 2).

In patients with prostate cancer, a positive correlation was found between PSA level and PD-L1 expression on BDCA2+/123+ cells ( $p = 0.020$ ). Patients in all risk groups of biochemical recurrence showed higher PD-L1 expression on BDCA2+/123+ cells than patients in the control group. Patients in the clinical stage of T2/T3 had significantly higher PD-L1 expression on BDCA2+/123+ compared to the control group ( $p = 0.011$ ). Patients in the Gleason 6 and Gleason 7 histological groups showed a higher PD-L1 expression on BDCA2+/123+ cells than patients in the control group ( $p = 0.023$ ,  $p = 0.027$ ).



**Figure 1.** Comparison of PD-L1 expression on BDCA2+123+ dendritic cells in the study and control groups ( $p = 0.015$ )

### PD-1 expression on peripheral blood mononuclear cells

There was no statistically significant difference in the level of PD-1 expression on peripheral blood mononuclear cells between the study group and the control group. Only in the case of CD19+ lymphocytes statistically higher expression of PD-1 was found in the control group ( $p = 0.003$ ). In the group of patients with prostate cancer, we found a relationship between PD-1 expression and the tumor aggressiveness potential. This is evidenced by the positive correlation between the expression of the PD-1 receptor on CD3+ T cells and PSA, risk group, and TNM. In patients with prostate cancer, a positive correlation was found between PSA levels and PD-1 expression on CD3+ cells in peripheral blood ( $p = 0.049$ ) (Fig. 2). A positive correlation was also shown between the cTNM stage and PD-1 expression on CD3+ cells ( $p = 0.050$ ) (Fig. 3). It has also been shown that the higher the risk of biochemical recurrence, the higher the expression of PD-1 on CD3+ ( $p = 0.030$ ) and the higher the number of CD3+PD-1+ and CD3+/4+PD-1+ lymphocytes ( $p = 0.002$  vs.  $p = 0.040$ ) (Fig. 4). No statistically significant correlations were found between the expression of PD-1 on mononuclear cells and the histological grade on the Gleason scale.

### CTLA-4 expression on peripheral blood mononuclear cells

We have demonstrated that men with prostate cancer have a significantly lower expression of CTLA-4 on CD3+ cells compared to people from the control group ( $p = 0.006$ ). Patients in the clinical stage of T2/T3 and N1/M1 have a significantly lower number of CD3+ ( $p = 0.007$ ) and CD3+8+ ( $p = 0.037$ ) cells showing CTLA-4 expression than patients in the control group. Patients with histological grade Gleason 6 have a significantly lower number of CD3+CTLA4+ cells ( $p = 0.002$ ) compared to men from the control group, and patients with Gleason 8-9 grade have a significantly lower number of CD3+ ( $p = 0.037$ ), CD3+/4+ ( $p = 0.025$ ) and CD3+/8+ cells ( $p = 0.025$ ) showing CTLA-4 expression compared to the control group. A correlation was found

**Table 2.** PD-L1 expression on peripheral blood dendritic cells in the study and control groups

Parameter	Group						Mann-Whitney	
	Prostate cancer			Control			U test	
	M	Me	SD	M	Me	SD	Z	p
%PD-L1 on BDCA-1+/19+	1.45	1,04	1,31	1,31	0,49	1,74	-1,427	0,153
%PD-L1 on BDCA-2+/123+	5.23	4,18	5,03	2,35	2,10	1,89	-2,423	<b>0,015*</b>
BDCA1+/19+PD-L1+	0.45	0,31	0,51	0,49	0,14	0,69	-1,111	0,267
BDCA2+/123+PD-L1+	0.30	0,21	0,27	0,17	0,11	0,23	-2,263	<b>0,024*</b>

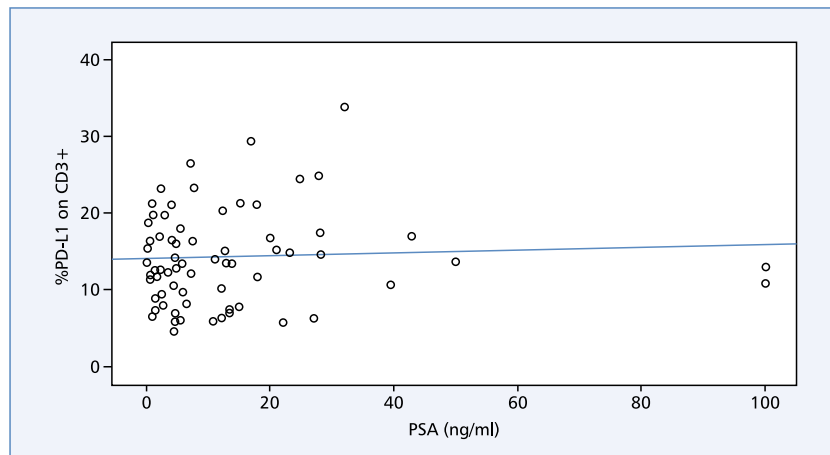


Figure 2. Relationship between PD-1 expression on CD3+ lymphocytes and PSA level ( $p = 0.049$ )

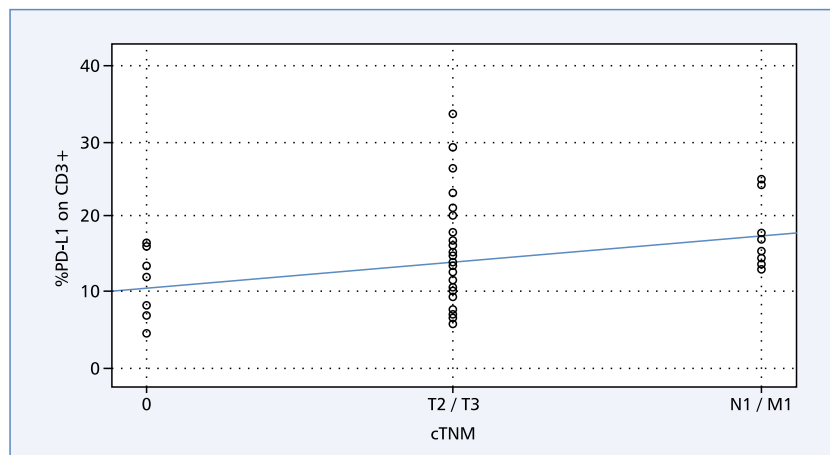


Figure 3. Relationship between PD-1 expression on CD3+ lymphocytes and cTNM stage ( $p = 0.050$ )

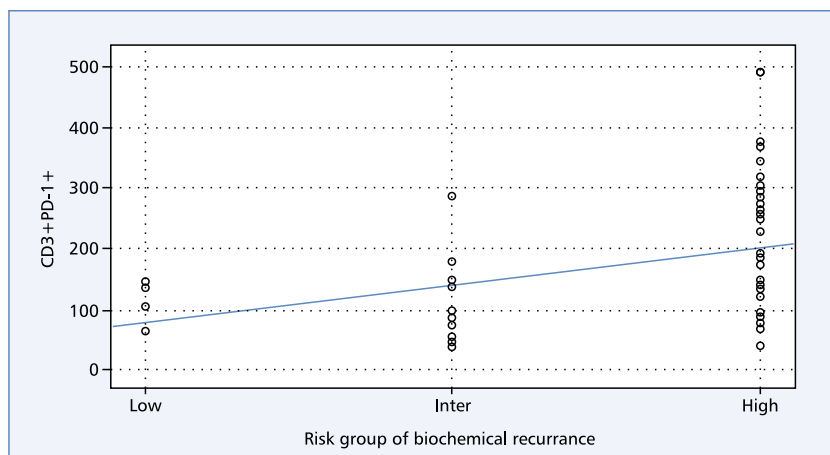


Figure 4. Relationship between the number of CD3+PD-1+ lymphocytes and the risk group of biochemical recurrence ( $p = 0.002$ )

according to which the higher the PSA level, the lower the expression of CTLA-4 on CD3+ ( $p = 0.001$ ) and CD3+/8+ ( $p = 0.020$ ) cells in the peripheral blood. Moreover, patients in the high-risk group are characterized by significantly lower CTLA-4 expression on CD3+ ( $p = 0.003$ ) and CD3+/8+ ( $p = 0.019$ ) cells compared to the control group.

## Discussion

### Expression of PD-L1 on antigen presenting cells

Previous studies on PD-L1 expression in prostate cancer have shown that tumor and antigen presenting cells (APC) surrounding cells show high PD-L1 expression [17]. An important group of APCs is dendritic cells (DCs). High PD-L1 expression on DCs can be detected both in the tumor environment and in the peripheral blood. Therefore, the expression of PD-L1 was examined on populations of myeloid dendritic cells (BDCA1+/19+) and lymphoid dendritic cells (BDCA2+/123+) in the peripheral blood. To our knowledge, this is one of the first studies to evaluate PD-L1 expression on APCs in the peripheral blood in prostate cancer.

### PD-L1 expression on peripheral blood mononuclear cells

It was found that the expression of PD-L1 on BDCA2+/123+ is significantly higher in patients with prostate cancer than in men from the control group. Also, the number of BDCA2+/123+ cells expressing PD-L1 was significantly higher in patients with prostate cancer compared to the control group. A similar correlation was described by Bishop et al. [15]. They demonstrated an increased number of DCs expressing high-level PD-L1 in mCRPC (metastatic castration-resistant prostate cancer) patients refractory to treatment with Enzalutamide. High PD-L1 expression has been found not only in the immune cells surrounding the tumor but also in the peripheral blood of patients. A similar correlation was also found in the study of Ness et al. [12]. High PD-L1 expression was found on tumor stromal immune cells (including DCs) in 66% of patients with prostate cancer. Also, the publication by Massari et al., in which the level of PD-L1 expression on prostate cancer cells and tumor stromal cells in the material after radical prostatectomy was assessed, confirms the above observations. PD-L1 expression was demonstrated in 50% of patients from the study group, and in 19% of patients, the expression level was assessed as high [18].

PD-L1 expression on dendritic and tumor cells and PSA level, Gleason score, TNM scale, and cancer characteristic

In patients with prostate cancer, a positive correlation was found between PSA level and PD-L1 expression on BDCA2+/123+ cells. A similar correlation between the PD-L1 expression and the level of PSA was also found in the study by Calagua et al. This study assessed the histopathological material after radical prostatectomy from 174 patients with prostate cancer. 44 patients received neoadjuvant therapy with abiraterone acetate, prednisone, and leuprorelin, while the remaining 130 patients did not receive neoadjuvant treatment. In the second group, a positive correlation was found between the amount of PSA and the level of PD-L1 expression [14]. Opposite results were presented in the study by Bass et al. They performed immunohistochemistry for PD-1, PD-L1, and CD3 and scored from 0 to 5 on prostatectomy/biopsy tissue samples taken from 25 men with high-grade prostate cancer. A score of 3 to 5 on the semiquantitative 0 to 5 score was deemed "high" expression whereas a score of 0 to 2 was deemed "low" expression. Of the 25 samples, 2 (8%) scored high for PD-1 expression, 2 (8%) scored high for PD-L1 expression. They also found no relationship between PD-L1 expression level and PSA level and other disease characteristics [19].

We found no linear correlation between PD-L1 expression and Gleason grade. But we have observed that patients in the Gleason 6 and Gleason 7 histological group showed a higher PD-L1 expression on BDCA2+/123+ cells than patients in the control group. Moreover, patients with Gleason 7 grade had significantly more BDCA2+/123+ cells with high PD-1 expression compared to men from the control group. We have also found that patients in the clinical stage T2/T3 had significantly higher PD-L1 expression on BDCA2+/123+ cells compared to the control group. The positive correlation between PD-L1 expression and the Gleason score, as well as the clinical stage, was confirmed in the study by Haffner et al., which assessed tumor tissue in 539 patients with primary prostate cancer [20]. They showed higher PD-L1 expression in patients with higher grade groups ( $p = 0.08$ ). Particularly high PD-L1 expression was found in patients with Gleason 4 and 5.

Patients in all risk groups of biochemical recurrence showed higher PD-L1 expression on BDCA 2+123+ cells than patients in the control group. The results of our study correlate with the results of the study by Li et al., which included a group of 127 patients with prostate cancer in the high-risk group after radical prostatectomy and ADT (androgen deprivation therapy) [17]. In this study, higher PD-L1 expression was found on cancer and

stromal cells in high-risk patients. The level of PD-L1 expression was an independent recurrence prognostic factor after radical prostatectomy. Patients with high PD-L1 expression had a shorter BCR (biochemical recurrence)-free survival compared to patients with low PD-L1 expression (13 vs. 25 months). It was also found that high PD-L1 expression is associated with a worse prognosis.

The results of our study on PD-L1 expression on BDCA2+123+ cells in the peripheral blood confirm the observation that in more advanced and malignant cancers there is a higher expression of PD-L1 on APCs. This is consistent with the results of multicenter studies in large groups of patients in which the expression of PD-L1 on tumor and stromal cells was assessed. The KEYNOTE-028 multicenter study evaluated PD-L1 expression on cancer cells and immune cells. In a group of 245 men with mCRPC, 14% of patients showed high PD-L1 expression [21,22]. The KEYNOTE-199 study included 539 patients with prostate cancer. It was found that mCRPC patients show significantly higher PD-L1 expression (31.6%) compared to patients with primary prostate cancer (7.7%) [20]. In a study by Petitprez et al. in a group of 51 patients with prostate cancer and lymph node metastases, it was found that patients with > 1% of tumor cells with PD-L1 expression have shorter metastasis-free survival than patients with expression < 1% [23]. The same study found that patients with high PD-L1 expression have a four-fold higher risk of metastasis than patients with low PD-L1 expression. Also, Gevensleben et al. showed that mCRPC patients have high PD-L1 expression [24, 25]. They not only demonstrated increased PD-L1 expression on stromal and prostate cancer cells but also proved that high PD-L1 expression is an independent and negative prognostic factor for relapse-free survival in patients undergoing radical prostatectomy.

The correlations shown in our study regarding PD-L1 expression on peripheral blood DCs in prostate cancer confirm the observation in other studies that patients with a more advanced and aggressive form of cancer show higher PD-L1 expression on tumor cells and immune cells compared to healthy patients and patients with less advanced and aggressive cancer [26]. However, it should be taken into account that, unlike our research, other studies assessed mostly the expression of checkpoints on tumor tissue and not on peripheral blood cells.

#### Expression of PD-1 on peripheral blood lymphocytes

There are very few studies that assess PD-1 expression on peripheral blood lymphocytes in patients with prostate cancer. Most studies to date on prostate cancer have evaluated PD-1 expression on cells in the tumor

tissue. However, PD-1 expression in peripheral blood lymphocytes has already been studied many times in other cancers. Most of the available publications assessed mainly PD-1 expression in the CD3+, CD8+, and CD4+ lymphocytes. This is justified because the above-mentioned lymphocyte populations play a key role in the destruction of cancer cells. There are scientific reports confirming that CD8+ T cells specific for cancer tissue and expressing PD-1 can be successfully detected in the peripheral blood [12]. This means that testing the expression of immune checkpoints on peripheral blood cells makes sense and may be a viable alternative to testing this expression in cancer tissue. In our study, there was no statistically significant difference in the level of PD-1 expression on peripheral blood lymphocytes between patients with prostate cancer and healthy men. But in the group of patients with prostate cancer, we found a correlation between PD-1 expression and the tumor aggressiveness potential.

#### PD-1 expression on lymphocytes and PSA, TNM, and Gleason score

One of the key results of our study is the finding that the expression of PD-1 on lymphocytes in patients with prostate cancer positively correlates with the risk group of biochemical recurrence. The obtained results are analogous to those published by Ness et al. In 535 patients undergoing radical prostatectomy, they found that high density of PD-1(+) lymphocytes is a significant, independent, and negative prognostic factor for clinical failure-free survival (CFFS) [7]. Similarly, Hansen et al. [21] revealed in their study that high PD-1 expression on T lymphocytes in the tumor stroma is associated with faster progression of prostate cancer. Also, Kwek et al. [27] showed that a higher number of CD4+PD-1+ T cells in the peripheral blood is associated with shorter overall survival (OS) in patients with prostate cancer.

#### Expression of CTLA-4 on peripheral blood lymphocytes

In our study, men with prostate cancer have a significantly lower expression of CTLA-4 on CD3+ cells compared to people from the control group. This correlation is in contrast to most of the available publications. This may be due to the small size of the study group (22 patients) and the fact that in our own study, CTLA-4 expression was examined on peripheral blood lymphocytes, and not in cancer tissue, as was the case in most studies to date. So far, very few authors have undertaken studies of CTLA-4 expression on T lymphocytes in prostate cancer. The assessment of the level of CTLA-4 expression on peripheral blood lymphocytes in prostate cancer requires further studies on larger groups of patients.



## Conclusions

We have demonstrated that prostate cancer patients exhibit high PD-L1 expression on peripheral blood dendritic cells, which suggests that checkpoint inhibitors may be useful in the treatment of prostate cancer. Moreover, we identified specific groups of patients (patients with high PSA levels, patients at high risk of biochemical recurrence, and patients with high TNM stage) that show high expression of PD-1 and PD-L1 on peripheral blood cells and may be potential beneficiaries of this type of immunotherapy. The positive correlation between the expression of PD-1 and PD-L1 on mononuclear peripheral blood cells and the cancer aggressiveness proves that the expression of immune checkpoints can also be used as a prognostic biomarker in prostate cancer.

## Conflict of interest

There are no competing financial interests in relation to the work described.

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