Inflammatory and autoimmune predictive markers of response to anti-PD-1/PD-L1 therapy in NSCLC and melanoma

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Received May 8, 2022; Accepted June 17, 2022

DOI: 10.3892/etm.2022.11495

Abstract. Immune checkpoint inhibitors (ICI) are a standard in cancer therapy, but few patients respond to the treatment. The aim of the present study was the determination of immunological markers for monitoring response to ICI. The present study included 74 patients receiving ICI in subsequent [group 1; non-small cell lung cancer (NSCLC)] and first-line setting (group 2; melanoma) and 30 patients with NSCLC receiving first-line chemotherapy. In groups 1 and 2 β-2 microglobulin (B2-MG), neopterin (NPT), IL-6, IL-18, *HLA-DRB1* and autoantibodies were assessed after two months of ICI, and before the start of next administration in group 3. In group 1 low level of B2-MG (P<0.0001), NPT (P<0.0001), IL-6 (P<0.0001), IL-18 (P=0.0003), *HLA-DRB1*03* (P=0.016) and anti-TPO antibodies (P=0.016) were associated with response >six months. In group 2 high level of B2-MG (P=0.0001), NPT (P=0.0016), IL-6 (P=0.013) and IL-18 (P=0.032) were associated with early disease progression (<six months). Univariate analysis demonstrated that immune-related adverse events were predictive marker of prolonged progression-free survival (PFS) in group 1 (P=0.038) and 2 (P=0.020). Neutrophil-lymphocyte ratio ≥5 before immunotherapy was correlated with shorter PFS in melanoma in multivariate analysis (P=0.007). B2-MG ≥2.5 mg/ml (P=0.006) and NPT ≥12 nmol/l (P=0.027) were predictors of shorter PFS in group 1. B2-MG ≥2.5 mg/ml was predictor of shorter PFS (P=0.008) in group 2. In group 1 levels of B2-MG, NPT, IL-6 and IL-18 were higher than in group 3. In summary, immunological markers are promising predictive markers for immunotherapy; however, it requires further prospective studies.

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Key words: immune checkpoint inhibitors, immune-related adverse events, autoantibodies, HLA-DRB1, neutrophil-to-lymphocyte ratio, peripheral blood biomarker, β-2 microglobulin, neopterin, interleukin-6, interleukin-18

Introduction

Immune checkpoint inhibitors (ICI) demonstrate unprecedented results in the treatment of variety advanced solid tumors, such as non-small cell lung cancer (NSCLC) and melanoma (1,2). Immunotherapy has led to an advantage in progression-free survival (PFS) and overall survival (OS) over conventional chemotherapy; unfortunately, 60-70% of patients do not respond to ICI (3). Given the high cost of ICI and the high possibility of severe immune-related adverse events (irAEs), there is a growing need of immunotherapy predictive biomarkers, as well as biomarkers for monitoring response to the therapy.

PD-L1 expression on tumor and immune cells is a potential predictor of sensitivity to ICI and was initially approved by the FDA in 2015 (4). In the KEYNOTE-024 trial it was shown that patients with metastatic NSCLC with high PD-L1 expression (≥50%) had longer PFS and OS, when using anti-PD-1 antibodies compared with chemotherapy (4). However, PD-L1 expression is useful for only a few tumors: NSCLC, bladder cancer, gastric cancer, cervical cancer, head and neck squamous cell carcinoma, esophageal cancer and triple negative breast cancer (5). In addition, patients with low and negative PD-L1 expression also can response to the therapy (5). Thus, the main challenge is to find additional universal predictive markers to determine the indications for ICI in various malignant tumors.

Tumors with microsatellite instability (MSI) demonstrate high sensitivity to anti-PD-1 therapy regardless of a histological

type (6). MSI is the first approved indication for tissue-agnostic treatment (6). Another FDA-approved marker, which allows to select patients for ICI, is high tumor mutational burden (TMB-h) (7). However, low prevalence of MSI or TMB-h leads to limitations for ICI appointment for most of patients.

Clinical evaluation of predictive markers in tumors is limited by intratumoral heterogeneity and subsequent biopsies. Blood-based biomarkers are more accessible and could be used for non-invasive therapeutic monitoring of the treatment efficacy. In addition, blood could provide a holistic view on the patient's immune response, which is one of the key factors of the effectiveness of cancer immunotherapy (8).

Systemic inflammation causes tumor growth and progression and, as a result, is associated with poor survival in various types of cancer (3). Changes in ratios of peripheral blood biomarkers, for example, based on changes in the number of lymphocytes [neutrophil-lymphocyte (NLR) and platelet-lymphocyte ratio (PLR)] and level of cytokines can serve as a reflection of this process among patients with malignant tumors (8). In particular, a high level of NLR has been described as a predictor of poor survival regardless of treatment in various tumors (3). A number of studies have shown that immunological markers, such as NLR, PLR and IL-6, are also predictors of the effectiveness of ICI (8-10). The present study also suggested that peripheral markers of T-cell immune response activation [in particular, β-2 microglobulin (B2-MG) and IL-18], and markers of macrophages activation, the best known of which is neopterin (NPT), could be used for monitoring the response to ICI. As irAEs are associated with the response to ICI, the present study performed the determination of various autoantibodies, which could serve as early sign of autoimmune reactions during ICI, as well as the study of the most well-known gene in the mosaic of autoimmunity, human leukocyte antigen (*HLA*)-*DRB1*. The aim of the present study was to define novel immunological markers for monitoring the response to ICI in advanced NSCLC and melanoma (Fig. 1).

Materials and methods

Study population, treatment and response evaluation. The present retrospective study included 74 patients with advanced malignant tumors who received ICI (groups 1 and 2). The present study also included 30 patients with advanced NSCLC (aNSCLC), who received initial platinum chemotherapy (group 3).

The present study was conducted from September 2018 to July 2021 at Pavlov First Saint Petersburg State Medical University. Patients eligible for the study had to be >18 years old, histologically confirmed NSCLC or cutaneous melanoma, stage IIIC-IV for cutaneous melanoma and stage IIIB-IVB for NSCLC and Eastern Cooperative Oncology Group Performance Status (ECOG PS) 0, 1 or 2 (4) All patients with NSCLC and cutaneous melanoma were classified based on Tumor Node Metastasis staging and the American Joint Committee on Cancer (8th edition, 2017) (11). Patients who had a concomitant infection including human immunodeficiency virus or hepatitis, received systemic steroids, had previous immunotherapy, concomitant or previous radiotherapy and previous or ongoing autoimmune disease were excluded. Patient characteristics are summarized in Table I.

Radiological assessment was performed according to the criteria RECIST 1.1 (12). The primary endpoint for all groups was response rate over a six-month follow-up. Response was defined as the patient's disease control rate (DCR) in the course of treatment. This way, the responder subgroup comprised patients, who showed signs of clinical benefit within the first six months of treatment, which included a complete response (CR), a partial response (PR) and stable disease (SD). Objective response was defined as the cases with CR and PR. The non-responder group included every patient who discontinued treatment due to disease progression within the first six months of the treatment. Progression was defined as a measurable increase in tumor size according to the criteria RECIST 1.1 or a presence of new metastatic sites. The secondary endpoint for all groups was the assessment of PFS. OS has not been assessed due to the immaturity of the data. For group 1, the median follow-up was 7.8 months [interquartile range (IQR): 6.5-11.8 months]. For group 2, the median follow-up was 8.4 months (IQR: 6.1-9.7 months), for group 3, 7.6 months (IQR: 6.3-9.1 months).

For all of the patients a measurement of immunological markers in peripheral blood was performed. All serum samples were collected by venipuncture of arm and stored as minimum of 2 ml aliquots at -80°C until analysis at Pavlov First Saint Petersburg State Medical University as previously described (10). The present study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Pavlov First Saint Petersburg State Medical University (approval no. 246-2021). All participants signed informed consent forms.

Group 1 included 45 patients with locally advanced and metastatic NSCLC which were treated with ICI in subsequent-line setting: anti-PD-1 (nivolumab, pembrolizumab) or anti-PD-L1 therapy (atezolizumab; Table I). The patients had previously progressed on a platinum-based chemotherapy (n=40) or EGFR/ALK inhibitors (n=5). Group 1 was divided into subgroups based on the duration of response to ICI: responders (n=26), and non-responders (n=19).

Group 2 included 29 patients with unresectable locally advanced (stage IIIC and IIID) or metastatic melanoma who received first-line nivolumab as monotherapy. According to the results of radiological evaluation, the group was also divided into 2 subgroups: responders (n=22), and non-responders (n=7).

Measurement of tissue and serum markers. PD-L1 expression was assessed in paraffin tissue sections (4-μm thick) by the Dako PD-L1 IHC clone 22C3 pharmDx (Agilent Technologies, Inc.) and the Ventana PD-L1 IHC clone SP142 (Ventana Medical Systems, Inc.) assay according to manufacturer's instructions. NLR and PLR were also calculated before and after two months the start of the treatment in groups 1 and 2. All patients participated the study had no history of autoimmune diseases before starting ICI. Among patients from groups 1 and 2 level of thyroid-stimulating hormone was within the range 0.4-4.0 mU/l before starting anti-PD-1/PD-L1 therapy.

In groups 1 and 2 peripheral blood samples were taken after two months from the start of ICI. Blood samples were taken both before the start of the next cycle of ICI, and immediately after the completion of the ICI infusion. The last

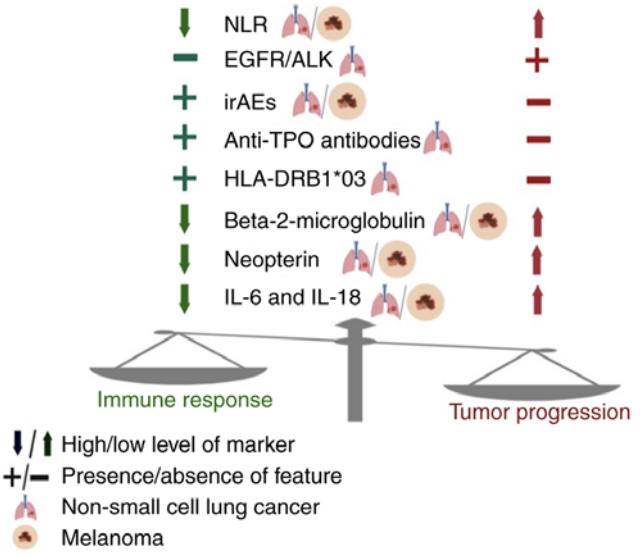

Figure 1. Graphical abstract. In advanced NSCLC and melanoma the following immunological and genetic features could predict response to immunotherapy: low level of NLR, presence of irAEs, high level of β-2 microglobulin, neopterin, IL-6 and IL-18. Absence of EGFR/ALK mutation, presence of anti-TPO autoantibodies and HLA-DRB1*03 are also predictors of response to anti-PD-(L)1 therapy in NSCLC. NLR, neutrophil-to-lymphocyte ratio; irAE, immune-related adverse event; anti-TPO-anti-thyroid peroxidase antibodies; IL-interleukin.

sampling point was chosen in order to assess the direct effect of ICI on cytokine levels. Due to retrospective nature of the study some patients from group 1 and all patients from group 2 did not have preserved in advance baseline blood samples that were taken before the initiation of ICI. In group 1, 16 patients also had additional points of taking serum samples: before the start of ICI and six months after the start of therapy. These points were used to study the possible dynamic changes in the level of markers. In group 3, peripheral blood was taken before the start of the infusion of three or subsequent cycles of the chemotherapy.

B2-microglobulin (B2-MG) was determined by an immunoturbidimetric method, using a test system manufactured by Biosystems S.A. Due to the fact that the level of B2-MG in the blood depends on the renal function, the value of serum creatinine was studied among patients from the three groups before the start and two months after treatment. The study of neopterin (NPT) was performed using enzyme-linked immunosorbent assay (ELISA) kit of IBL International GmbH (cat. no. RE59321) according to the manufacturer's instructions. The level of cytokines, IL-6 (cat. no. A8768) and IL-18 (cat. no. A8770) were determined by ELISA with test systems of the Vector-Best also according to the kit instructions.

The majority of autoantibodies were determined by ELISA using commercial test systems of Orgentec Diagnostika GmbH: antibodies to extractable nuclear antigens (cat. no. 416-5140), anticardiolipin antibodies (IgG and IgM; cat. no. 416-5150), anti-MCV antibodies (cat. no. 416-5480). Anti-thyroid peroxidase autoantibodies (anti-TPO) (cat. no. EA 1012-9601 G), antibodies to thyroid stimulating hormone receptor (cat. no. EA 1015-9601 G), antibodies to β-2-glycoprotein (cat. no. EA 1632-9601 G) were evaluated using commercial ELISA kits manufactured by Euroimmun Medizinische Labordiagnostika AG. Anti-neutrophil cytoplasmic antibodies IgG (cat. no. FA 1200-1005), antinuclear antibodies (diagnostic titer >1: 160) (cat. no. FA 1510-0010-1), anti-mitochondrial antibody, anti-liver kidney microsomal antibody and anti-smooth muscle antibody (cat. no. FA 1300-1005-8) were determined by indirect immunofluorescence using a commercial kit of Euroimmun Medizinische Labordiagnostika AG. The detection of the listed autoantibodies was carried out according to manufacturer's instructions.

Determination of allelic variant of the *HLA-DRB1* gene was performed by reverse transcription PCR using a commercial test system HLA-DNA-TECH (DNA-Technology LLC; cat. no. R1-H001-S3/5EU) also according to kit instructions.

Statistical analysis. Statistical data processing was performed using GraphPad Prism (version 9.3.1; GraphPad Software Inc.). Fisher's exact test was applied for the comparative analysis of qualitative characteristics. Evaluation of differences in quantitative parameters between two compared groups was performed using the Mann-Whitney U-test. Optimal cut-off values for immunological markers and the level of PD-L1 expression were determined using receiver operating characteristic curve (ROC) analysis for the subsequent study of PFS. Differences in PFS between two compared groups were analyzed using a log-rank test with hazard ratio analysis, as well as a graphical presentation using the Kaplan-Meier method. In group 1 and 2 univariate and multivariate Cox regression analysis were used to assess the effect of clinical, morphological data and immunological markers on PFS. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical and morphological parameters of ICI efficiency

NSCLC. There were no significant differences between responders and non-responders in sex (P=0.891), age (P=1.0), ECOG PS (P=1.0), smoking status (P=0.068), body mass index (BMI) (P=0.719), histological type (P=0.283), PD-L1 expression levels on tumor cells (P=0.152) and the presence of EGFR/ALK mutations (P= 0.146) in group 1. No differences were observed between responders and non-responders in NLR before (P=0.546) and two months after initiation of therapy (P=0.132), as well as PLR before (P=0.244) and two months after initiation (P=0.428).

Using ROC analysis optimal cut-off level of PD-L1 expression to determine efficacy of ICI was ≥50%. However, PD-L1 ≥50% was not significantly associated with longer PFS in univariate Cox regression analysis [hazard ratio (HR): 0.28, 95% confidence interval (95% CI) 0.04-0.99; P=0.091]. In univariate regression analysis, the presence of EGFR/ALK mutations (HR: 5.18, 95% CI 0.75-22.68; P=0.045) and NLR ≥5 (HR: 8.02, 95% CI 1.21-32.24, P=0.009) were associated with shorter PFS (Table II). In multivariate analysis, only the presence of EGFR/ALK mutations (HR: 8.13, 95% CI 1.13-64.97; P=0.018) was a predictor of short PFS (Table II).

Melanoma. No differences were observed between the responders and non-responders in age (P=0.811), sex (P=0.665), ECOG PS (P=1.0), disease stage (P=1.0), category M (P=0.690), level of serum LDH and PD-L1 expression

Table I. Clinical and epidemiological data of patients included in three groups.

Characteristics	Group 1 (n=45)	Group 2 (n=29)	Group 3 (n=30)
Sex, n (%)			
Male	30 (66.7)	16 (55.2)	21 (70.0)
Female	15 (33.3)	13 (44.8)	9 (30.0)
Age, median (IQR), n (%)	62 (59-69)	57 (53-62)	64 (59-70)
<60	25 (55.6)	14 (48.3)	12 (40.0)
>60	30 (44.4)	15 (51.7)	18 (60.0)
Histology, n (%)			
Squamous cell lung cancer	27 (60)	0 (0.0)	18 (60.0)
Adenocarcinoma of the lung	18 (40)	0 (0.0)	12 (40.0)
Cutaneous melanoma	-	29 (100)	-
Stage, n (%)			
Locally advanced	9 (20)	2 (6.9)	6 (20.0)
Metastatic	36 (80)	27 (93.1)	24 (80.0)
Disease progression within the first six months, n (%)			
Yes	19 (42.2)	7 (24.1)	17 (56.7)
No	26 (57.8)	22 (75.9)	13 (43.3)
Immunotherapy, n (%)			
Nivolumab	12 (26,6)	29 (100.0)	-
Pembrolizumab	30 (66,7)	0 (0.0)	-
Atezolizumab	3 (6,7)	0 (0.0)	-
Systemic therapy, n (%)			
First-line	0 (0,0)	29 (100.0)	-
Second-line	36 (80)	0 (0.0)	-
Third-line	9 (20)	0 (0.0)	-
First-line therapy, n (%)			
Chemotherapy	0 (0,0)		30 (100.0)
ALK inhibitors	36 (80)		0 (0.0)
EGFR inhibitors	9 (20)		0 (0.0)
Mutational status, n (%)			
EGFR$^+$	2 (4,4)	0 (0.0)	0 (0.0)
ALK$^+$	3 (6,7)	0 (0.0)	0 (0.0)
EGFR/ALK-	21 (46,7)	0 (0.0)	5 (16.7)
No data	19 (42,2	29 (100.0)	25 (83.3)
PD-L1 expression, n (%)			
<1%	16 (35,6)	9 (31.1)	7 (23.3)
1-49%	20 (44,4)	13 (44.8)	3 (10.0)
>50%	9 (20)	0 (0.0)	0 (0.0)
No data	0 (0,0)	7 (24.1)	20 (66.7)

IQR, interquartile range; ALK, anaplastic lymphoma kinase.

(P=0.792) in group 2. NLR ≥5 before initiation of ICI was significantly associated with progression of disease <six months (P=0.007). There were no significant differences between responders and non-responders in NLR after two months of starting ICI (P=0.068), as well as PLR before (P=0.922) and two months after initiation (P=0.546).

In melanoma optimal cut-off level of PD-L1 expression to determine efficacy of therapy was ≥5%. At the same time, the level of PD-L1 ≥5% was also not correlated with longer PFS in univariate Cox regression analysis (HR: 0.28, 95% CI 0.01-1.59; P=0.237) (Table III). Only NLR ≥5 before initiation of ICI was associated with short PFS when using univariate (HR: 8.95, 95% CI 2.45-32.67; P=0.0006), as well as multivariate regression analysis (HR: 7.93, 95% CI 1.80-40.91; P=0.007; Table III).

Autoimmune markers and ICI efficiency. In group 1 irAEs developed in 37.8% of cases. All cases were represented by

Table II. Univariate and multivariate regression analysis of clinical, morphological and immunological parameters associated with PFS in NSCLC.

	Univariate analysis		Multivariate analysis	
Characteristic	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥75 vs. <75 years)	1.41 (0.49-3.20)	0.332	-	-
Sex (male vs. female)	1.03 (0.36-2.65)	0.914	-	-
ECOG PS (0/1 vs. 2)	1.11 (0.56-2.49)	0.834	-	-
Smoking status (former/current vs. never)	1.53 (0.43-4.25)	0.452	-	-
BMI (≥25 vs. <25 kg/m^2)	0.85 (0.60-1.45)	0.623	-	-
Histology (non-squamous vs. squamous)	0.45 (0.16-1.17)	0.110	-	-
None vs. presence EGFR/ALK mutation	5.18 (0.75-22.68)	**0.045**	8.13 (1.13-64.97)	**0.018**
Level of PD-L1 expression (<50 vs. ≥50)	0.28 (0.04-0.99)	0.091	-	-
irAEs (presence vs. none)	2.88 (1.10-8.45)	**0.038**	3.46 (1.01-14.78)	0.064
NLR before initiation of therapy (<5 vs. ≥5)	8.02 (1.21-32.24)	**0.009**	8.36 (0.78-91.11)	0.068
B2-MG (≥2.5 vs. <2.5)	0.27 (0.09-0.69)	**0.009**	0.13 (0.03-0.40)	**0.006**
Neopterin (≥12 vs. <12)	0.23 (0.07-0.64)	**0.007**	0.35 (0.13-0.87)	**0.027**
IL-6 (≥10 vs. <10)	0.46 (0.18-1.16)	0.091	-	-
IL-18 (≥273 vs. <273)	0.23 (0.05-1.06)	0.056	-	-
Anti-TPO (none vs. presence)	0.31 (0.05-1.09)	0.118	-	-

Values in bold are significant. PFS, progression-free survival; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; BMI, body mass index; irAEs, immune-related adverse events; NLR, neutrophil-lymphocyte ratio; B2-MG, β-2 microglobulin; anti-TPO, antibodies to thyroperoxidase; HR, hazard ratio; 95%CI, 95% confidence interval.

1-2 grade and included the following diseases: Autoimmune thyroiditis (n=7), rash (n=4), hepatitis (n=3), pneumonitis (n=2), colitis (n=1). The presence of irAEs was associated with the duration of the response >6 months: 53.9% of cases (14/26) in responders compared with 15.8% (3/19) in non-responders (P=0.013). Univariate regression analysis showed that irAEs was associated with longer PFS (HR: 2.88, 95% CI 1.10-8.45; P=0.038). However, this relationship was not found in multivariate analysis (P=0.064).

In group 2 irAEs developed in 44.8% of cases and represented the following diseases: rash (n=6), autoimmune thyroiditis (n=5), hepatitis (n=1), pneumonitis (n=1). Only the case of hepatitis was represented by grade 3 of toxicity, the rest were grade 1-2. The appearance of irAEs during ICI was associated with prolonged PFS in univariate (HR: 4.72, 95% CI 1.42-21.36; P=0.020), but not in multivariate regression analysis (HR: 5.21, 95% CI 1.07-38.67; P=0.058).

Autoantibodies. Anti-TPO antibodies (according to the manufacturer's instructions the positive threshold value was >50 IU/ml) were detected in all cases of autoimmune thyroiditis (n=7) in group 1. The appearance of antibodies after two months from the start of ICI was significantly associated with a response ≥six months: in responders a presence of the marker was observed in 26.9% (7/26) of cases and in non-responders-0% (0/19) (P=0.016). Using univariate regression analysis, no association between anti-TPO antibodies and PFS was demonstrated in group 1 (P=0.118). In group 2, anti-TPO antibodies were also detected in all cases of autoimmune thyroiditis (n=5). No association was demonstrated between the appearance of anti-TPO antibodies and duration of response ≥6 months and also PFS (P>0.05).

Diagnostic titer of antinuclear antibodies and antibodies to extractable nuclear antigens was detected in all cases of immune-related hepatitis in group 1 (n=3) and in group 2 (n=1). In group 1 and 2 none of studied autoantibodies was detected in other immune-related adverse events, such as rash, pneumonitis and colitis.

In group 1 and 2 there was no statistically significant association between the duration of response to anti-PD-1/PD-L1 therapy, PFS and the presence of one of the following autoantibodies: Antinuclear antibodies, antibodies to extractable nuclear antigens, anticardiolipin antibodies, anti-MCV antibodies, antibodies to thyroid stimulating hormone receptor, antibodies to β-2-glycoprotein, anti-neutrophil cytoplasmic antibodies, anti-mitochondrial antibody, anti-liver kidney microsomal antibody and anti-smooth muscle antibody (P>0,05).

HLA-DRB1. The *HLA-DRB1*03* genotype was associated with response to therapy among patients in group 1: 26.9% of cases (7/26) were responders, 0% (0/19) non-responders (P=0.016). At the same time, six patients with the *HLA-DRB1*03* in group 1 had a partial response after six months from the start of therapy, and one patient had a complete response. Among patients with the *HLA-DRB1*03* PFS was statistically significantly longer compared with patients with other variants of alleles of the *HLA-DRB1* gene in group 1: median was not reached compared with 224 days, respectively (HR=3.6; 95% CI 1.2-11.2; P=0.0276; Fig. 2). However, no association

Table III. Univariate and multivariate regression analysis of clinical, morphological and immunological parameters associated with PFS in melanoma.

	Univariate analysis		Multivariate analysis	
Characteristic	HR (95% CI)	P-value	HR (95% CI)	P-value
Melanoma (group 2)				
Age (≥65 vs. <65)	0.78 (0.11-4.78)	0.792	-	-
Sex (male vs. female)	0.82 (0.04-9.58)	0.873	-	-
ECOG PS (0/1 vs. 2)	2.60 (0.51-11.36)	0.209	-	-
Disease stage (III vs. IV)	0.87 (0.11-5.68)	0.882	-	-
Category M (M1a-b vs. M1c)	0.43 (0.02-2.72)	0.443	-	-
Serum LDH (elevated vs. normal)	1.95 (0.55-9.43)	0.343	-	-
Level of PD-L1 expression (≤5 vs. >5)	0.28 (0.01-1.59)	0.237	-	-
irAEs (presence vs. none)	4.72 (1.42-21.36)	**0.020**	5.21 (1.07-38.67)	0.058
NLR before initiation of therapy (<5 vs. ≥5)	8.95 (2.45-32.67)	**0.0006**	7.93 (1.80-40.91)	**0.007**
B2-MG (≥2.5 vs. <2.5)	0.10 (0.02-0.39)	**0.003**	0.09 (0.01-0.44)	**0.008**
NPT (≥12 vs. <12)	0.62 (0.14-1.21)	0.184	-	-
IL-6 (≥10 vs. <10)	0.25 (0.07-0.84)	**0.015**	0.57 (0.31-1.67)	0.516
IL-18 (≥273 vs. <273)	1.69 (0.47-8.31)	0.459	-	-
Anti-TPO (none vs. presence)	0.21 (0.01-1,07)	0.133	-	-

Values in bold are significant. PFS, progression-free survival; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; BMI, body mass index; irAEs, immune-related adverse events; NLR, neutrophil-lymphocyte ratio; B2-MG, β-2 microglobulin; NPT, neopterin; anti-TPO, antibodies to thyroperoxidase; HR, hazard ratio; 95%CI, 95% confidence interval.

was observed between this genotype and PFS when using univariate Cox regression analysis (P>0.05).

Immunological markers and ICI efficiency

B2-MG. Among patients with aNSCLC receiving ICI, a level of B2-MG after two months was significantly lower in responders compared with non-responders in group 1: Median was 1.7 mg/l (95% CI 1.6-2.3 mg/l) compared with 2.9 mg/l (95% CI 2.5-3.3 mg/l), respectively (P<0,0001; Fig. 3A). There were no significant differences between cases with objective response and SD (P=0.284). In group 1, in patients with B2-MG ≥2.5 mg/l, PFS was lower than among patients with a level of less than 2.5 mg/l: Median was 168 days and not reached, respectively (HR 2.8; 95% CI 1.2-6.9; P=0.017; Fig. 3C). Also, B2-MG ≥2.5 mg/ml was associated with shorter PFS in univariate (HR: 0.27, 95% CI 0.09-0.69; P=0.009) and multivariate regression analysis (HR: 0.13, 95% CI 0.03-0.40; P=0.006; Table II).

In 16 patients with NSCLC receiving ICI no association was observed between pretreatment level of B2-MG and PFS (P=0.805). The change in B2-MG from baseline to measurements after two months was associated with PFS. In 16 patients increased level of marker during two months of treatment >2.7 times was associated with short PFS: Median was 319 days compared with not reached, respectively (P=0.021). Also in group 1, 16 patients with NSCLC median level of the marker after two and six months were comparable (P=0.912).

B2-MG was also statistically significantly lower in responders than in non-responders in group 2: median was 1.8 mg/l (95% CI 1.6-2.3 mg/l) compared with 3.6 mg/l (95% CI 3.1-4.0 mg/l), respectively (P=0.0001) (Fig. 3B).

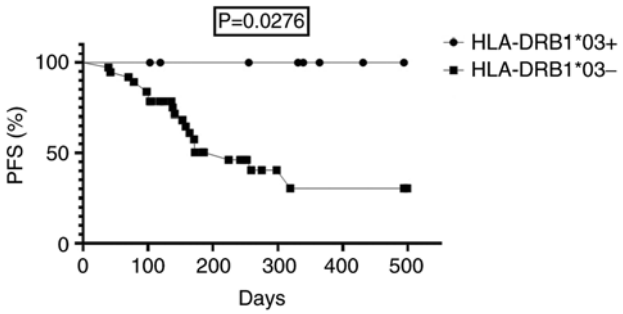

Figure 2. PFS depending on the presence of the *HLA-DRB1*03* in group 1. PFS, progression-free survival.

There were no significant differences between the cases with objective response and SD (P=0.094). Among patients with melanoma with B2-MG ≥2.5 mg/l PFS was shorter than among patients with the level of the marker <2.5 mg/l: median was 178 days and not reached, respectively (HR 10.0; 95% CI 2.9-34.4; P=0.0002) (Fig. 3D). B2-MG ≥2.5 mg/l was associated with shorter PFS in both univariate (HR: 0.10, 95% CI 0.02-0.39; P=0.003) and multivariate analysis (HR: 0.09, 95% CI 0.01-0.44; P=0.008).

Among patients from groups 1 and 2 creatinine corresponded to the reference value both before the start and after two months of ICI. This indicates the absence of effect of renal function on the level of the marker in two groups.

NPT. Median of NPT was lower in responders compared with non-responders in group 1: 8.6 nmol/l (95 % CI 7.6-10.0 nmol/l)

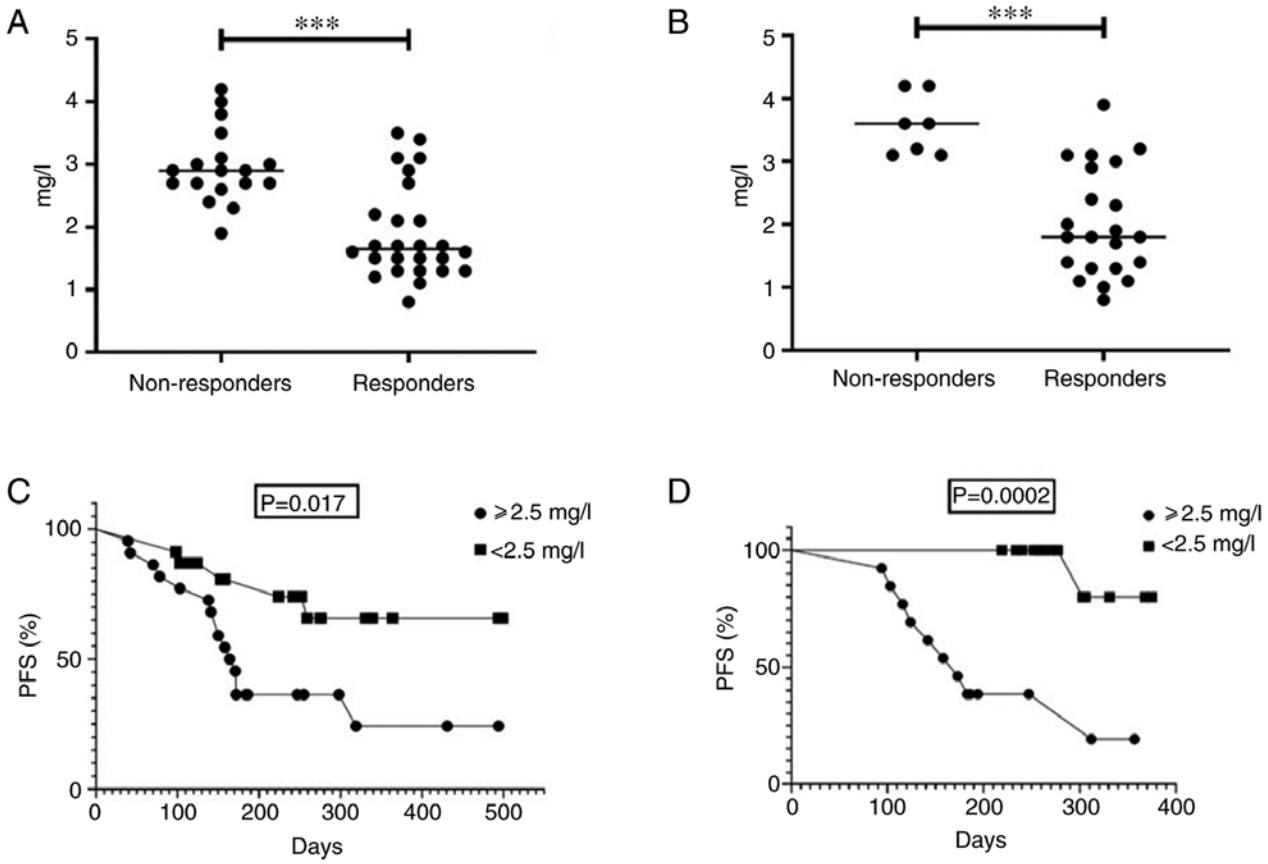

Figure 3. B2-MG among patients with advanced non-small cell lung cancer and melanoma received immune checkpoint inhibitors. (A-C) Median of B2-MG in responders and non-responders in (A) group 1 and in (B) group 2. PFS depending on the level of B2-MG in (C) group 1 and (D) group 2. B2-MG, β-2 microglobulin; PFS, progression-free survival; ***P<0.001.

compared with 13.4 nmol/l (95% CI 13.0-23.0 nmol/l), respectively (P<0.0001; Fig. 4A). There was also no significant difference in the level of the marker between patients with objective response and SD (P=0.151). Among patients with aNSCLC with NPT ≥12 nmol/l PFS was significantly lower than among patients with NPT <12 nmol/l: median was 164 days compared with not reached, respectively (HR=4.8; 95% CI 1.9-12.3; P=0.0007) (Fig. 4C). NPT ≥12 nmol/l was associated with shorter PFS in both univariate (HR: 0.23, 95% CI 0.07-0.64; P=0.007) and multivariate analysis (HR: 0.35, 95% CI 0.13-0.87; P=0.027).

In group 1 in 16 patients with NSCLC high baseline level of NPT (defined as >6.8 nmol/l) was associated with short PFS: Median was 224 days compared with not reached (P=0.0018). No association was observed between the change in NPT from baseline to measurements after two months of therapy initiation and PFS (P=0.067). Also, 16 patients demonstrated no differences in the level of the marker at two control points: after two and six months of the start of ICI (P=0.736).

There was a statistically significant difference of the level of NPT in responders and non-responders in group 2: Median was 8.7 nmol/l (95% CI 7.6-10.3 nmol/l) compared with 14.2 nmol/l (95% CI 10.9-17.0 nmol/l), respectively (P=0.0016) (Fig. 4B). There was also no significant difference in the level of the marker between patients with objective response and SD (P=0.151). However, there was no statistically significant relationship between NPT ≥12 nmol/l and shorter PFS while using log-rank test (HR: 2.88, 95% CI 0.86-9.64; P=0.052) and univariate Cox regression analysis (HR: 0.62, 95% CI 0.14-1.21; P=0.184).

IL-6. A level of IL-6 after two months of the start of ICI was lower in responders compared with non-responders in group 1: Median was 3.9 pg/ml (95% CI 2.8-5.0 pg/ml) compared with 14 pg/ml (95% CI 6.0-18.4 pg/ml), respectively (P<0.0001) (Fig. 5A). There were no statistically significant differences between patients with objective response and SD (P=0.217). Using univariate regression analysis there was no significant association between a level of IL-6 and PFS (P=0.091).

In group 1 in 16 patients with NSCLC no significant association was observed between lower IL-6 at baseline (P=0.209), decreasing the level of the marker during first two months of ICI (P=0.091) and PFS. Also, levels of the marker were comparable after two and six months in 16 patients in group 1 (P=0.334).

A level of IL-6 after two months was lower in responders compared with non-responders in group 2: Median was 1.8 pg/ml (95% CI 1.8-5.0 pg/ml) and 6.7 pg/ml (95% CI 4.2-12.9 pg/ml), respectively (P=0.013; Fig. 5B). There were no differences in the level of the marker among patients with objective response and SD (P=0.891). Among patients with melanoma with a level of IL-6 ≥10 pg/ml PFS was lower than among patients with IL-6 <10 pg/ml: median was 133 and 312 days, respectively (HR=4.9; 95% CI 0.5-49.7; P=0.006; Fig. 5C). A level of IL-6 ≥10 pg/ml

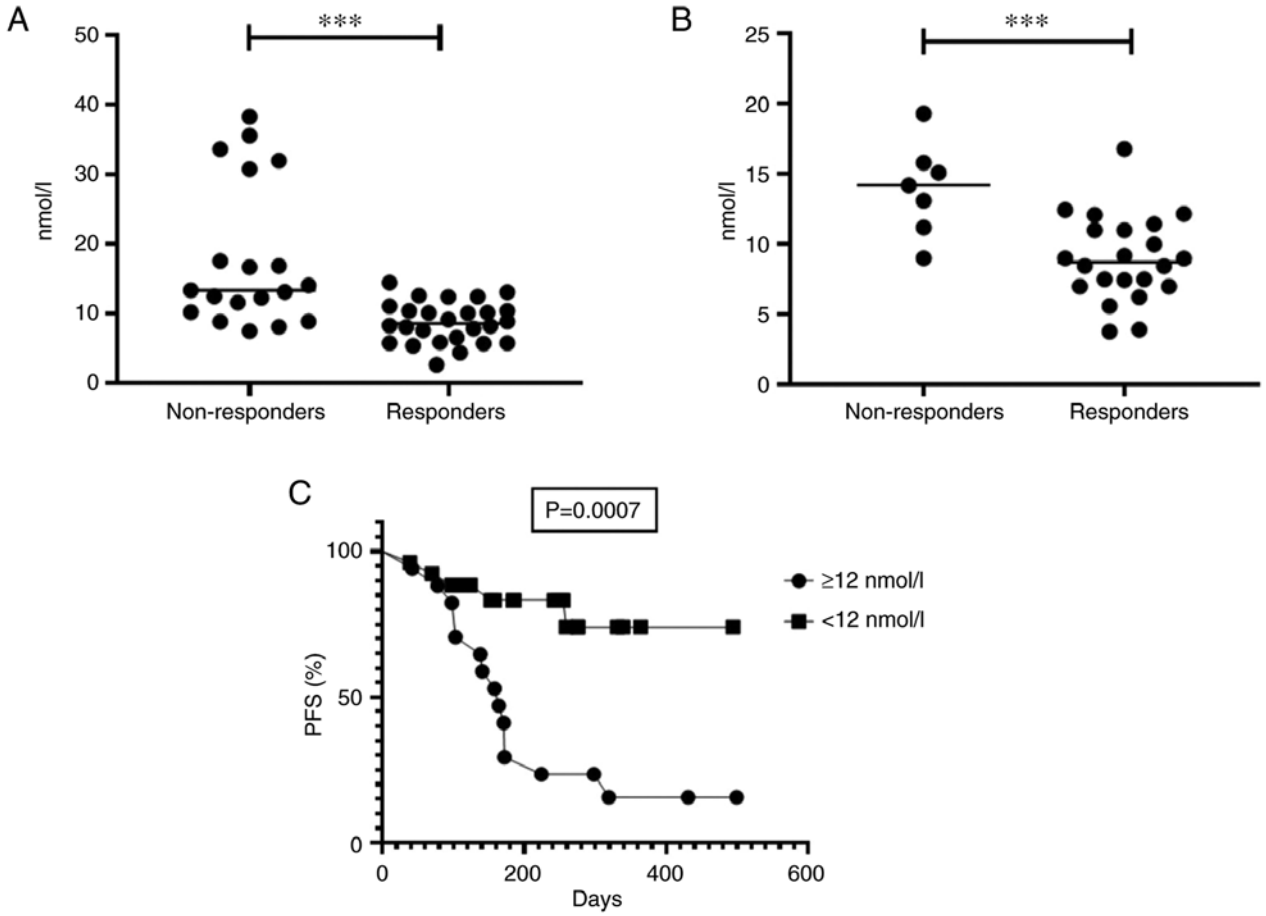

Figure 4. NPT among patients with advanced non-small cell lung cancer and melanoma received immune checkpoint inhibitors. Median of NPT in responders and non-responders in (A) group 1 and (B) group 2. (C) PFS depending on the level of NPT in group 1. NPT, neopterin; PFS, progression-free survival; ***P<0.001.

after two months of initiating ICI was associated with low PFS in univariate Cox regression analysis (HR: 0.25, 95% CI 0.07-0.84; P=0.015), but not in multivariate analysis (HR: 0.57, 95% CI 0.31-1.67; P=0.516).

IL-18. The level of IL-18 in responders was significantly lower compared with non-responders in group 1: Median was 233.3 pg/ml (95% CI 198.9-271.8 pg/ml) compared with 327.4 pg/ml (95% CI 300.5-405.5 pg/ml), respectively (P=0.0003; Fig. 6A). There was no significant difference in the level of this marker among patients with objective response and SD in group 1 (P=0.432). Using univariate analysis, the relationship between the level of IL-18 and PFS has not been demonstrated (P=0.056).

In 16 patients with NSCLC receiving ICI no association was observed between the baseline level of IL-18 (P=0.641), change in the level of the marker during two months of treatment (P=0.067) and PFS. As for IL-6, 16 patients had similar levels of IL-18 at two and six months after therapy initiation (P=1.0).

The level of IL-18 was significantly lower in responders compared with non-responders in group 2: Median was 206.3 pg/ml (95% CI 183.0-287.1 pg/ml) and 314.0 pg/ml (95% CI 222.0-347.7 pg/ml), respectively (P=0.032) (Fig. 6B). Using univariate analysis, no association between a level of IL-18 and PFS was demonstrated (P=0.459).

Immunological markers in chemotherapy and ICI. In group 3 there were no differences between responders and non-responders in sex (P=1.0), age (P=0.411), ECOG PS (P=0.892), smoking status (P=0.384), body mass index (P=1.0), histological type (P=0.460) and stage of disease (P=1.0), NLR before (P=0.196) and two months after initiation of therapy (P=0.104), as well as PLR before (P=0.627) and two months after initiation (P=0.359). Also, there were no differences between two subgroups in B2-MG (P=1.0), NPT (P=0.233), IL-6 (P=0.893) and IL-18 (P=1.0). None of the studied autoantibodies was found during the treatment. For the group 3, no statistical significance was observed between any of allelic variants of the *HLA-DRB1* and the duration of the response to the chemotherapy (P>0,05). In univariate analysis no association between clinical and laboratory parameters and PFS was shown (Table IV).

Group 3 allowed the assessment of the specificity of changes in immunological parameters in relation to ICI in groups 1 and 2. Measurement of these markers in group 3 could help to indirectly assess the level of markers before the start of ICI among patients in group 1 who previously had received chemotherapy. Group 1 and group 3 did not statistically differ in sex (P=0.806), age (P=0.655), histology (P=1.0) and TNM stage (P=1.0).

Among patients from group 1, previously treated with platinum-containing chemotherapy, a level of B2-MG after

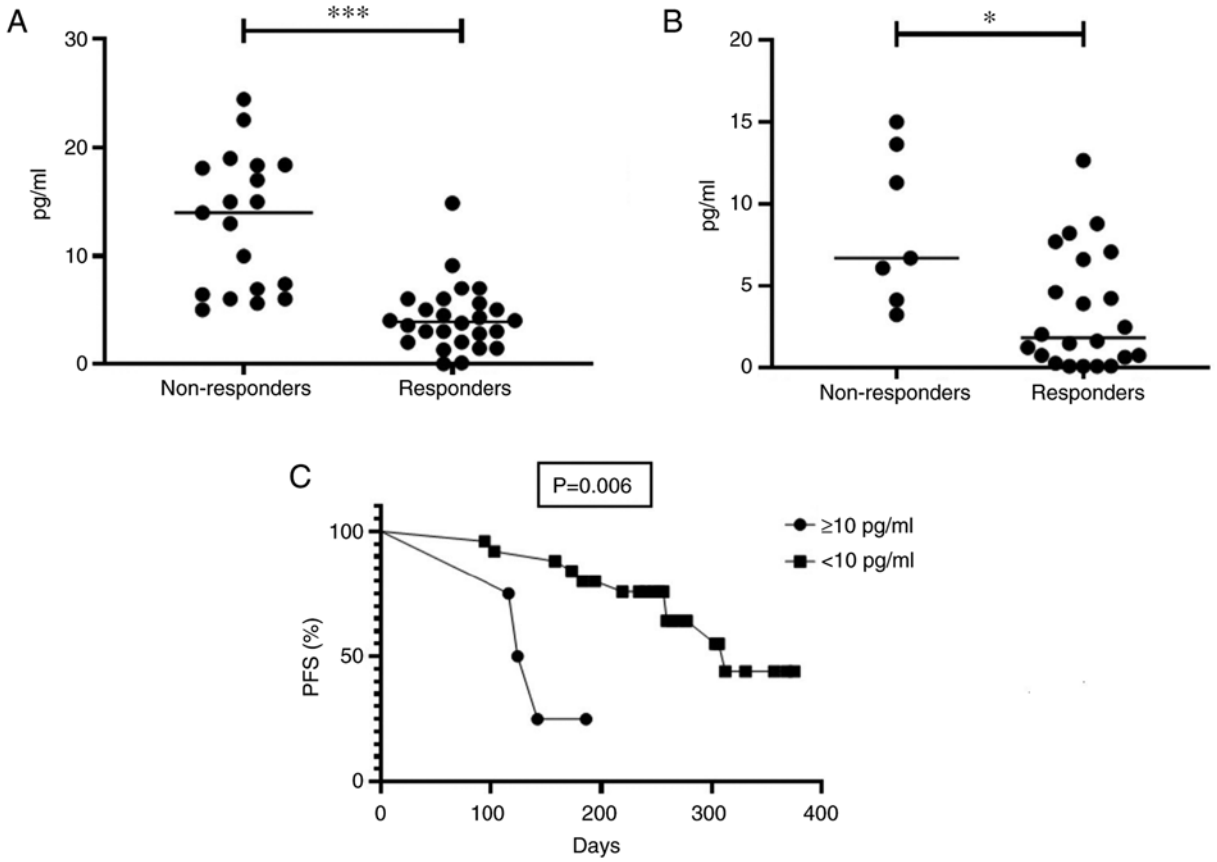

Figure 5. Median level of IL-6 in responders and non-responders in (A) group 1 and (B) group 2. (C) PFS depending on the level of IL-6 after two months in group 2. PFS, progression-free survival; *P<0.05; ***P<0.001.

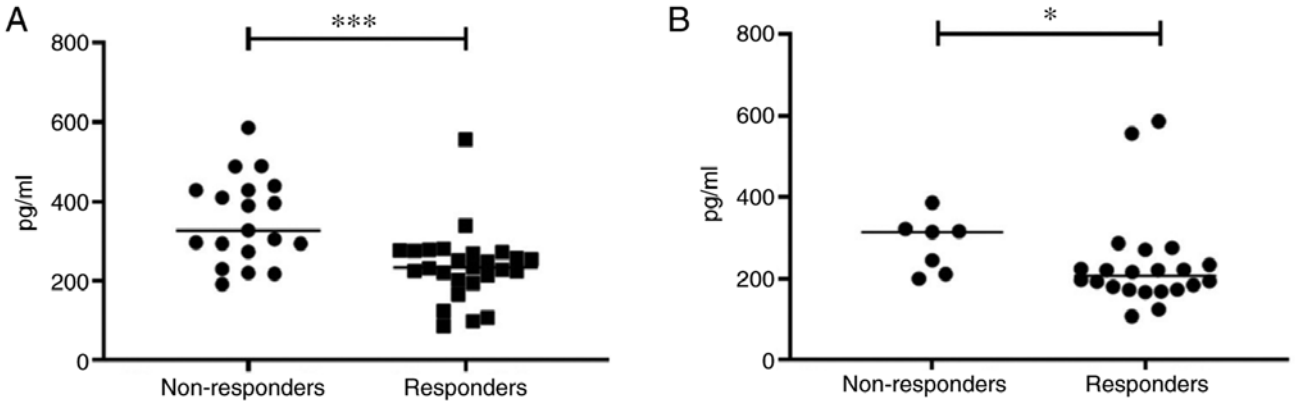

Figure 6. Median level of IL-18 in responders and non-responders in in (A) group 1 and (B) group 2; *P<0.05; ***P<0.001.

the initiation of ICI was significantly higher compared with patients from group 3 who received chemotherapy: Median was 2.1 mg/l (95% CI 2.0-2.5 mg/l) compared with 1.1 mg/l (95% CI 1.0-1.2 mg/l), respectively (P<0.0001). During the study of another inflammatory marker NPT, a significant difference was also demonstrated among patients from group 1 and group 3: median was 9.8 nmol/l (95% CI 10.4-14.3 nmol/l) and 6.2 nmol/l (95% CI 5.7-7.5 nmol/l), respectively (P<0.0001). A statistically significant difference in the level of IL-6 and IL-18 was observed among patients with aNSCLC, receiving ICI and chemotherapy (P<0.0001).

For 16 patients from group 1, median level of B2-MG and NPT evaluated before the start of ICI was 1.2 mg/l (95% CI 1.0-1.4 mg/l) and 5.9 nmol/l (95% CI 5.2-6.9 nmol/l), respectively. These values were comparable to the same in group 3. In 16 patients median of IL-6 before the start of ICI was similar to level of the marker in the comparison group receiving platinum-based chemotherapy in group 1: 1.9 pg/ml (95% CI 1.4-2.6) and 2.0 pg/ml (95% CI 1.7-3.0), respectively. Similar values of IL-18 were shown in two groups: ICI (group 1)-158.4 pg/ml (95% CI 141.3-169.8 pg/ml), chemotherapy (group 3)-165.5 pg/ml (95% CI 142.9-181.3 pg/ml).

Table IV. Univariate regression analysis of clinical, morphological and immunological markers associated with PFS.

	Univariate analysis	
Characteristics	HR (95% CI)	P-value
Age (≥75 vs. <75)	0.83 (0.48-1.66)	0.485
Sex (male vs. female)	0.97 (0.92-1.18)	0.672
ECOG PS (0/1 vs. 2)	1.32 (0.83-1.92)	0.213
Smoking status (former/current vs. never)	1.24 (0.91-1.71)	0.153
Histology (non-squamous vs. squamous)	0.91 (0.85-1.83)	0.532
NLR before initiation of therapy (<3 vs. ≥3)	1.26 (0.85-1.93)	0.196
NPT (≥10 vs. <10)	0.61 (0.42-1.19)	0.145

PFS, progression-free survival; ECOG PS, Eastern Cooperative Oncology Group Performance Status; NLR, neutrophil-lymphocyte ratio; NPT, neopterin.

Discussion

The present study demonstrated a predictive role of some markers of chronic inflammation in NSCLC and cutaneous melanoma for the first time, to the best of the authors' knowledge. It has shown that NLR, B2-MG, NPT, IL-6 and IL-18 were associated with response to ICI. An association between autoimmune reactions and the effectiveness of ICI was also demonstrated.

According to the study of potential predictive tumor markers, only the presence of EGFR/ALK mutations was an independent predictor of shorter PFS among patients with NSCLC. In the present study, no relationship was found between PD-L1 expression and response to therapy in NSCLC and melanoma.

ICI suppresses negative co-stimulatory signals of T cells, thereby enhancing the antitumor response (13). ICI can also activate autoreactive T cells through the immunostimulating mechanism. This, in turn, can lead to impairment of tolerance of T cells to autoantigens and to the activation of autoreactive B cells. This ultimately leads to the formation of autoantibodies that are early predictors of irAEs (13). In the present study, the appearance of irAEs among patients with NSCLC and melanoma was associated with longer PFS only in univariate analysis. Hussaini *et al* (14) demonstrated in meta-analysis that irAEs were independent predictors of increased survival regardless of tumor type and the type of ICI. The present study also found that the presence of a diagnostic titer of anti-TPO antibodies was associated with the response to the therapy only among patients with NSCLC during ICI, while the generation of the autoantibodies was not observed in non-responders. However, no relationship was shown between anti-TPO antibodies and PFS among patients with NSCLC and melanoma. Music *et al* (15) also showed that an increase in the level of anti-TPO antibodies before starting the 3rd cycle of therapy was associated with an increase OS among patients receiving pembrolizumab.

Another autoimmune marker of ICI efficacy is poorly studied allelic variants of gene HLA class II *HLA-DRB1*. HLA class II molecules play a role in cross-priming, T and B cell modulation, and the development of autoimmunity (16). The present study, for the first time to the best of the authors' knowledge, showed the relationship between the *HLA-DRB1*03* allele and the response to ICI. The frequency of *HLA-DRB1*03* allele in patients with NSCLC receiving ICI was 26.9%. The similar prevalence of the allele in patients with NSCLC receiving chemotherapy was 23,3% (7/30). Kapustin *et al* (17) analyzed 200 healthy individuals from the same region and showed that the frequency of the *HLA-DRB1*03* allele in general population was 16.0%.

Among patients with aNSCLC receiving ICI, *HLA-DRB1*03* was associated with response duration ≥6 months compared with other allelic variants of the gene. All patients with *HLA-DRB1*03* achieved objective response within six months. Moreover, *HLA-DRB1*03* was associated with longer PFS in the log-rank test, but not in Cox regression analysis.

NLR is a convenient blood marker of systemic inflammation that reflects the balance of the antitumor immune response (18). Traditionally, neutrophils demonstrate tumor-promoting activity in the tumor microenvironment, while lymphocytes are effective suppressors of tumor growth (18). An increase of level of NLR indicates a high level of absolute neutrophil count and/or a low level of absolute lymphocyte count and, as a result, a decrease in the antitumor response (19). The present study found that a high level of NLR, measured before starting ICI, was associated with shorter PFS among patients with NSCLC in univariate analysis and among patients with melanoma in multivariate analysis. Similar results are demonstrated in a number of other studies (18-20).

B2-MG is a non-glycosylated protein that is a component of HLA class I (21). After release from the cell surface, B2-MG is separated from the complex and circulates as a monomer (21). Serum levels of B2-MG are markers of cellular activation of the immune system, as well as markers of poor prognosis in certain lymphoproliferative disorders such as multiple myeloma (22). The present study showed that B2-MG could be used as a marker for monitoring response to anti-PD-(L)1 therapy. Responders with NSCLC and melanoma, who received first-line and subsequent ICI, had significantly lower B2-MG levels than non-responders. At the same time, no differences were observed in the level of B2-MG among patients with objective response and SD. It was shown that a high level of B2-MG (≥2.5 mg/l) among patients

with aNSCLC and cutaneous melanoma that received ICI was associated with shorter PFS in multivariate analysis. Also, in 16 patients with NSCLC increased level of the marker during ICI was associated with short PFS. A possible explanation of the relationship between B2-MG and the resistance to ICI is that high concentrations of serum B2-MG *in vitro* lead to a decrease in the antigen-presenting ability, as well as to inhibition of the T-cell immune response (23). Also, high protein levels lead to retardation in the differentiation of monocytes into functional dendritic cells (23). Moreover, it has been shown that high concentrations of B2-MG can also promote tumor growth and survival by increasing the production of cytokines such as IL-6 and IL-10 by monocytes (23).

NPT is a marker of macrophage activation (24). In malignant tumors, NPT production is a result of chronic stimulation of macrophages and reflects an inability of immune surveillance to inhibit tumor growth (25). In the present study the possibility of using NPT was investigated for predicting response to ICI. NPT was significantly lower in responders with NSCLC and melanoma, who received ICI, than in non-responders. At the same time, the level of the marker did not differ in objective response and stable disease. In 16 patients with NSCLC the high baseline level of the marker during treatment was associated with short PFS. Also, a high level of NPT (≥12 nmol/l) after two months was independent predictor of short PFS only among patients with aNSCLC, receiving subsequent-line ICI monotherapy.

Cytokines are potential markers of response to ICI. IL-6 is one of the most promising predictive cytokine markers. A high level of IL-6 production is associated with the formation of an immunosuppressive tumor microenvironment due to myeloid-derived suppressor cells, M2 macrophage cells and Treg cells (26). Also, IL-6 is responsible for the immune evasion by tumor cells via enhancing of PD-L1 expression (26). The present study found that level of IL-6 after two months of initiating ICI was statistically significantly higher in responders with NSCLC and melanoma that received ICI, than in non-responders. No differences were observed in the level of IL-6 between cases of objective response and stable disease. Also, a high level of IL-6 among patients with melanoma was associated with short PFS only in univariate analysis. Laino *et al* (27) analyzed CheckMate-064, 066 and 067 studies and found that high IL-6 levels were associated with poor response and shorter survival among patients with melanoma treated with nivolumab.

A similar association was shown between a high level of IL-18 during ICI and early disease progression among patients with NSCLC and melanoma who received ICI. However, no association was shown between a high level of the marker and PFS in both groups of patients. Although preclinical and some clinical studies suggest that IL-18 has antitumor activity, other studies shown that IL-18 plays a dual role in tumors, as it can exhibit pro-invasive and pro-angiogenic activity in various tumors (28-30). In a recent retrospective study of patients with aNSCLC, Wang *et al* (29) demonstrated that a high level of IL-18 before starting ICI was associated with response to the therapy. However, the same study noted a decreased level of IL-18 among patients who achieved a partial response (29). A possible explanation for the data obtained in the present study, as well as in the work of Wang *et al* (29) is that IL-18 produced by tumor cells reduces the antimetastatic activity of NK cells in a PD-1-dependent manner (30).

A limitation of the present study was the absence of an index that included some of the studied markers, which allows the most accurate determination of patients who will respond to immunotherapy. Different combination of predictive markers before initiation of treatment and also during immunotherapy did not show greater statistically significance by univariate Cox regression analysis than using single markers.

Despite the fact that the dynamic changes in B2-MG, NPT, IL-6 and IL-18 were not assessed among all patients who received ICI before the start and two months after initiation of the treatment, these markers significantly change only during immunotherapy. It was shown that levels of B2-MG, NPT, IL-6 and IL-18 were higher among patients with aNSCLC who received ICI (regardless of response or progression) than among patients with the same disease who received chemotherapy. Moreover, the levels of these markers in 16 patients with aNSCLC before initiating ICI were similar to those among patients receiving chemotherapy. Thus, a platinum-based chemotherapy does not affect the level of the inflammation markers. Also, 16 patients showed no significant changes in differences of B2-MG, NPT, IL-6, IL-18 after two and six months of starting ICI.

The use of immunological markers, such as NLR, B2-MG, NPT, IL-6, IL-18, *HLA-BRB1*, anti-TPO antibodies, could determine the efficacy of ICI among patients with NSCLC and melanoma. However, further prospective study is warranted.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AAM was responsible for development of research design, review of publications on the topic of the article, analysis of the data obtained, design of illustrative material, statistical analysis and article writing. SVL was responsible for development of research design, methodology and analysis of the data obtained. MAU, SVOd, IVC and AMU were involved in curation of patients, performed experiments and curated data. ALA and SVOr were responsible for idea and design development, scientific editing, and research management. AAM and SVOr confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the Pavlov First Saint Petersburg State Medical University (approval no. 246-2021). All participants signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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