



# The role of tumor-infiltrating lymphocytes (TILs) as a predictive biomarker of response to anti-PD1 therapy in patients with metastatic non-small cell lung cancer or metastatic melanoma

Anton Uryvaev<sup>1</sup> · Maria Passhak<sup>1</sup> · Dov Hershkovits<sup>3</sup> · Edmond Sabo<sup>2,4</sup> · Gil Bar-Sela<sup>1,4,5</sup> 

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## Abstract

Immunotherapy plays an important role in cancer treatment. Biomarkers that can predict response, including tumor-infiltrating lymphocytes (TILs), are in the spotlight of many studies. This cohort study was designed to evaluate the role of CD4+ and CD8+ TILs as predictive factors for response to anti PD-1 treatment in patients with metastatic non-small cell lung cancer (NSCLC) or metastatic melanoma. We evaluated the expression of CD4+ and CD8+ TILs in tissue samples of 56 patients with metastatic NSCLC or melanoma treated with anti-PD1 immunotherapy. The study included 30 patients with melanoma and 26 with NSCLC. An association was found between CD8+/CD4+ TILs ratio and response to anti-PD1 treatment in both cancers. Regarding melanoma patients, ratios of CD8+/CD4+ lower than 2 predicted lack of response to treatment (0%) ( $p = 0.006$ ), while CD8+/CD4+ ratios higher than 2.7 had an 81.3% response rate ( $p = 0.0001$ ). In addition, we found that the presence of more than 1900/mm<sup>2</sup> of CD8+ lymphocytes in the melanoma tumor predicted a 90% response to therapy. In the metastatic NSCLC group, tumors with CD8+ lymphocyte count under 886/mm<sup>2</sup> showed low response rates (16.7%,  $p = 0.046$ ). When the CD8+ lymphocyte count was in the range of 886-1899/mm<sup>2</sup>, the response rate was high (60%,  $p = 0.017$ ). In CD8+/CD4+ ratios lower than 2, the response rate was low (13.3%), and in ratios higher than 2, response rates ranged between 43 and 50% ( $p = 0.035$ ). The use of CD8+/CD4+ TILs ratios in tumor biopsies may predict response to anti-PD1 treatment in metastatic melanoma and NSCLC.

**Keywords** Tumor-infiltrating lymphocytes (TILs) · CD8+ lymphocyte · CD4+ lymphocyte · Melanoma · Lung cancer

## Background

The immune system identifies and acts against tumor cells by adaptive cell reactions of cytotoxic T lymphocytes (CTL) and the cells of the innate immune system, natural killer

(NK) cells. Over 80% of NK cells are positive to CD8 receptors. A significant presence of CTL and NK cells inside the tumor usually correlates with tumor elimination and better prognosis. The function of B and T helper lymphocytes is ambiguous. All cell types inside the tumors are classified as tumor-infiltrating lymphocytes (TILs) [1].

The significance of TILs on prognosis has been examined in many clinical research studies. According to a meta-analytic study published in 2011, there is no clear conclusion regarding the prognostic significance of lymphocytes present in the tumor environment and it is probable that the quantitative ratio between the population of different cells is more significant than their presence in this environment [2]. Recently, several major research studies have been published on melanoma and non-small cell lung cancer, exploring the prognostic significance of TIL cells for these types of tumors. In these studies, it was found that there is good prognostic significance for disease-specific survival and overall survival in the presence of CD4+ and CD8+

✉ Gil Bar-Sela  
g\_barsela@rambam.health.gov.il

<sup>1</sup> Division of Oncology, Rambam Health Care Campus, Haifa, Israel

<sup>2</sup> Pathology Department, Rambam Health Care Campus, Haifa, Israel

<sup>3</sup> Department of Pathology, Sourasky Medical Center, Tel Aviv, Israel

<sup>4</sup> Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

<sup>5</sup> Integrated Oncology and Palliative Care Unit, Rambam Health Care Campus, POB 9602, 31096 Haifa, Israel

lymphocytes in the tumor area [3–8]. In a few studies on melanoma, the presence of TILs in the primary tumor was a significant prognostic factor for overall survival in multivariate analyses, adjusting for other risk factors [3, 4]. Moreover, studies on non-small cell lung cancer (NSCLC) have shown that the high numbers of TILs (CD4+ and CD8+) were independent positive prognostic factors for disease-specific survival [5–8].

Several studies with anti-PD1 treatment in melanoma and lung cancer also tried to find predictive factors for response; PD-L1 overexpression was one of the main factors [9–12]. Early-stage trials have suggested that PD-L1 expression on tumor cells and probably on TILs may increase the likelihood of response to PD-1-directed therapies in metastatic NSCLC [13–15].

A recently published randomized phase 3 trial in advanced NSCLC compared pembrolizumab with cytotoxic chemotherapy as first-line treatment in the population of patients with membranous PD-L1 expression in at least 50% of tumor cells and revealed a significantly longer progression-free survival (PFS) and overall survival (OS) in patients in the immunotherapy arm, suggesting a relevant predictive value for high PD-L1 expression in this population [16]. No validated predictive biomarkers of response to immunotherapy exist for metastatic melanoma [17].

In a few studies that tried to evaluate the significance of TILs as a predictor of immunotherapy response, the outcomes were controversial relative to the small size of the studies [18, 19]. The relation between the different TIL cell populations was not thoroughly examined. However, CD8+ cells or the CD8/CD4 ratio could have a predictive value in evaluating the possible benefit of anti PD1 treatment [20–23].

The aim of this study was to preliminarily examine CD4+ and CD8+ TIL expression in metastatic NSCLC and metastatic melanoma in patients treated with anti-PD1 antibodies to evaluate the possible relation between the expression volume of these cells and treatment response.

## Methods

### Study design

After approval of the study protocol by the institutional ethics committee (Rambam Health Care Campus Ethical Committee, #0360-15-RMB), a retrospective analysis of medical records of adult patients treated in the Division of Oncology at Rambam Health Care Campus (RHCC) in Haifa, Israel, for metastatic NSCLC or metastatic melanoma from October 2015 to January 2017 was undertaken. Eligibility

for this study included having metastatic disease, an initial tumor sample or biopsy sample in the archives of RHCC's Pathology Institute, treatment with anti-PD1 immunotherapy (pembrolizumab or nivolumab) and complete oncological follow-up from diagnosis until research completion. Patients were de-identified after data collection from digital and non-digital records. Data extracted from the medical files included demographic and clinical data (age, sex, performance status, disease sites, histological type, previous treatment, type of anti-PD1 antibody), as well as response to anti-PD1 treatment, PFS and OS.

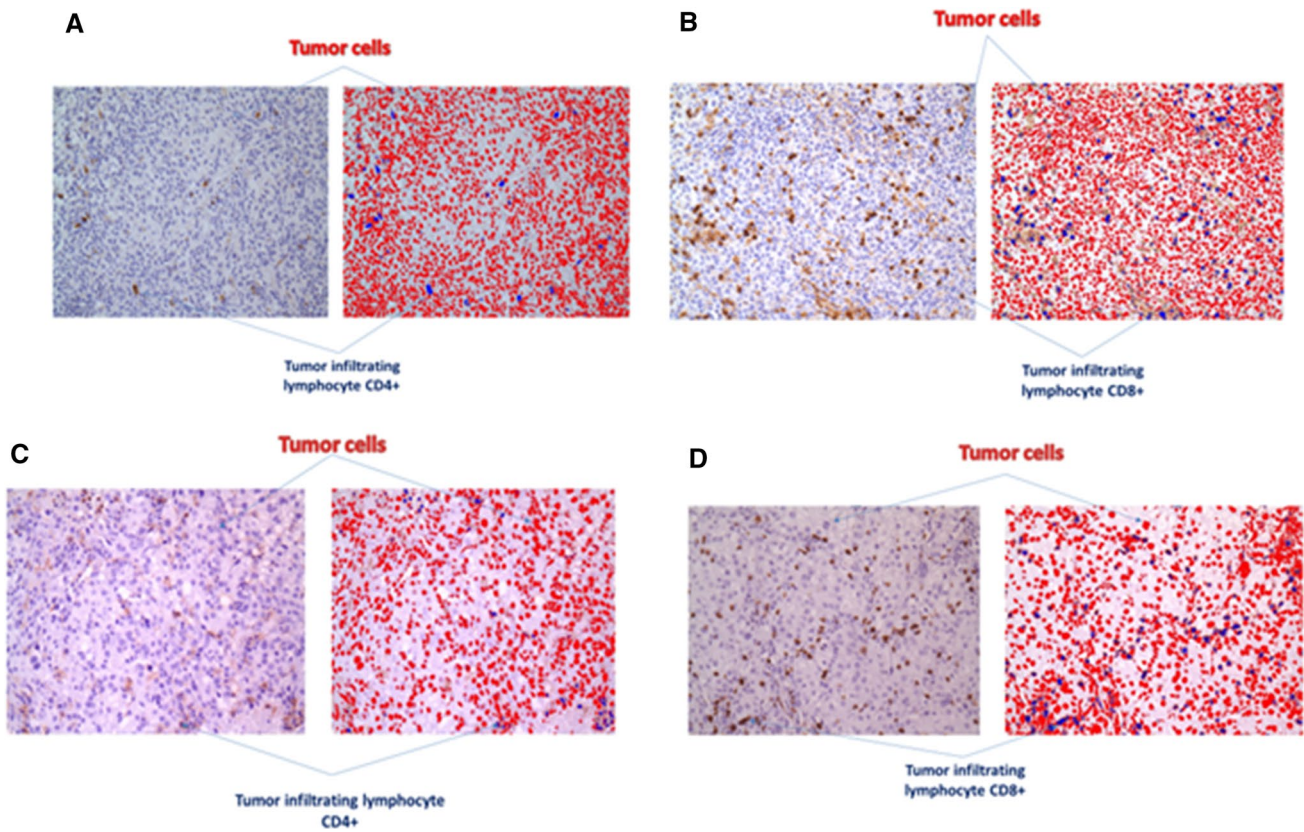
### Staining of the pathology samples

The study included 56 samples (26 NSCLC and 30 melanoma tumors). Four-micron-thick slides were sampled from the blocks containing tumor tissue. After deparaffinization, immunohistochemical staining was performed with anti-CD4/IgG 1:40, monoclonal antibodies (SP35), and anti-CD8 antibodies/IgG 1:40, monoclonal (SP16), rabbit antibodies (Spring Bioscience, Pleasanton CA, USA). The slides were stained by the automatic coloring system produced by Ventana Benchmark Company (Ventana Medical Systems, Tucson AZ).

Quantitative analyses were done with high-resolution microscope fields of view X40 pictures followed by semi-automatic counting with the image processing program Image Pro Plus 6.3 (MediaCybernetic Massachusetts, USA). Accumulated tumor tissues infiltrated by intra-tumor lymphocytes were chosen to be pictured according to the “hot spot” method, where areas with the maximum lymphocytes infiltration are pictured. The image processing program was tuned to identify the tumor cells and the lymphocytes inside the tumors according to different color shades (lymphocytes with darker color). The tumor tissues were marked in red, and the lymphocytes were marked in blue. Automatic counting of the elements marked on the picture was performed. In this manner, the absolute number of CD4 and CD8 lymphocytes was determined in every view field, as well as the quantitative ratio between CD8 and CD4 lymphocytes in every field (Fig. 1).

### Statistical analysis

Bivariate Cox regression was used for calculation of the odds ratios (OR) with 95% confidence intervals (95% CI), and *p* values in bivariate analysis were used to determine associations between tumor characteristics, number of CD4 and CD8 lymphocytes, CD8/CD4 ratio and the likelihood of response to anti-PD1 treatment or PFS. Variables with  $p \leq 0.1$  were selected as candidates for the multivariable



**Fig. 1** Staining of the pathology samples and computer analysis. **a** Case melanoma (CD4+). **b** Case melanoma (CD8+). **c** Case NSCLC adenocarcinoma (CD4+). **d** Case NSCLC adenocarcinoma (CD8+)

analysis. Multivariable forward stepwise logistic regression analysis was performed. The area under the receiver operating characteristic (ROC) curve was used as a measure of model discrimination. Two-tailed *p* values of 0.05 or less were considered as statistically significant. Statistical analyses were performed with Statistics Products Solutions Services (SPSS) 21.0 software for Windows.

## Results

In the study period, 56 patients were included, 30 diagnosed with metastatic melanoma and 26 with metastatic NSCLC. All the patients were treated with anti-PD1 therapy (pembrolizumab or nivolumab) as a first-, second- or third-line therapy. Statistical data processing was performed separately for each cancer due to the biology differences of these two diseases and tumor immunogenicity which results in different responses to immunotherapy and different survival periods.

## Melanoma

The 30 patients with metastatic melanoma included 21 males and 9 females with a median age of 65 (range 32–93 years). Patient characteristics are summarized in Table 1. Only three (10%) patients were initially diagnosed with stage IV. Four patients had interferon alpha as adjuvant therapy. For the 27 patients with early stages, disease-free survival period ranged from 2 to 140 months, with an average of 37.7 months. At the time of anti-PD1 treatment, only three patients had ECOG performance status (PS) of 2, and all others were at PS 0 or 1. Ten patients had BRAF-positive tumors; however, only three started anti-BRAF and anti-MEK treatment as first line. Six patients were given ipilimumab, and 21 patients were treated with anti-PD1 as first-line therapy. Seventeen patients were treated with pembrolizumab and 13 with nivolumab (Table 1). The response rate is summarized in Table 2. The rate of response was 53.3%, and only 30% had progressive disease. Some patients' characteristics had statistically significant influence on therapy results. Patients with ECOG-PS of 0 had 73% response rate to anti-PD1 (*p* = 0.02, OR 8.25). Patients

**Table 1** Baseline demographic and disease characteristics of patients with melanoma

Age at diagnosis (year)	
Median	64.8
Range	32–93
Gender, <i>n</i> (%):	
Male	21 (70)
Female	9 (30)
Stage of disease at diagnosis, <i>n</i> (%)	
I	4 (16.6)
II	13 (43.3)
III	8 (26.3)
IV	3 (10)
Unknown	1 (3.3)
ECOG performance status in metastatic disease, <i>n</i> (%)	
0	15 (50)
1	12 (40)
2	3 (10)
Biopsy site, <i>n</i> (%)	
Skin	13 (43.3)
Lymph node	10 (33.3)
Lung	3 (10)
Liver	1 (3.3)
Brain	1 (3.3)
Other	2 (6.6)
BRAF status, <i>n</i> (%)	
Mutant	10 (33.3)
Wild	16 (53.3)
Unknown	4 (13.3)
LDH level, <i>n</i> (%)	
Above ULN	15 (50)
Under ULN	15 (50)
Adjuvant high-dose interferon alpha, <i>n</i> (%)	
Yes	4 (13.3)
No	18 (60)
Not relevant	8 (26.7)
Prior systemic therapy, <i>n</i> (%)	
Yes	9 (30)
No	21 (70)
Type of anti-PD1 therapy, <i>n</i> (%)	
Pembrolizumab	17 (56.7)
Nivolumab	13 (43.3)

**Table 2** Response to anti-PD1 therapy—melanoma

	Number of patients	% of patients
Complete response (CR)	5	16.6
Partial response (PR)	11	36.6
Stable disease (SD)	5	16.6
Progressive disease (PD)	9	30

without BRAF mutation had significantly higher response rates of 62% ( $p = 0.004$ , OR 6.67).

### TILs as predictive of response in melanoma

An intensive infiltration of melanoma tumor by CD4+ and CD8+ lymphocytes predicts a statistically significant high response to anti-PD1 therapy. A lower count of CD4+ lymphocytes inside the tumor predicted a lower response rate (RR) (Table 3, Fig. 2). A CD8+ lymphocyte count lower than 866/mm<sup>2</sup> predicted a relatively low response to immunotherapy (RR 30%). In tumors with CD8+ lymphocytes count inside the tumor above 1900/mm<sup>2</sup>, the RR was 90% with a  $p$  value of 0.0001 for response. In addition, CD8+/CD4+ lymphocytes ratio also predicted response to anti-PD1 treatment. In tumors with CD8+/CD4+ lymphocytes ratios lower than 2, no response to therapy was observed (RR 0%). Most of the patients with CD8+/CD4+ lymphocytes ratio above 2.7 responded to immunotherapy (RR 81.3%) with a  $p$  value of 0.0001 (Table 3, Fig. 1). A statistically significant relationship between RR to anti-PD1 treatment and PFS was observed, with 81% PFS in 1 year for responders ( $p = 0.0001$ ).

### Non-small cell metastatic lung cancer (NSCLC)

Twenty-six patients with metastatic NSCLC participated in the study. Patient characteristics are summarized in Table 4. Eighteen were diagnosed with adenocarcinoma, and the others with squamous cell carcinoma. The group includes 19 men and 7 women with an age range of 46–82 years (median 62.5). Stage IV disease was observed at the time of diagnosis in 15 (57.7%) patients. The remaining 11 patients were in stages I–III with disease-free intervals of 3.7–67.9 months (average 20.6 months). Only four patients had tumor EGFR mutations. For 19 (73.3%) patients, chemotherapy was the first-line therapy for metastatic disease. Two patients were treated with the biological therapy EGFR TKIs as first line, and only five (19.2%) patients started immunotherapy without any previous systemic treatment. All 26 patients were treated with nivolumab (Table 4). The response rate was 23.1% (6 patients, 2 with complete response). Twelve (46.1%) patients had progressive disease (Table 5). Statistically significant influence of clinical parameters, such as performance status, histological type or EGFR mutation, was not found.

### TILs as predictive of response in NSCLC

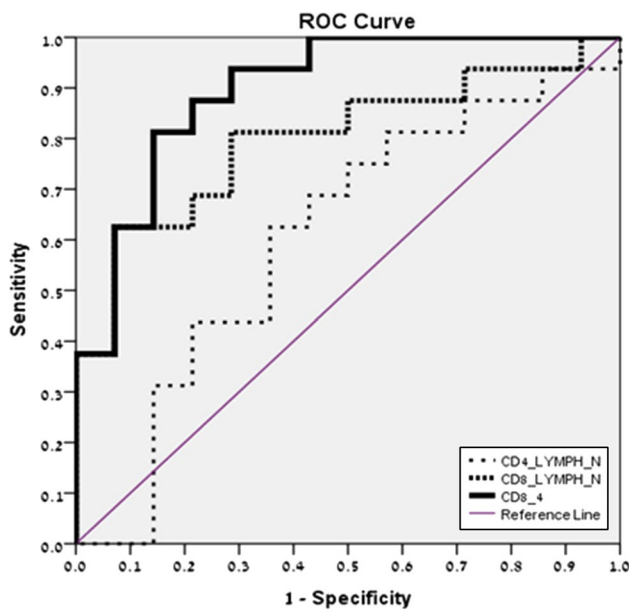
No statistically significant relationship between the presence of CD4+ lymphocytes and response rate to immunotherapy was observed (Table 6, Fig. 3). However, statistical analysis of data showed a significant relationship



**Table 3** Relation between response rate to anti-PD1 treatment and the presence of CD4+ or CD8+ lymphocytes in tumor or CD8+/CD4+ ratio in melanoma patients

	Number of patients	Response rate (RR) [%]	<i>p</i> value	Odds ratio	CI 95% lower	CI 95% upper
CD4+—number of lymphocytes per mm <sup>2</sup> of tumor, melanoma						
< 300	9	33.3	0.087	1.0	—	—
300–479	6	50.0	0.364	2.0	0.45	8.94
480 +	15	66.7	<b>0.028</b>	4.0	1.16	13.82
CD8+—number of lymphocytes per mm <sup>2</sup> of tumor, melanoma						
< 886	10	30.0	<b>0.001</b>	1.0	—	—
886–1899	9	33.3	0.825	1.17	0.3	4.59
1900 +	11	90.9	<b>0.0001</b>	23.33	4.1	132.9
CD8+/CD4+ ratio, melanoma						
< 2	5	0.0	<b>0.006</b>	1.0	—	—
2–2.69	9	33.3	0.9	—	8.18	—
< 2.7	14	21.4	0.01	1.0	—	—
2.7 +	16	81.3	<b>0.0001</b>	15.89	4.48	56.35

Significant values are highlighted in bold



**Fig. 2** The area under the receiver operating characteristic (ROC) curve for response rate to anti-PD1 treatment and the presence of C4+ or CD8+ lymphocytes in tumor or CD8+/CD4+ ratio in melanoma patients. CD4\_LYMPH\_N—CD4+ number of lymphocytes per mm<sup>2</sup> of tumor. CD8\_LYMPH\_N—CD8+ number of lymphocytes per mm<sup>2</sup> of tumor. CD8\_4—CD8+/CD4+ ratio

between infiltration of the NSCLC tumor by CD8+ TILs and response to immunotherapy. Among patients with metastatic NSCLC, there were 12 cases with tumors

infiltrated by CD8+ lymphocytes count under 886/mm<sup>2</sup>. In this group, the average response rate to the therapy was low (16.7%, *p* = 0.046). When the CD8+ lymphocytes count was in the range of 886–1899/mm<sup>2</sup>, the response rate was high (60%, *p* = 0.017). However, a higher count of CD8+ did not predict response (Table 7, Fig. 2). The CD8+/CD4+ ratio was statistically significant in three categories of patients as a predictor of response. When the ratio was lower than 2, a very low response rate (13.3%) was found, and in ratios higher than 2, response rates ranged between 43 and 50% (*p* = 0.035) (Table 7, Fig. 2).

### Discussion

Immune checkpoint therapy has been the most important oncology breakthrough therapy in the past decade. Immunotherapy with anti-PDL1/anti-PD1 antibodies was examined and proved to be effective in almost every field in oncology, including melanoma, head and neck tumors, lung cancer and tumors of the digestive system and the urinary system. Identifying and developing specific biological markers that can predict response to immunotherapy and survival are important and challenging. The goal in searching for biomarkers is developing a clinical algorithm based on pathology parameters.

In many research studies, PD-L1 receptors in tumor tissue and in lymphocytes inside the tumor were examined. The level of the receptor expression was examined at different cutoff values of 1, 5, 10 and 50% to define positive immunohistochemical staining to PD-L1. Different cutoff values showed differences in rates of response to treatment,

**Table 4** Baseline demographic and disease characteristics of patients with NSCLC

Age at diagnosis (year)	
Median	62.5
Range	46–82
Gender, <i>n</i> (%)	
Male	19 (73)
Female	7 (27)
Histological type of the tumor, <i>n</i> (%)	
Adenocarcinoma	18 (69.2)
Squamous cell carcinoma	8 (30.8)
Stage of disease at diagnosis, <i>n</i> (%)	
I	2 (7.7)
II	1 (3.8)
III	8 (30.8)
IV	15 (57.7)
ECOG performance status in metastatic disease, <i>n</i> (%)	
0	5 (19.2)
1	16 (61.6)
2	5 (19.2)
Brain metastasis, <i>n</i> (%)	
Yes	3 (11.5)
No	23 (88.5)
Biopsy site, <i>n</i> (%)	
Lung	11 (42.3)
Lymph node	7 (26.9)
Liver	1 (3.8)
Bone	3 (11.5)
Other	4 (15.5)
EGFR status, <i>n</i> (%)	
Mutant	4 (15.4)
Wild	16 (61.6)
Unknown	6 (23)
EML4–ALK translocation, <i>n</i> (%)	
Positive	0 (0)
Negative	17 (65.4)
Unknown	9 (34.6)
Prior concurrent chemoradiotherapy, <i>n</i> (%)	
Yes	8 (30.8)
No	18 (69.2)
Prior systemic therapy, <i>n</i> (%)	
Yes (chemotherapy)	19 (73.1)
Yes (TKIs)	2 (7.7)
No	5 (19.2)
Type of anti-PD1 therapy <i>n</i> (%)	
Pembrolizumab	0 (0)
Nivolumab	26 (100)

ECOG Eastern Cooperative Oncology Group, EGFR epidermal growth factor receptor, EML4 echinoderm microtubule-associated protein-like 4, ALK anaplastic lymphoma kinase, TKIs tyrosine kinase inhibitors

**Table 5** Response to anti-PD1 therapy—NSCLC

Response to anti-PD1 therapy—NSCLC	Number of patients	% of patients
Complete response (CR)	2	7.7
Partial response (PR)	4	15.4
Stable disease (SD)	8	30.8
Progressive disease (PD)	12	46.1

but, in some studies, results were ambiguous [12, 24–26]. However, the research studies KEYNOTE-001 and KEYNOTE-010 showed significant predictive and prognostic importance of the number of cells with PD-L1 receptors in metastatic NSCLC tumor tissue, with PD-L1 expression in 50% or more of the cells. For treatment-naive patients, response to immunotherapy with pembrolizumab was 58.3% with a progression-free survival of 12.5 months and an overall survival of 60.6% for 2 years [14, 15].

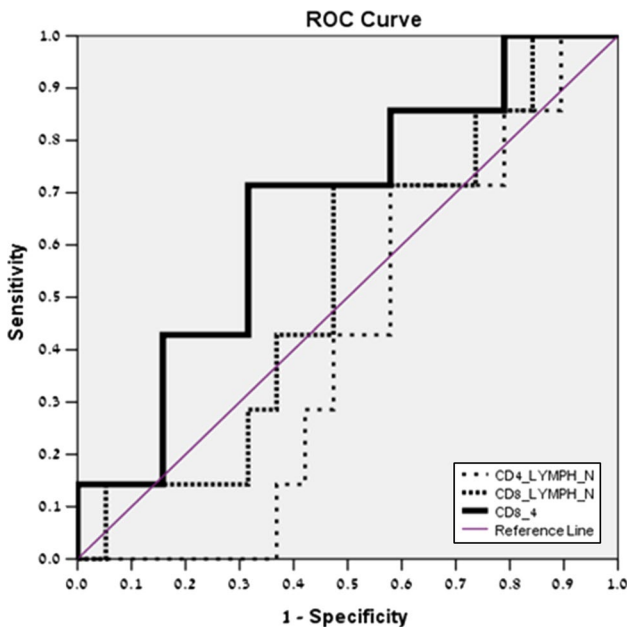
Another step in developing an algorithm based on biomarkers is probably the test for the presence of cytotoxic lymphocytes CD8+ and/or regulator lymphocytes (mostly CD4+). The presence of CD4+ cells is complicated for prediction, as it was recently reported that subtypes of lymphocytes that express CD4+, such as CD4+ Th1 subtype which secretes interleukin-2 and interferon gamma, have cytotoxic influence on tumor cells. However, subtype CD4+ Th17 secretes interleukin-4, interleukin-13 and interleukin-10 which speed up tumor progression [19]. In the current study, the level of CD8+ cells was more significant than CD4+ and had the most prediction power to the CD8/CD4 cells ratio.

In several studies, the influence of enzyme indoleamine 2,3-dioxygenase (IDO) on T cells function was examined. Overexpression of the enzyme inside the melanoma tumor caused suppression of CD8+ lymphocytes and natural killer (NK) cells and an increase in regulator cells function [13]. Several studies are currently ongoing with IDO inhibitor combined to other immunotherapy checkpoint inhibitors for metastatic melanoma treatment [27, 28], raising the importance of CD8+ TILs for immune response.

An additional biomarker that has potential to predict response to anti-PD1 treatment is the BCL-2-interacting mediator of cell death (BIM). This protein encourages interaction between the PD-1 and PD-L1 receptors. In a study where blood samples of patients suffering from metastatic melanoma were taken, the expression of BIM protein in peripheral CD8+/PD-1 T cells was examined, and a relation between overexpression of BIM protein and higher response to anti-PD1 treatment was found. The method is convenient, noninvasive and allows tracking the response

**Table 6** Relation between response rate to anti-PD1 treatment and the presence of CD4+ lymphocytes in tumor in NSCLC patients

CD4+—number of lymphocytes per mm <sup>2</sup> of tumor—NSCLC	Number of patients	Response rate (%)	<i>p</i> value	Odds ratio	CI 95% lower	CI 95% upper
< 300	9	22.2	0.302	1.00	–	–
300–479	7	42.9	0.218	2.63	0.57	12.18
480 +	10	20.0	0.867	0.88	0.18	4.17



**Fig. 3** The area under the receiver operating characteristic (ROC) curve—NSCLC. CD4\_LYMPH\_N—CD4+ number of lymphocytes per mm<sup>2</sup> of tumor. CD8\_LYMPH\_N—CD8+ number of lymphocytes per mm<sup>2</sup> of tumor. CD8\_4—CD8+/CD4+ ratio

to immunotherapy by PD-1 CD8+ T cells count and the expression of BIM protein in the blood sample of patients [29].

In the current study, among the melanoma patients, the presence of more than 1900/mm<sup>2</sup> of CD8+ lymphocytes in the tumor predicted response to therapy of 90%. CD8+/

CD4+ ratio lower than 2 predicted lack of response to the treatment (0%) (*p* = 0.006), while CD8+/CD4+ ratio higher than 2.7 showed a response rate of 81.3% (*p* = 0.0001). This new biomarker should be tested in a larger study. However, these results may help to identify the melanoma patients with low chances of responding to anti-PD1 treatment, such as patients with CD8+/CD4+ ratios lower than 2. In such cases, it may be possible to consider combined immunotherapy with anti-PD1 and anti-CTLA4 (nivolumab + ipilimumab), which has a higher response rate to immunotherapy and a higher overall survival [10].

Regarding metastatic NSCLC, a routine immunohistochemical test for PD-L1 receptors during the diagnosis of the metastatic disease is performed. If the expression rate of receptors is equal or higher than 50%, there is a place to consider pembrolizumab as first-line therapy [16]. In the lung cancer group, negative and positive predictive values for the CD8+/CD4+ ratio in response rates to anti-PD1 therapy were found. When the ratio of CD8+/CD4+ was lower than 2, a response rate of 13.3% (*p* = 0.046) was observed. A CD8+/CD4+ ratio higher than 2 predicted response rates of 43–50% (*p* = 0.038). In the group of lung cancer patients, there was no significant predictive meaning to the absolute count of lymphocytes CD4+ and CD8+ inside the tumor. It is possible that the ratio between the lymphocyte populations can help to predict higher chances of response in an advanced line of lung cancer therapy.

The main limitations of our research should be considered—retrospective staining of a relatively small number of samples. Therefore, the results should be treated as exploratory only and not as proven clinical results. We are currently

**Table 7** Relation between response rate to anti-PD1 treatment and the presence of CD8+ lymphocytes in tumor or CD8+/CD4+ ratio in NSCLC patients

	Number of patients	Response rate (%)	<i>p</i> value	Odds ratio	CI 95% lower	CI 95% upper
CD8+—number of lymphocytes per mm <sup>2</sup> of tumor—NSCLC						
< 886	12	16.7	<b>0.046</b>	1.0	–	–
886–1899	5	60.0	<b>0.017</b>	7.5	1.43	39.41
1900 +	9	22.2	0.65	1.43	0.31	6.7
CD8+/CD4+ ratio—NSCLC						
< 2	15	13.3	<b>0.046</b>	1.0	–	–
2–2.69	4	50.0	<b>0.035</b>	6.5	1.14	37.05
2.7 +	7	42.9	<b>0.038</b>	4.88	1.1	21.69

Significant values are highlighted in bold

gathering additional cases with two kinds of tumors to create a wider base for the results.

## Conclusion

The use of CD8+/CD4+ TILs ratio in tumor biopsies may predict response to anti-PD1 treatment in metastatic melanoma and NSCLC.

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## Compliance with ethical standards

**Conflict of interest** None.

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