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# Role of tumor-infiltrating immune cells and HLA expression in influencing tumor progression and the effectiveness of anticancer treatment

**PhD thesis**


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**List of Abbreviations**

| | |
|---|---|
| AE | adverse event |
| AJCC | American Joint Committee on Cancer |
| ALM | acrolentiginous melanoma |
| APC | antigen presenting cell |
| APM | antigen processing machinery |
| B2M | beta 2 microglobulin |
| BOR | best overall response |
| BRAF | B-Raf and v-Raf murine sarcoma viral oncogene homolog B |
| CAF | cancer-associated fibroblast |
| CD | cluster of differentiation |
| CDK4 | cyclin-dependent kinase 4 |
| CPS | combined positive score |
| CR | complete remission |
| CT | computer tomography |
| CTL | cytotoxic T lymphocyte |
| CTLA-4 | cytotoxic T-lymphocyte associated protein 4 |
| ECOG | Eastern Cooperative Oncology Group |
| FDA | Food and Drug Administration |
| FOXP3 | forkhead box P3 |
| gp100 | glycoprotein 100 |
| $H_2O_2$ | hydrogen peroxide |
| HLA | human leukocyte antigen |
| HMB-45 | human melanoma black 45 |
| HR | hazard ratio |

| | |
|---|---|
| ICI | immune checkpoint inhibitor |
| IDO | indoleamine 2,3-dioxygenase |
| IFN-γ | interferon gamma |
| IHC | immunohistochemistry |
| IL-2 | interleukin 2 |
| irRC | immune related response criteria |
| LAG-3 | lymphocyte-activation gene 3 |
| LDH | lactate dehydrogenase |
| LLM | lentigo maligna melanoma |
| mAb | monoclonal antibody |
| MAGE-1 | human melanoma antigen 1 |
| MAPK | mitogen-activated protein kinase |
| MEK | mitogen-activated extracellular signal-regulated kinase |
| MHC | major histocompatibility complex |
| mPFS | median progression-free survival |
| mut | mutant |
| NK | natural killer |
| NM | nodular melanoma |
| NRAS | neuroblastoma RAS viral oncogene homolog |
| NS | not significant |
| NSCLC | non-small cell lung cancer |
| ORR | overall response rate |
| OS | overall survival |
| PD | progressive disease |
| PD-1 | programmed death protein 1 |
| PD-L1 | programmed cell death ligand 1 |

| | |
|---|---|
| PET | positron emission tomography |
| PFS | progression-free survival |
| PR | partial remission |
| Pt | patient |
| SD | stable disease |
| SLNB | sentinel lymph node biopsy |
| SSM | superficial spreading melanoma |
| TCR | T cell receptor |
| TIL | tumor-infiltrating lymphocytes |
| TME | tumor microenvironment |
| TNM | tumor, node, metastasis |
| TPS | tumor proportion score |
| UV | ultraviolet |
| WHO IARC | World Health Organization's International Agency for Research on Cancer |
| wt | wild type |

# Introduction

## 1. Scientific background

### 1.1. Epidemiology of melanoma malignum

Melanoma is a life-threatening malignancy, originating from melanocytes located in the basal layer of the epidermis. It affects different primary sites, with cutaneous, ocular, and mucosal sites.

According to the estimated incidence and mortality data from the GLOBOCAN project run by the World Health Organization's International Agency for Research on Cancer (WHO IARC), melanoma is the 18th most common malignant disease worldwide and the 23rd most common cause of death. It is important to mention that in two decades, the number of new cases has approximately doubled and the number of deaths has increased by almost one and a half times internationally (1).

Regarding the epidemiological situation of malignant tumors in Hungary, the National Cancer Registry publishes incidence values on a reporting basis, while mortality statistics are compiled by the Central Statistical Office (KSH). According to the Cancer Registry database, in 2019, melanoma was the 9th most common malignant tumor in Hungary in terms of new cases, with new cases approaching 3,000 in recent years, and the increase in incidence is in line with international trends. When the data is standardized, the increase per 100,000 people still appears to be significant. In terms of the male-female distribution of cases, the disease appears to be more common among women, however, when the data is standardized and projected per 100,000 people, there is a male dominance in terms of both incidence and mortality (2).

The increase in incidence is explained by the changed quality of sunlight due to environmental pollution and the unhealthy sunbathing habits (3).

In terms of mortality, the annual number of cases in Hungary is between 300-400, which has not changed significantly over the past two decades, and all this has resulted in a significant decrease in the mortality/incidence ratio, meaning that the survival of diagnosed patients has improved which was also confirmed by direct analysis of survival data (2).

The survival advantage experienced can be attributed to two main factors. On the one hand, due to the strengthening of secondary prevention, such as intensive screening campaigns, melanoma patients are being detected at an increasingly lower stage, thus the

outcome of the disease is also more favorable (4). On the other hand, there has been significant progress in the treatment of the disease in the past 15 years, with the advent of targeted therapies and immunotherapies, which have significantly improved overall survival both internationally and with regard to the patients of our institute (4, 5).

## 1.2. Melanoma classification, diagnostic features and prognostic factors

### *1.2.1. Clinical classification of melanoma malignum:*

- *In situ melanoma* is the form with the best prognosis, the tumor cells infiltrate only the epidermis, therefore metastasis cannot develop.
- *Superficial spreading melanoma (SSM)* is the most common subtype, accounting for 60-70% of cases in Caucasians. It is characterized by initial superficial, radial tumor growth, at which time the cells do not yet have the ability to metastasize. Over time, it enters a vertical growth phase, and the deeply penetrating cells already have metastatic potential.
- *Nodular melanoma (NM)* is the second most common subtype in the Caucasian race. In contrast to SSM, this type immediately begins with vertical infiltration of the skin, grows rapidly, and has a worse prognosis than SSM.
- *Lentigo maligna melanoma (LLM)* occurs primarily on sun-exposed body surfaces, most commonly in the head and neck region, and the transition from in situ melanoma (lentigo maligna) to LMM usually occurs slowly.
- *Acrolentiginous melanoma (ALM)* occurs on the palms, soles, or subungual areas. It is often hypopigmented or amelanotic, which may delay clinical diagnosis. It is the most common type of melanoma in African and Asian populations, and is rare in Caucasians.
- *Desmoplastic melanoma* is usually amelanotic, with a pronounced connective tissue component. It has a lower metastatic potential and a higher local recurrence rate than the above subtypes.
- The majority of *extracutaneous melanomas* are ocular melanomas, they are usually discovered accidentally during a routine eye examination, and their prognosis is worse than that of cutaneous melanomas. The other large group, mucosal melanoma, occurs most often on the anal and vaginal mucosa, the oral

cavity, the nasal cavity and the paranasal sinuses. Due to the location of the tumor, it is usually diagnosed late, mostly only when the tumor size causes symptoms (6).

### *1.2.2. Clinical staging and prognostic factors of melanoma*

In addition to the above-mentioned histological and genetic prognostic factors, various clinical factors also influence the prognosis of disease: primary melanomas of the head/neck and trunk have a worse prognosis than other anatomic locations, and older age and male gender are also independent predictors of shorter survival (7). One of the most significant clinical prognostic factors is the TNM status of the tumor, which classification is based on the 8th edition of the AJCC (American Joint Committee on Cancer). The TNM classification depends on the thickness of the primary tumor, its ulceration status, the number of lymph nodes involved, the presence of satellite, in-transit and microsatellite metastases, the location of distant metastases and the LDH level, which indicates tumor burden (8).

### *1.2.3. Histological and genetic features and prognostic factors of melanoma*

The gold standard for the diagnosis of melanoma is currently histological examination, which requires the maximum vertical diameter of the tumor in mm (Breslow value) and the level of invasion (Clark value) as well as the main additional histological prognostic factors: tumor growth phase, regression, exulceration, satellites, presence of lymphatic and blood vessel invasion, mitotic index, and presence of tumor-infiltrating lymphocytes (TIL) in the dermis (9).

In case of diagnostic difficulties, melanoma-associated antigens can be determined by immunohistochemistry. Most often, the differentiation antigen gp100 or the Melan-A/Mart-1 and S-100 proteins are determined to differentiate amelanotic melanomas from other malignant tumors (9).

In cutaneous melanoma, mutations in three oncogenes predominate: the activating mutation in BRAF/exon15/codon600 (~50%), the activating mutation in NRAS oncogene/exon3/codon61 (~20%), and various mutations in the KIT oncogene (~15%) (10, 11). The pathway that is genetically affected in melanoma is the dominant

melanocyte receptor pathway, the KIT receptor, which also follows the golden rule that only one oncogene can be mutated in a pathway at a time. BRAF and NRAS mutations are characteristic of SSM/NM melanomas, while KIT mutations predominate in lentiginous forms (12).

As can be seen from the above, the prognosis of melanoma is influenced by numerous histological and genetic parameters in addition to clinical data, which will likely expand further in the future and which also play a significant role in determining therapy.

## 1.3. Melanoma treatment options

### *1.3.1. Local therapeutic options – surgery and radiation therapy*

The gold standard treatment of primary melanoma is surgical excision with an appropriate safety zone, which means a 5 mm surgical margin for in situ melanoma, a 1 cm safety zone for invasive melanomas thinner than 2 mm, and a 2 cm safety zone for melanomas thicker than 2 mm (13). According to the current international guidelines (NCCN, ESMO), sentinel lymph node biopsy (SLNB) should be offered to the patient if the tumor size is T1b and tumor thickness is between 0.8-1.0 mm or the tumor is ulcerated. Above T2a tumor size (Breslow thickness > 1mm) sentinel lymph node biopsy is clearly justified. SLNB positivity has a prognostic role and is important in assessing the need for further systemic treatment. Currently, in the case of a positive SLNB, routine CLND (completion lymph-node dissection) is not recommended (based on MSLT-II trial), because surgery did not improve patients overall survival compared to surveillance (14, 15).

Radiation therapy, which is common for many other cancer types, has not gained widespread use in melanoma, as skin tumors are usually radioresistant. Adjuvant irradiation is an option if adequate local control cannot be achieved surgically. In the case of symptomatic metastases, irradiation may also be indicated for palliative purposes, to reduce tumor mass (13).

### *1.3.2. Systemic treatment*

The systemic treatment of malignant melanoma has undergone significant development in the last 15 years. As late as 2010 only two FDA-approved agents were

registered for the treatment of advanced melanoma: the chemotherapy dacarbazine and high-dose IL-2 treatment, for which the response rate was between 10-16%, and was not proven to improve survival, the average survival was around 8 months (16, 17).

Currently, with the appearance of various immunotherapies and targeted treatments, there has been a significant improvement in the outcome of the disease, the median survival for patients with advanced melanoma using combined immunotherapy after 10 years of follow-up is 71.9 months, a huge improvement over the results of 15 years ago (18).

*Chemotherapy*

Cytostatic therapy in metastatic melanoma is now only used in multiple lines, with maximal palliative goals but the response rate for both monotherapies and combinations is less than 15% and survival does not reach 1 year (19).

*Targeted therapy*

About 40-50% of melanomas have a mutation in the BRAF gene at the V600 site, and several studies have addressed the use of a targeted BRAF inhibitor in the treatment of melanoma versus the standard of care chemotherapy (BREAK-3 trial dabrafenib vs dacarbazine mPFS 6.9 vs 2.7), and although it improved progression-free survival, the development of resistance over time severely limited the applicability of BRAF inhibitors (20).

Both BRAF and MEK are genes that function in the mitogen-activated protein kinase (MAPK) signaling pathway, which leads to increased cell division and proliferation, and when mutated, they become overactive, resulting in tumor growth. Acquired resistance to BRAF inhibitors is often due to aberrant reactivation of the MAPK pathway. Studies have shown that the combination of dabrafenib with the MEK inhibitor trametinib results in improved response to treatment in this patient population due to delayed development of resistance (COMBI-d trial) (21). Investigators countered by adding MEK inhibition to BRAF inhibition in trials that created a new targeted standard of care for patients with BRAF-mutated melanoma.

Treatment with dabrafenib (Tafinlar) and trametinib (Mekinist), vemurafenib (Zelboraf) and cobimetinib (Cotellic) (coBRIM trial) (22), and encorafenib (Braftovi) and

binimetinib (Mektovi) combinations are all associated with longer OS than BRAF inhibitor monotherapy (COLUMBUS trial) (23).

Due to the above results, BRAF and MEK inhibitor combination therapy is currently one of the mainstays of melanoma treatment, which improves patient survival not only in advanced stages, but also during adjuvant use (24).

*Immune checkpoint inhibitor therapy*

The past few years brought a dramatic breakthrough of immunotherapeutic modalities in cancer treatment. Advances in understanding the mechanisms regulating antitumor immune response led to development of a new class of immunotherapeutic agents targeting molecular interactions blocking T-cell activation, the so called immune checkpoint inhibitors (ICIs) (25). Monoclonal antibodies directed at programmed cell death protein 1 receptor (PD-1) and its ligand (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3) have provided robust activation of the adaptive immune system, restoring immune surveillance leading to host tumor recognition and destruction. Immune checkpoint inhibitors have opened a new chapter in cancer treatment (26).

Their first representative was the CTLA-4 inhibitor ipilimumab (Yervoy), which was approved in 2011 for the treatment of advanced melanoma patients after two phase III trials, in which the overall response rate was 10.9% in previously treated melanoma patients. It resulted in a durable response in a smaller percentage of patients and extended overall survival by 4 months overall compared to the control group (median OS, ipilimumab-gp100 10.1 months vs 6.4 months with gp100 peptide vaccine alone) (27, 28).

The FDA approval of the PD-1 inhibitors nivolumab (Opdivo) and pembrolizumab (Keytruda) in 2014 represented a major breakthrough in the field of immunotherapy. In the KEYNOTE-001 phase 1 clinical trial, both therapy-naive and pre-treated patients were included, the ORR was 41% in the overall population and 52% in the case of therapy naive patients. Results from the phase 3 KEYNOTE-006 study showed that after a ten year follow-up, median OS was 51.9 months in patients receiving pembrolizumab and 17.2 months in patients receiving ipilimumab (29-31).

Nivolumab was compared to standard-of-care dacarbazine in a previously untreated population of patients with advanced melanoma in CheckMate 066. In this

population, nivolumab demonstrated significant improvement in overall survival (1 year overall survival 72.9% vs. 42.1%) and progression-free survival (5.1 months vs. 2.2 months) (32).

Currently, both nivolumab and pembrolizumab monotherapy (31), as well as the double ICI combination ipilimumab-nivolumab, are considered the gold standard in the treatment of advanced melanoma (18, 33). Based on the efficacy of immune checkpoint inhibitor therapy in advanced melanoma patients, the treatment has been extended to the adjuvant treatment of high-risk disease. Randomized, controlled studies (KEYNOTE-054, CheckMate 238) have proven that the therapy can improve recurrence-free survival, but it clearly could not add to distant metastasis-free and overall survival (34-36).

Following the clinical success of nivolumab utilized in combination with ipilimumab, other combinations of immune checkpoint inhibitors have been explored. One such combination has utilized an inhibitor of the lymphocyte-activation gene 3 (LAG-3) relatlimab in addition to nivolumab (37). RELATIVITY-047 was a phase 2/3 trial that evaluated the combination of relatlimab and nivolumab compared to standard-of-care nivolumab in patients with untreated unresectable stage III or IV disease. Results demonstrated that the combination therapy improved progression-free survival with a median PFS of 10.1 months with relatlimab and nivolumab compared to 4.6 months with nivolumab monotherapy. Grade ≥ 3 adverse events occurred in 18.9% of the relatlimab–nivolumab group compared with 9.7% of patients in the nivolumab group (37).

Below is the evolution of melanoma immunotherapy treatment over the past 15 years (Figure 1). Currently, there are several studies underway using different immunotherapeutic mechanisms to treat advanced melanoma patients.

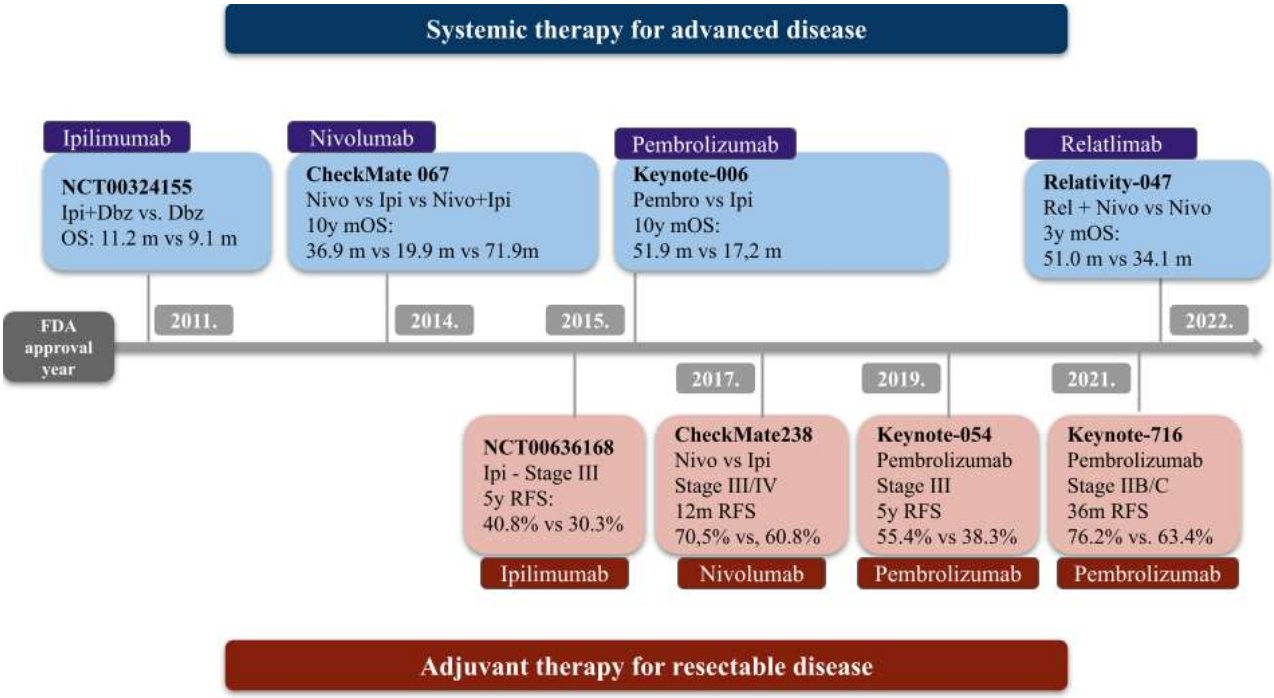


**Figure 1** | Immune checkpoint inhibitor clinical trials and FDA approval year in melanoma treatment (18, 28, 31, 35, 36, 38-40)

*Source: Knight A, Karapetyan L, Kirkwood JM. Immunotherapy in Melanoma: Recent Advances and Future Directions. Cancers. 2023;15(4):1106.* (41). *CC BY license (https://creativecommons.org/licenses/by/4.0/).*

## 1.4. Antitumor immune response

Tumor cells are usually immunologically distinguishable from healthy cells due to the genetic changes that have occurred in them, so that in an optimal case the immune system is able to find and destroy them through the activation of both the innate and adaptive immune systems. Tumor-specific antigens that are generated by different mutations involved in tumor development occur exclusively on tumor cells and they are called neoantigens. The other group is the significantly more common tumor-associated antigens, which are proteins that appear at an abnormal time, in tissue or in quantity, such as melanoma antigen-1 (MAGE-1) and tyrosinase, which is required for melanin biosynthesis and is found only in melanocytes and melanoma cells (42).

An effective antitumor immune response requires several steps, collectively referred to as the tumor immunity cycle (43). The extent of the immune response is regulated by the interaction of costimulatory and inhibitory signaling pathways.

Tumor antigens are taken up by antigen-presenting cells (APCs), typically dendritic cells (DCs), circulating in peripheral tissues, and transported to lymph nodes, where antigen fragments are presented via the MHC molecules of the APCs to T lymphocytes, resulting in their activation and clonal expansion, with the contribution of costimulatory mechanisms(44). Upon activation, T cells leave the lymph node and travel through the circulation to the tumor tissue, where they recognize tumor cells and develop into tumor-specific effector and memory T cells, which then cooperate with other immune cells and the humoral immune response that occurs in parallel to destroy the tumor (43).

The first signal leading to T cell activation is the recognition of the HLA/antigen peptide complex by the T cell receptor. The second signal of the activation process is the interaction of the CD80 and CD86 molecules on the surface of the APC and the CD28 molecule on the surface of the T cell (45).

In order to properly regulate the effector function exerted by T cells, various immune checkpoint inhibitor molecules are expressed, which are responsible for ensuring immune homeostasis (46). Peripheral tolerance is an immune regulatory process that takes effect through the activation of various immune checkpoint inhibitory mechanisms, the best known of which are cytotoxic T cell-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG-3), and T-cell

immunoglobulin-3 (TIM-3), Their therapeutic inhibition has brought about a huge breakthrough in the treatment of several extremely aggressive tumors (47).

Immunotherapy has been particularly successful in the treatment of melanoma, with currently approved agents including the CTLA-4 inhibitor ipilimumab, the PD-1 inhibitors nivolumab and pembrolizumab, the PD-L1 inhibitor atezolizumab, and the LAG-3 inhibitor relatlimab. The efficacy of these therapies has been further enhanced by the use of combinations, such as ipilimumab-nivolumab or relatlimab-nivolumab, the mechanism of action of these therapies is illustrated in the figure below (Figure 2).

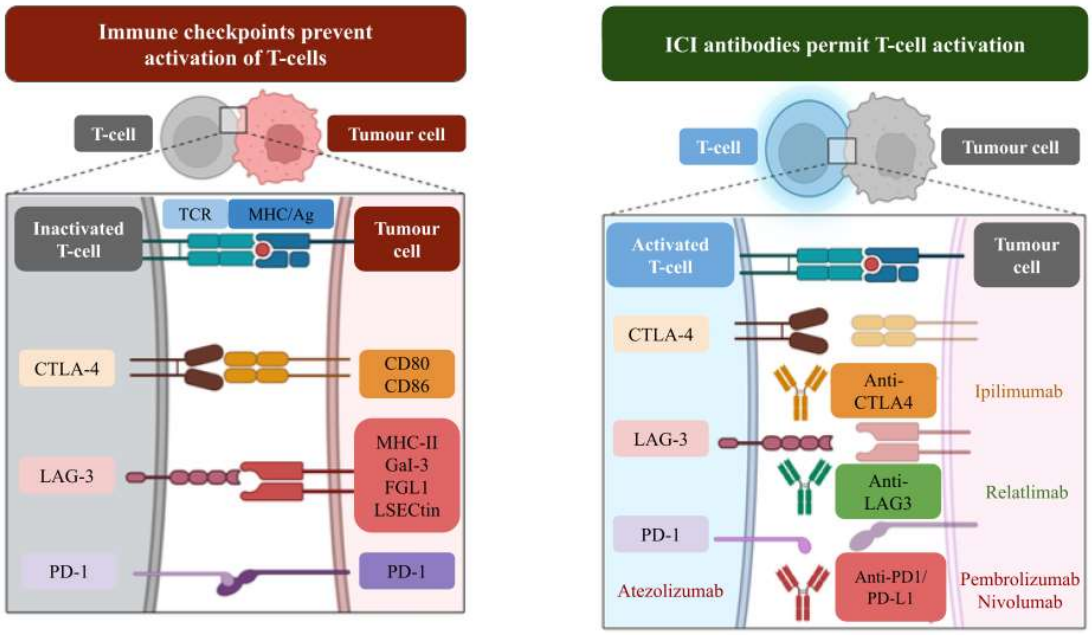


**Figure 2** | Mechanism of action of immune checkpoints and immune checkpoint inhibitors (ICIs) in melanoma treatment: releasing the 'brakes' on the immune system, allowing T-cells to kill cancer cells.

*Source: Chen Y, Kovács T, Ferdinandy P, Varga Z. Treatment options for immune-related adverse events associated with immune checkpoint inhibitors. British Journal of Pharmacology. 2024; DOI: 10.1111/bph.16405* (48).

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## 1.5. Resistance mechanisms

Although the survival of patients with advanced melanoma has improved significantly with the introduction of immunotherapy in the last 15 years, a significant proportion of patients do not benefit from treatment and experience early relapse or progression after initial response (49).

Resistance mechanisms to immune checkpoint inhibitors can be divided into the following groups based on clinical course: primary resistance, when there is no response to therapy at all; adaptive resistance, when a functional antitumor response is established, but is limited by immunosuppressive mechanisms; and acquired resistance, which represents progression after an initial therapeutic response (50).

Currently, we distinguish between intrinsic (intracellular or intra-tumoral) and extrinsic (systematic) factors in the background of ICI resistance. The former group includes factors present in tumor cells, such as oncogenic signaling pathways, tumor mutational burden, defects of antigen-processing machinery, HLA downregulation, PD-L1 expression, T-cell exhaustion and negative immune checkpoints, neoantigen affinity and heterogeneity, genomic instability as well as certain components of the TME responsible for immunosuppression, such as Tregs and tumor-infiltrating myeloid cells, which are responsible for blocking the effective T-cell response. Among extrinsic factors, there is evidence of the role of the aging of the immune system (immunosenescence), as well as the existing chronic inflammation through the inhibition of T- and NK-cell migration into the tumor, and the role of the composition of the intestinal flora has also been raised (49).

As mentioned above, the loss of HLA class I expression by tumor cells can be a possible option for the development of the resistance in melanoma to ICI therapy, during our research, we examined this option, among others. This mechanism is presented in detail below.

## 1.6. The role of HLA in the antitumoral immune response

The presence of tumor antigens and the cells involved in their recognition, the expression of HLA class I antigens by tumor cells, as well as the expression of components of the so-called antigen-processing machinery (APM), is essential for the

development of an effective antitumor response. During the cell-mediated immune response, molecules released from damaged tumor cells are taken up by antigen-presenting cells (APCs) found in many parts of the body, and the protein fragments are presented to T lymphocytes in the lymph nodes in association with their MHC (major histocompatibility complex) molecules. Presentation to CD8+ T cells is done by MHC-I (in humans, it is called human leukocyte antigen, HLA-I), while presentation to CD4+ T cells is done by MHC-II (in humans, it is called HLA-II). The MHC-I complex consists of a polymorphic α-chain (HLA-A, -B or -C alleles) and the 2-microglobulin (B2M) molecule, while the MHC-II complex consists of polymorphic α- and β-chains (HLA-DP, -DQ, DR alleles) (51).

T-lymphocytes are able to bind to the MHC-antigen peptide complex with the help of their specific receptors (T-cell receptor, TCR), and the T cell that recognizes the corresponding antigen epitope undergoes activation and clonal proliferation after binding. Activated antigen-specific CD8+ T cells can enter the tumor and destroy tumor cells carrying the appropriate antigen, and restart the process by releasing additional antigenic molecules from the destroyed tumor cells. Defects in the expression of any of the components of this process are likely to have a negative impact on the clinical course of the disease and on the outcome of T-cell based immunotherapies (43).

Since antigen presentation by MHC class I molecules is the prerequisite of tumor cell recognition by cytotoxic T lymphocytes, considered to be major players in antitumor immune responses, it could be logically expected that MHC-I expression by tumor cells would be required for the efficacy of T cell-based immunotherapies, including ICIs. Human cancer cells frequently downregulate the components of HLA class I antigen processing machinery (APM), which was found to be associated with poor prognosis in several tumor types (52). The mutation or loss of heterozygosity of β2-microglobulin (B2M) was identified as a mechanism of resistance to immunotherapies, including PD-1 inhibitors. However, decreased HLA class I expression is most frequently caused by epigenetic or transcriptional mechanisms (53). The potential association of HLA class I protein expression loss with ICI therapy resistance has been explored only to a limited extent (54).

Surprisingly, the studies demonstrated the predictive role of tumor cell HLA class I expression in melanoma patients treated with the CTLA-4 inhibitor ipilimumab but not

in those receiving PD-1 blocking agents. Conversely, HLA class II expression on tumor cells proved to be predictive of anti-PD-1 efficacy but not anti-CTLA-4 effects (55, 56).

### 1.7. Difficulties with predictive biomarkers for ICI therapy

Several potential predictive markers have been investigated for ipilimumab, primarily serum factors detectable in peripheral blood and the proportion of certain white blood cell populations (57) but the predictive potential of tumor-infiltrating immune cells has been studied less. In the case of antibody therapies targeting the PD-1 pathway, most studies have focused on the predictive role of PD-L1 ligand expression (58), which has generally shown a correlation with treatment efficacy, but the correlation is not absolute and some PD-L1-negative tumors also respond to treatment, therefore, the expression of the ligand by itself is not a sufficient marker for patient selection, although it is used as eligibility criteria in some clinical trials (59).

Reliable assessment of PD-L1 expression and comparison of results between different assays are complicated by several factors, such as the use of different reagents and variable cut-off values for expression across assays. In addition, PD-L1 expression is dynamic, can be different in the primary tumor and metastases, and is heterogeneous even within the same tumor, leading to sampling error in small biopsies (60, 61).

In addition, CD8+ T-cell density in pretreatment biopsies predicts response to pembrolizumab in melanoma patients, but there has been no such association between the amount of infiltrating T-cell subsets and clinical activity with other anti-PD-1/PD-L1 agents (62). Interestingly, the frequency of somatic mutations and the proportional amount of neoantigens in tumors – which, according to some genome-based analyses, correlated with the amount of cytotoxic T cells present in tumors and with cell killing activity – showed a correlation with the efficacy of both CTLA-4 and PD-1 inhibitory therapies (63, 64).

Most of the biomarkers listed above have been investigated in only one or a few studies, and none of them have been validated for use in routine clinical practice. One of our research goals was to identify biomarkers predictive of ICI therapy.

## 1.8. Previous results of our research group

In a retrospective study our research group investigated the tumor infiltrating immune cells as potential biomarkers predicting response to treatment and survival in patients with metastatic melanoma receiving ipilimumab therapy.

When analyzing the intratumoral density of different immune cells, it showed a different trend in lymph node and cutaneous/subcutaneous metastases.

The prevalence of most of the studied immune cell types (most significantly, CD8+ T lymphocytes and FOXP3+ cells) was higher in lymph node metastases of responders compared to non-responders. In contrast, there was a significant difference only in the case of CD16+ and CD68+ cells determined in cutaneous and subcutaneous metastases. All these observations showed a correlation with patient survival (65).

The research team's aim for another retrospective study was to test HLA class I and II expression in melanoma metastases as potential biomarkers of response to ipilimumab and survival in patients with metastatic melanoma.

HLA class I and II antigen expression levels were correlated to intratumoral density of different type lymphocytes (CD8+, CD45RO+, CD4+, FOXP3+ and PD-1+), to clinical response to treatment, and to patients' survival.

The HLA class I antigen expression level in lymph node metastases was found correlated to the density of $CD8^+$ and $CD45RO^+$ T cells and of lymphocytes expressing PD-1, as well as to response to ipilimumab treatment and survival (55).

The results obtained during the studies suggest that the degree of presence of certain immune cells in the tumor and HLA I expression are related to the response to ipilimumab treatment, which suggests the role of these factors as predictive biomarkers.

## Objectives

The advent of immune checkpoint inhibitors has significantly improved outcomes for patients with advanced, inoperable, and high-risk operable melanoma. However, there remains a portion of patients who have progression of disease despite this treatment. In order to increase the rate of responders to therapy, research into biomarkers that can predict therapeutic effect is of primary importance.

Based on previous results of the research group, it was confirmed that the density of certain tumor-infiltrating immune cells is associated with the response to ipilimumab therapy. In addition, HLA I expression was associated with the intratumoral density of certain immune cell markers, as well as the response to ICI therapy and patient survival.

Based on these results, the aim of our further research was to determine whether the loss of HLA I expression could be a mechanism of resistance to ipilimumab therapy. In addition, we examined the predictive role of HLA I and II expression in metastatic melanoma patients receiving PD-1 inhibitor therapy.

### Study I

- Thesis: Could the loss of HLA class I expression be behind the resistance that develops with ipilimumab therapy in melanoma patients?
- The first aim of the study was to explore potential changes in HLA-I expression level during the therapy, comparing pre-treatment and post-treatment metastatic samples.
- Since effective tumor antigen recognition relies on the interaction between CD8+ cytotoxic T lymphocytes and HLA class I molecules while HLA-I negative tumors may be sensitive to killing by natural killer (NK) cells, the second aim of the study was to examine infiltration of pre- and post-treatment tumor samples by CD8+ T cells and NK cells.

### Study II

- Thesis: Is HLA class I and II expression a predictive biomarker in melanoma patients receiving PD-1 inhibitors?
- The aim of the present study was to explore tumor cell HLA class I and class II expression as potential predictive markers in melanoma patients treated with PD-1 inhibitors, analyzing the association of expression results with therapeutic response and survival.

## Materials and methods

### Study I

### 1. Patients and tumor samples

We obtained archived paraffin blocks of sequential (pre- and post-treatment) tissue samples of patients with metastatic melanoma treated with ipilimumab between 2010 and 2015. Sample collection was restricted to metastases surgically removed within a 2 years range before or after ipilimumab treatment. A total of 29 metastasis samples from 6 patients met the above criteria in our institute during the study period. TNM classifications and stage grouping criteria were based on the 7th Edition of AJCC Staging System.

Five of the six patients received systemic treatment before ipilimumab therapy; all of them had chemotherapy while two also received radiotherapy, and one patient (Pt3) had already received ipilimumab therapy 32 months before the ipilimumab reinduction treatment evaluated in the present study.

Altogether 29 metastases were studied, 18 pre-treatment and 11 post-treatment surgical samples. Of the post-treatment samples, three were residual metastases from Pt1 while the other eight were progressing lesions.

Progression-free survival (PFS) and overall survival (OS) were calculated from the commencement of ipilimumab treatment till the last follow-up, tumor progression or death, respectively.

### 2. Monoclonal antibodies

The mouse monoclonal antibody (mAb) HCA2, recognizing B2M-free HLA-A (excluding -A24), -B7301, and -G heavy chains, the mAb HC10, which recognizes B2M-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73), and HLA-C heavy chains and the B2M-specific NAMB-1 were developed and characterized as described (66, 67). The mouse anti-human CD8 mAb and the mouse anti-human NKp46 mAb were purchased from Dako (Glostrup, Denmark) and from R&D Systems (Abingdon, United Kingdom), respectively. The dilution was 1:1000 for the HC10, HCA2 antibodies, 1:300 for the NAMB-1, 1:50 for the CD8 and 1:100 for the NKp46 antibody.

## 3. Immunohistochemical staining

Immunohistochemical staining of tissue sections of formalin-fixed, paraffin-embedded tumor samples was performed as described earlier (55, 65). Briefly, deparaffinated sections were treated with 3% $H_2O_2$ in methanol to block endogenous peroxidases, then antigen retrieval was performed by heating at 98°C for 40 min in citrate buffer (pH 6.0), followed by incubation with protein blocking solution (Protein Block, Serum-Free, Dako) for 10 min at room temperature, and incubation with the primary antibodies overnight at 4° C. For staining detection the SS™ One-Step Polymer-HRP IHC Detection System (BioGenex, Fremont, CA) and 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA) were used followed by counterstaining with hematoxylin. In the case of labeling with anti-HLA class I mAbs, the percentage of the area displaying stained melanoma cells was determined in the metastases. Intratumoral density of CD8+ and NKp46+ lymphocytes was assessed as described earlier (65); briefly, the number of labeled cells was counted within the metastases in at least 10 (median: 35, range: 10–120) randomly chosen fields per sample, using a graticule of 10×10 squares designating an area of 0.0625 $mm^2$ at ×400 magnification. For patients with more than one metastasis available the average values were also calculated for each marker, separately for pre- and post-treatment samples. The statistical significance of the differences between pre- and post-treatment samples was determined using the Mann-Whitney U test.

## 4. Computerized analysis of the staining intensity

The immunohistochemistry slides were acquired in TissueFaxs brightfield (Tissuegnostics, Vienna, Austria) system with a ×40 magnification dry lens coupled onto a Zeiss Axio Imager Z2 Microscope (Jena, Germany) and an eight slide automatic stage (Märzenhäuser, Wetzlar, Germany) using a Pixelink camera (Pixelink, Rochester, NY, United States). Regions of interest containing metastases without obvious artifacts were selected and analyzed using the HistoQuest (TissueGnostics) image cytometry software. “Cells” were identified on the basis of the hematoxylin stained nuclei and the immunohistochemical reaction was identified by ring mask (68). The cell nuclei area was used to distinguish among cell populations. The staining signal was quantified using a

single-reference-shade color deconvolution algorithm (69). Quantifications were confirmed visually by the backward connection function of the HistoQuest program.

### 5. Ethics statement

The study followed the Declaration of Helsinki and was approved by the Scientific and Ethical Committee of Medical Research Council, Hungary (2506-3/2017/EKU, 12120-1/2019/EKU). Informed consents from patients were not required by the board in case of retrospective studies where it is not possible to obtain consents from the majority of patients as in this case where most patients were deceased at the time of the study.

## Study II

### 1. Patients and tumor samples

We included in our study all patients who received at least 3 cycles of PD-1 inhibitor treatment (nivolumab 240 mg every two weeks or pembrolizumab 200 mg every 3 weeks) for stage IV melanoma in National Institute of Oncology between 2015-2022 and had a pre-treatment tumor sample of adequate quality.

Archived paraffin blocks of pretreatment surgical samples from 40 tumor resection samples of lymph node (n=70) and skin/subcutaneous metastases (n=42), obtained within 4 years of starting anti-PD-1 therapy (median 10 months, range 1–48), were selected; the study cohort consisted of 40 patients (1–9 examined lesions per patient). The primary site was cutaneous in 38 cases and occult in 2 cases. Most patients (n=28) received the anti-PD-1 treatment as first-line therapy. Response assessment was based on immune-related response criteria (irRC) (70); patients with complete or partial remission as best overall response were considered responders in the evaluation. Patients' follow-up was performed by CT every 3 months and if it was necessary, MRI or PET/CT was also performed. Among the clinical parameters of the patients, we examined the correlations of age, gender, BRAF mutation status, LDH level and ECOG status with the outcome of the disease.

### 2. Immunohistochemical staining

Immunohistochemical staining of tissue sections of formalin-fixed, paraffin-embedded tumor samples was performed utilizing standard methodology as described earlier (55, 65). Briefly, after deparaffination, sections were treated with 3% $H_2O_2$ in

methanol to block endogenous peroxidases, followed by antigen retrieval by heating at 98 ◦C for 40 min in citrate buffer (pH 6.0). After incubation with protein blocking solution (Protein Block, Serum-Free, Dako, Glostrup, Denmark) for 10 min at room temperature, primary antibodies were applied overnight at 4 ◦C. For detecting MHC class I molecules, HCA2 (Origene, Rockville, MD, USA), recognizing B2M-free HLA-A (excluding -A24), -B7301, and -G heavy chains; HC10 (Origene), recognizing B2M-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73) and HLA-C heavy chains; the β2-microglobulin (B2M) specific monoclonal antibody NAMB-1; and the anti-pan HLA class I EMR8-5 (Abcam) were utilized; HLA-DR,DQ,DP was detected using the monoclonal antibody LGII-612.14. The dilution was 1:1000 for the HC10, HCA2, EMR8-5, LGII-612.14 antibodies and 1:300 for the NAMB-1 antibody. Staining was detected using the EnVision+ System HRP Labeled Polymer Anti-mouse reagent (Dako), followed by staining with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA, USA) and hematoxylin counterstaining.

### 3. Evaluation of the immune reactions

HLA class I staining was scored as 0, 1, and 2 when the percentage of stained melanoma cells was <25%, 25–75%, and >75%, respectively, based on the criteria established by the 12th International Histocompatibility Workshop (1996). Expression on normal cells in the samples (e.g., immune cells, cells of the vasculature) served as positive control. In the case of HLA-DR,DQ,DP, the percentage of the area displaying positive tumor cell staining was determined in the metastases. We also calculated a combined score of HLA class I and class II expression (HLA I/II score) based on the number of anti-HLA-I antibodies showing higher positivity than the cutoff level, combined with HLA-II positivity in a given sample (score of 1 in the case of high labeling with at least 3 anti-HLA-I antibodies, and/or HLA-II expression ≥3%; score of 0 when neither of the above criteria are met). For patients with more than one metastasis available, the mean of the scores obtained for the different metastases were calculated for each marker. Cutoff levels were set up based on the median of the given variable in the whole patient cohort, with modifications for better discriminating power in some cases, and the proportion of patients with HLA expression score higher than the cutoff level was also determined for all markers. Immunohistochemical stainings were evaluated by two independent

examiners using a light microscope (Olympus BH-2). In case of disagreement, consensus was reached by re-evaluating the section and averaging the results.

## 4. Statistical analysis

For the statistical analysis of differences in HLA expression levels between different patient groups, we applied the Mann–Whitney U test, while Fisher's exact test was used to compare the proportions of cases in different groups. Correlations were analyzed by Pearson's nonparametric correlation method.

Progression-free survival (PFS) and overall survival (OS) were defined as the time from commencing anti-PD-1 treatment to disease progression or death or last follow-up, and death or last follow-up, respectively. Survival analysis was carried out using the Kaplan–Meier method and log-rank test. Differences were considered significant in the case of p values $\leq 0.05$. Statistics were calculated using the Statistica software version 12.5 (StatSoft, Tulsa, OK, USA).

## 5. Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Scientific and Ethical Committee of Medical Research Council, Hungary (12120-1/2019/EKU).

## Results

### Study I

Responses to therapy were evaluated based on immune-related response criteria (irRC) (70). One patient (Pt1) was scored as complete response (CR) with a few residual cutaneous papules, which showed minimal progression 11 months following initiation of ipilimumab therapy and were excised. Pt2 achieved stable disease (SD) lasting for 10 months, while Pt 3 showed short-term SD lasting for 4 months; the other three patients exhibited progressive disease (PD). Pt1 and Pt2 were classified as responders while the other four patients as nonresponders in the analysis.

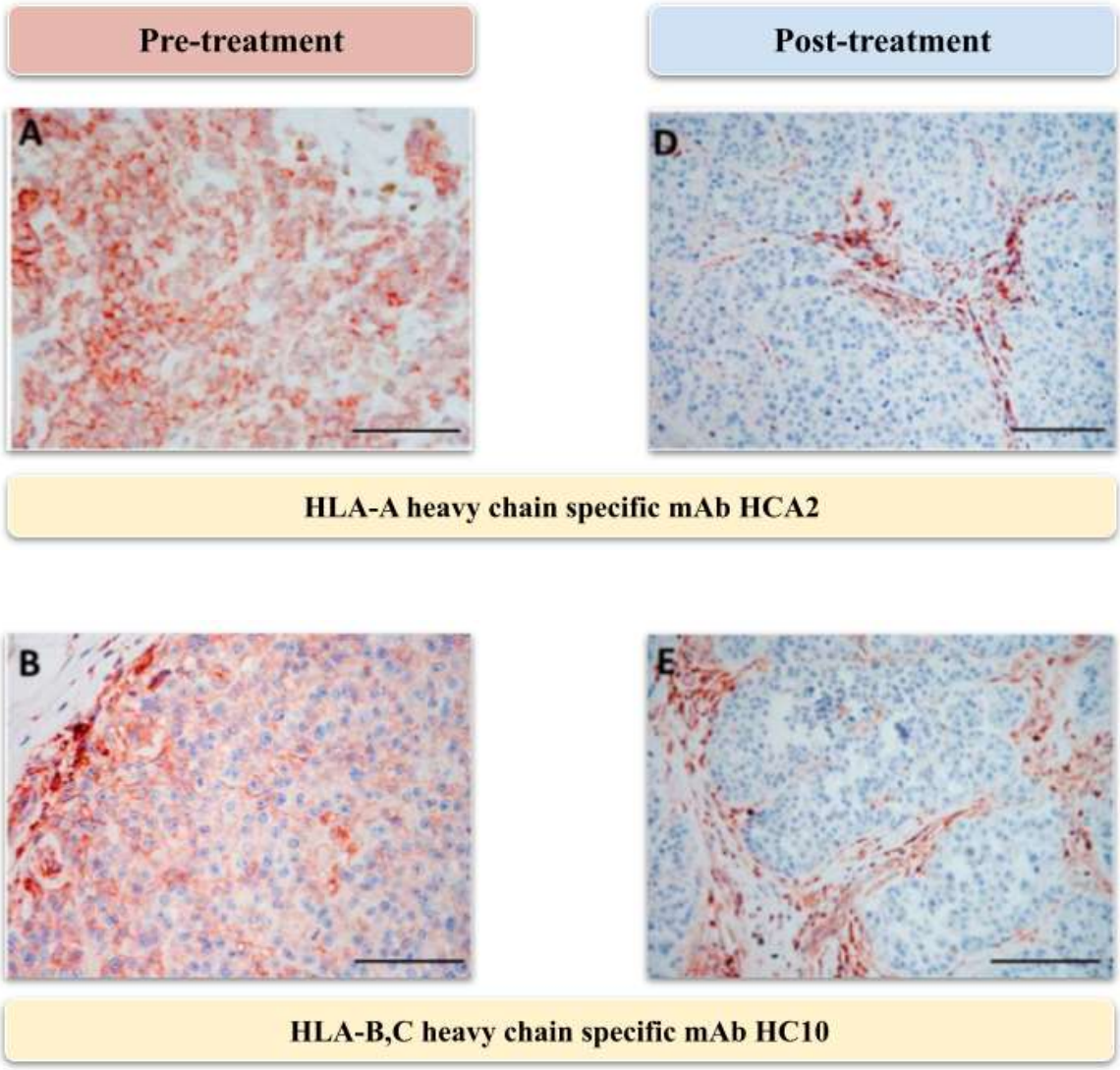
**Table 1**| Patients and samples characteristics

| | Pt1 | Pt2 | Pt3 | Pt4 | Pt5 | Pt6 |
|---|---|---|---|---|---|---|
| **Age (years)** | 52 | 51 | 73 | 53 | 59 | 57 |
| **Gender** | Female | Female | Male | Female | Male | Female |
| **Stage** | III N3c | IV M1c | IV M1a | IV M1b | IV M1c | IV M1c |
| **ECOG** | 0 | 0 | 0 | 0 | 1 | 0 |
| **BRAF** | mut | mut | wt | mut | wt | mut |
| **BOR** | CR | SD | SD | PD | PD | PD |
| **PFS (months)** | 11 | 10 | 4 | 4 | 3 | 3 |
| **OS (months)** | 67+ | 43 | 42 | 29 | 9 | 8 |
| **Pre samples analyzed** | 2 (cut.) | 1 (sc.) | 4 (LN/cut.) | 3 (LN, sc.) | 1 (sc.) | 7 (LN, breast) |
| **Post samples analyzed** | 3 (cut./sc. - res.) | 1 (sc. - prog.) | 2 (LN, sc. - prog.) | 3 (sc. -prog.) | 1 (cut. -prog.) | 1 (LN -prog.) |

Pt: patient, BOR: best overall response, PFS: progression-free survival, OS: overall survival, mut: mutant, wt: wild type, CR: complete remission, SD: stable disease, PD:

progressive disease, LN: lymph node, cut.: cutaneous, sc.: subcutaneous, res.: residual, prog.: progression.

Utilizing immunohistochemical staining with mAbs we analyzed the expression of HLA class I subunits in sequential metastasis samples from six melanoma patients treated with ipilimumab; the samples analyzed included 18 pre- and 11 post-treatment surgically excised metastases (Figure 3).



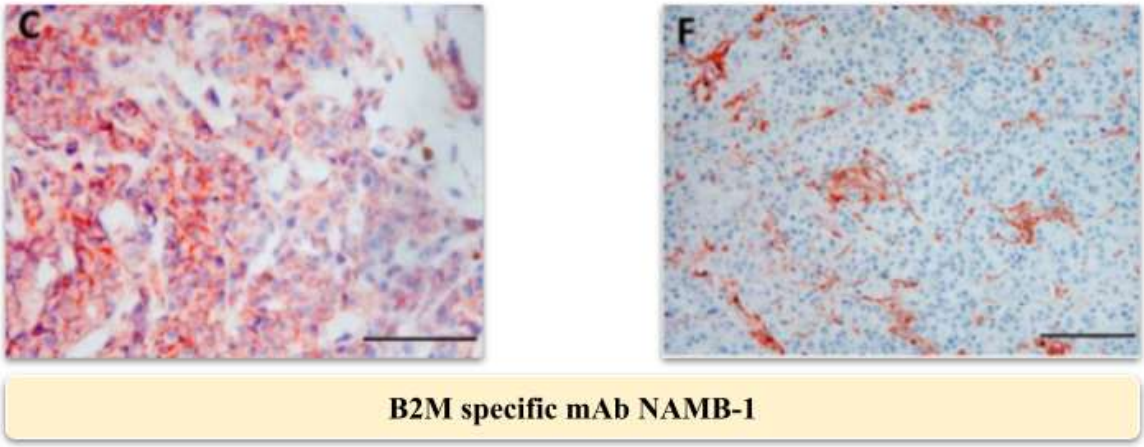



**Figure 3 |** Immunohistochemical staining of pre-treatment (A–C) and post-treatment (D–F) samples from the same patient (Pt3) with HLA-A heavy chain-specific mAb HCA2 (A,D), HLA-B,C heavy chain-specific mAb HC10 (B,E) and B2M-specific mAb NAMB-1 (C,F). Scale bars: 100 μm.

Comparing pre-treatment and post-treatment samples of all patients evaluated together, the expression of HLA class I subunits, as measured by the % of stained melanoma cells, was significantly lower in post-treatment metastases compared to pre-treatment ones (Figures 3, 4). The medians and ranges of the percentage values of melanoma cells stained by HLA-A heavy chain-specific mAb HCA-2, by HLA B,C heavy chain-specific mAb HC10 and by anti-B2M mAb NAMB-1 were 94.0 (5.1–100), 91.0 (4.5–100) and 90.5 (62.2–100) in the pre-treatment metastases, and 63.5 (0–83.6), 25.0 (0–84.2) and 57.6 (0–93.1) in the post-treatment metastases, respectively. The percentage of melanoma cells stained by all three mAbs tested was higher than 80 in the majority of the 18 pre-treatment metastases analyzed, compared to only 1 of the 11 post-treatment metastases. Metastases with a heterogeneous staining pattern displayed higher labeling at the margin of the tumors in the proximity of inflammatory cells, consistent with locally induced expression. Percentages of staining with the three antibodies were fairly consistent in the majority of cases, with discrepancies larger than 30% in only 7 of the 29 metastases. Furthermore, comparing tumor cell staining of different lesions with the same antibody, we detected a moderate level of intrapatient heterogeneity in most patients in the case of pre-treatment metastases and in two of the three patients with more than one post-treatment lesions (Table 2).

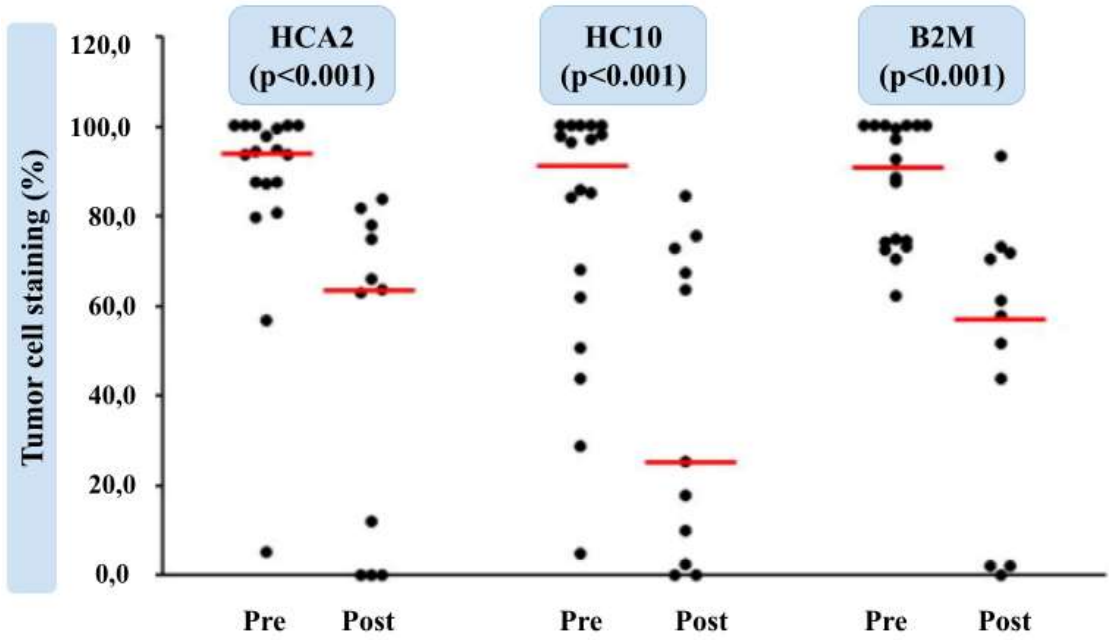



**Figure 4 |** HLA class I expression of melanoma cells (% of stained area) in pre-treatment (Pre, n=18) and post-treatment (Post, n=11) metastases from ipilimumab-treated patients. Circles: percentage values of individual samples; horizontal line: median.

Comparison of the HLA class I subunit expression levels in pre- and post-treatment metastases removed from each individual patient revealed HLA-I downregulation mainly in the case of progressing lesions of nonresponding patients; in contrast, minimal or no change was found in responding patients (Pt1 and Pt2). Interestingly, in the case of Pt1 exhibiting the best overall response and long-term survival, the baseline HLA class I subunit expression was high in the pre-treatment metastases and remained high in the post-treatment (residual) metastases (Figure 5, Table 2). In contrast, HLA class I subunit downregulation was maximal in the metastases from Pt5 and Pt6 exhibiting the shortest PFS and OS (Figure 5, Table 2). Results of quantitative evaluation of staining intensity in representative pre- and post-treatment samples of nonresponding patients with progressing lesions are in agreement with this finding (Figure 6).

**Table 2**| Patients' immunohistochemistry results

| | | | | HCA2 | HC10 | B2M | CD8+ T cells | NK cells |
|---|---|---|---|---|---|---|---|---|
| **Pt1** | Pre1 | cut. | -8 months | 100.0 | 100.0 | 100.0 | 107.2 | 6.4 |
| | Pre2 | cut. | -6 months | 56.6 | 61.8 | 74.1 | 232.0 | 0.0 |
| | Post1 | cut. | +11 months | 65.8 | 72.7 | 43.7 | 196.8 | 16.0 |
| | Post2 | sc. | +11 months | 83.6 | 84.2 | 93.1 | 857.6 | 17.6 |
| | Post3 | cut. | +18 months | 63.5 | 63.3 | 51.4 | 54.4 | 6.4 |
| **Pt2** | Pre1 | sc. | -2 months | 5.1 | 4.5 | 62.2 | 8.0 | 3.2 |
| | Post1 | sc. | +12 months | 11.8 | 9.8 | 57.6 | 4.8 | 0.0 |
| **Pt3** | Pre1 | LN | -13 months | 87.0 | 85.0 | 70.2 | 241.6 | 6.4 |
| | Pre2 | sc. | -9 months | 87.5 | 43.5 | 100.0 | 4.8 | 4.8 |
| | Pre3 | sc. | -9 months | 87.5 | 50.6 | 97.0 | 14.4 | 4.8 |
| | Pre4 | sc. | -9 months | 93.5 | 28.7 | 74.5 | 1.6 | 1.6 |
| | Post1 | LN | +7 months | 74.8 | 75.5 | 73.0 | 193.6 | 0.0 |
| | Post2 | cut./sc. | +7 months | 0.0 | 2.3 | 2.0 | 134.4 | 0.0 |
| **Pt4** | Pre1 | LN | -23 months | 97.5 | 96.2 | 87.4 | 2.9 | 0.0 |
| | Pre2 | sc. | -8 months | 100.0 | 100.0 | 100.0 | 84.8 | 9.6 |
| | Pre3 | LN | -2 months | 93.6 | 98.0 | 100.0 | 8.0 | 0.0 |
| | Post1 | sc. | +20 months | 81.5 | 67.2 | 70.4 | 22.4 | 0.0 |
| | Post2 | sc. | +20 months | 77.7 | 17.5 | 71.8 | 49.6 | 0.0 |
| | Post3 | sc. | +20 months | 62.7 | 25.0 | 61.1 | 57.6 | 0.0 |
| **Pt5** | Pre1 | sc. | -12 months | 80.7 | 68.0 | 73.0 | 52.8 | 3.2 |
| | Post1 | cut. | +4 months | 0.0 | 0.0 | 2.0 | 4.8 | 1.6 |
| **Pt6** | Pre1 | LN | -22 months | 79.6 | 85.7 | 74.8 | 128.0 | 1.6 |
| | Pre2 | LN | -22 months | 94.4 | 96.8 | 92.4 | 235.2 | 0.0 |
| | Pre3 | LN | -22 months | 99.5 | 100.0 | 99.5 | 329.6 | 4.8 |
| | Pre4 | LN | -22 months | 100.0 | 100.0 | 100.0 | 243.2 | 4.8 |
| | Pre5 | breast | -13 months | 100.0 | 100.0 | 100.0 | n.e. | n.e. |
| | Pre6 | breast | -13 months | 100.0 | 97.5 | 72.5 | 208.0 | 0.0 |
| | Pre7 | breast | -13 months | 94.5 | 84.0 | 88.5 | 385.6 | 0.0 |
| | Post1 | LN | +5 months | 0.0 | 0.0 | 0.0 | 238.4 | 6.4 |

The staining level with HLA-I antibodies (HC10, HCA2, B2M) was given in percentage (%). The unit of measurement for CD8+ T cell and NK cell density is cells/mm$^2$.

Pre: pre-treatment, Post: post-treatment, cut.: cutaneous, sc.: subcutaneous, LN: lymph node.

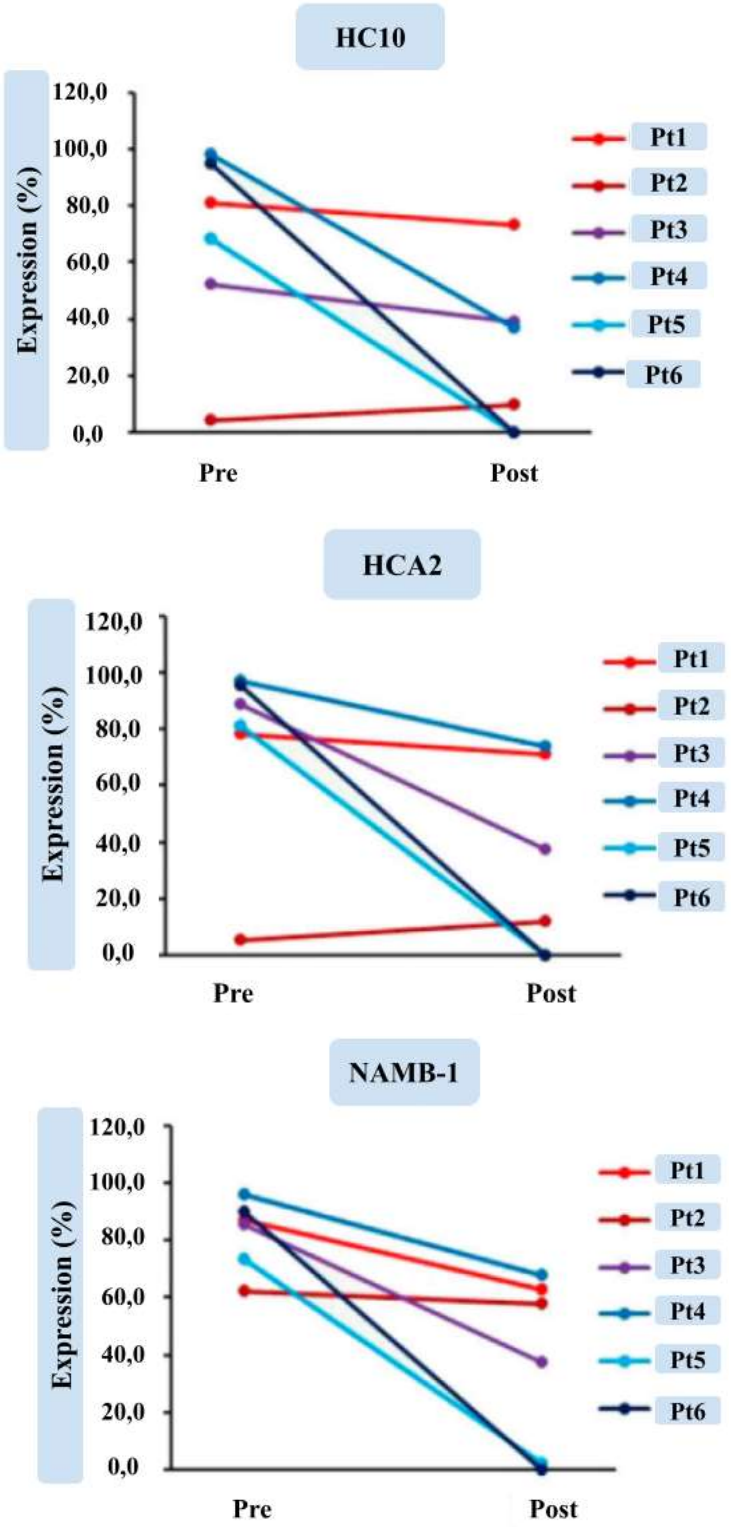



**Figure 5 |** Mean HLA class I expression of melanoma cells (% of stained area) in pre-treatment (Pre) and post-treatment (Post) metastases from ipilimumab-treated patients, labeled by HLA class I subunit-specific mAbs HC10, HCA2 and NAMB-1.

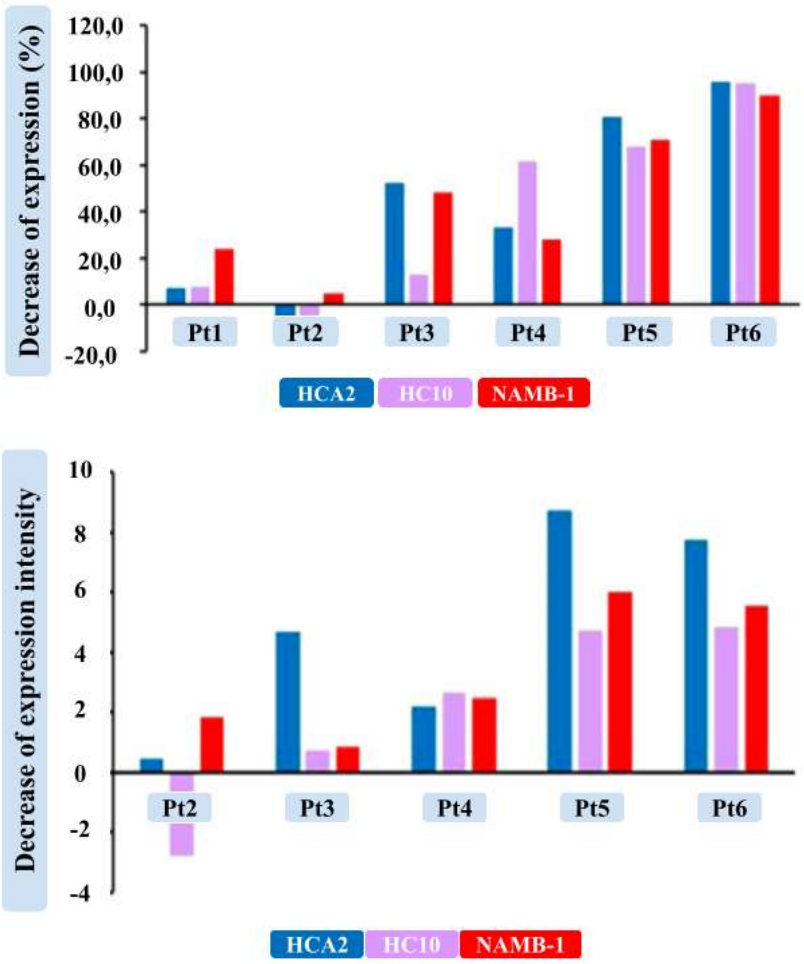



**Figure 6 |** Decrease of mean expression (% of stained area) and of staining intensity in post-treatment metastases as compared to autologous pre-treatment metastases from ipilimumab-treated patients, stained by HLA class I subunit-specific mAbs.

Since the efficacy of immune checkpoint inhibitors depends on the recognition of tumor antigen derived peptides by cytotoxic T lymphocytes in the context of HLA class I proteins, we also examined the extent of infiltration of CD8+ T cells in pretreatment vs. post-treatment tumors. The infiltration showed considerable intertumor variability and did not exhibit any consistent change between pre- and post-treatment time points (Figure 7).

Furthermore, the extent of NK cell infiltration was also tested because these cells are known to recognize HLA class I negative cells so their activity could possibly complement that of CD8+ T lymphocytes. Using NKp46 as a NK cell marker, we detected a very low number of NK cells infiltrating both pre-treatment and post-treatment tumors;

furthermore, no significant difference could be found between the two sample sets (Figure 7).

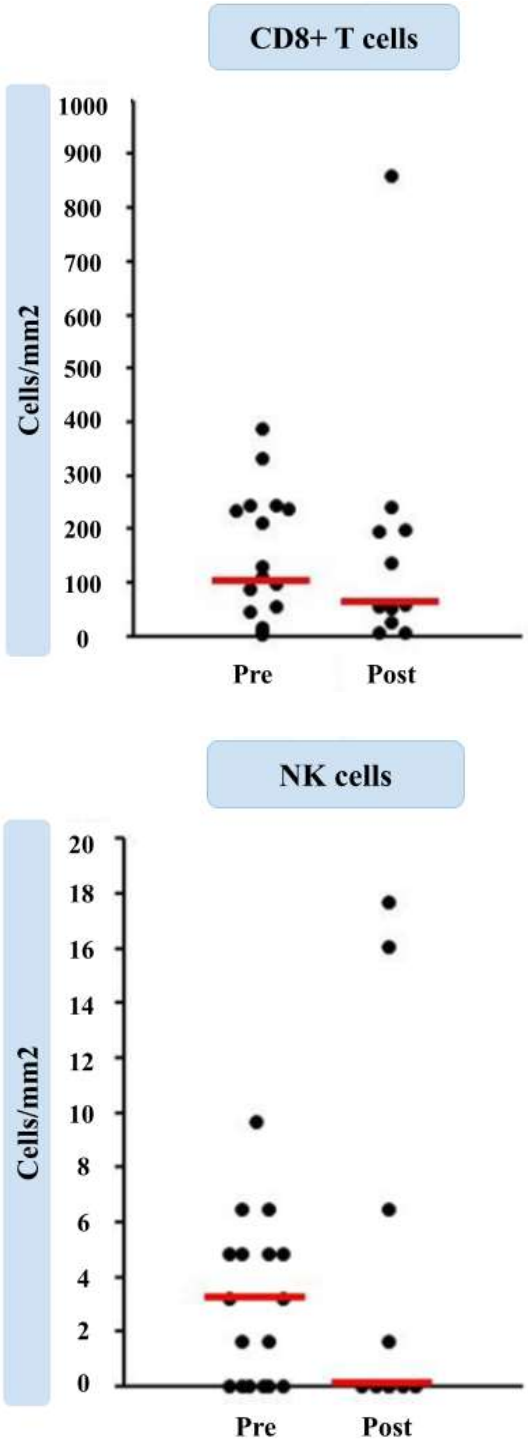



**Figure 7** | Density of CD8+ T cells and NKp46+ cells infiltrating pre-treatment (Pre, n=18) and post-treatment (Post, n=11) metastases from ipilimumab-treated patients. Circles: labeled cell density values of individual samples; horizontal line: median

**Study II**

Of the 40 stage IV melanoma patients involved in this study, 28 exhibited a complete (n=14) or partial response (n=14) (Table 3). Follow-up time of surviving patients was median 62.5 months (22–91). Among clinical parameters, the ECOG performance status and pretreatment LDH level showed an association with the response.

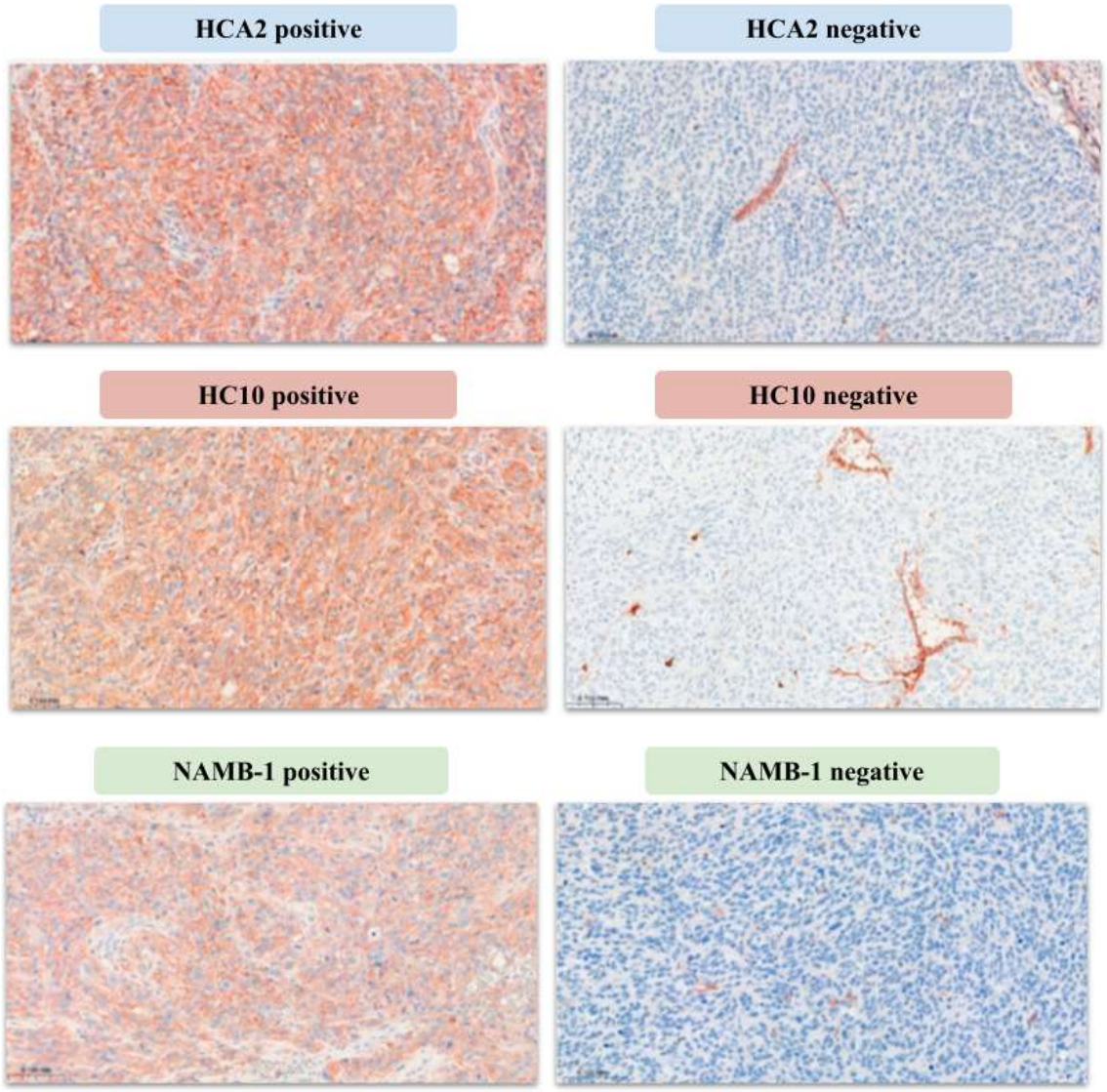
**Table 3**| Patients and samples characteristics

| | Responder (CR,PR) n=28 | Non-responder (SD, PD) n=12 | *p* value |
|---|---|---|---|
| **Age,** years: median (range) | 62 (27-87) | 53 (34-77) | NS[a] |
| **Gender** | | | |
| female | 11 | 2 | |
| male | 17 | 10 | NS[b] |
| **ECOG performance status** | | | |
| 0 | 27 | 8 | |
| 1 | 1 | 4 | 0.0223[b] |
| **BRAF mutation status** | | | |
| wild type | 20 | 10 | |
| mutant | 8 | 2 | NS[b] |
| **LDH level** | | | |
| normal | 23 | 4 | |
| >ULN | 5 | 8 | 0.0075[b] |
| **Anti-PD-1 drug** | | | |
| Nivolumab | 9 | 8 | |
| Pembrolizumab | 19 | 4 | NS[b] |
| **Line of therapy** | | | |
| 1 | 20 | 8 | |
| 2-5 | 8 | 4 | NS[b] |
| **PFS**, months: median (range) | 27 (4-90+) | 3 (1-11) | 0.0000[a] |
| **OS**, months: median (range) | 46 (13-91+) | 9 (4-62+) | 0.0002[a] |

[a]Mann-Whitney test, [b]Fisher's exact test. CR: complete response, PR: partial response,

SD: stable disease, PD: progressive disease, ECOG: Eastern Cooperative Oncology Group, BRAF: B-Raf proto-oncogene, LDH: lactate dehydrogenase, PFS: progression-free survival, OS: overall survival, ULN: upper limit of normal, NS: not significant.

Pretreatment surgical samples of lymph node and cutaneous/subcutaneous melanoma metastases were immunohistochemically analyzed for HLA expression (Figure 8), evaluating it using scores between 0 and 2 for HLA class I and determining the percentage of positive tumor area in the case of HLA class II.



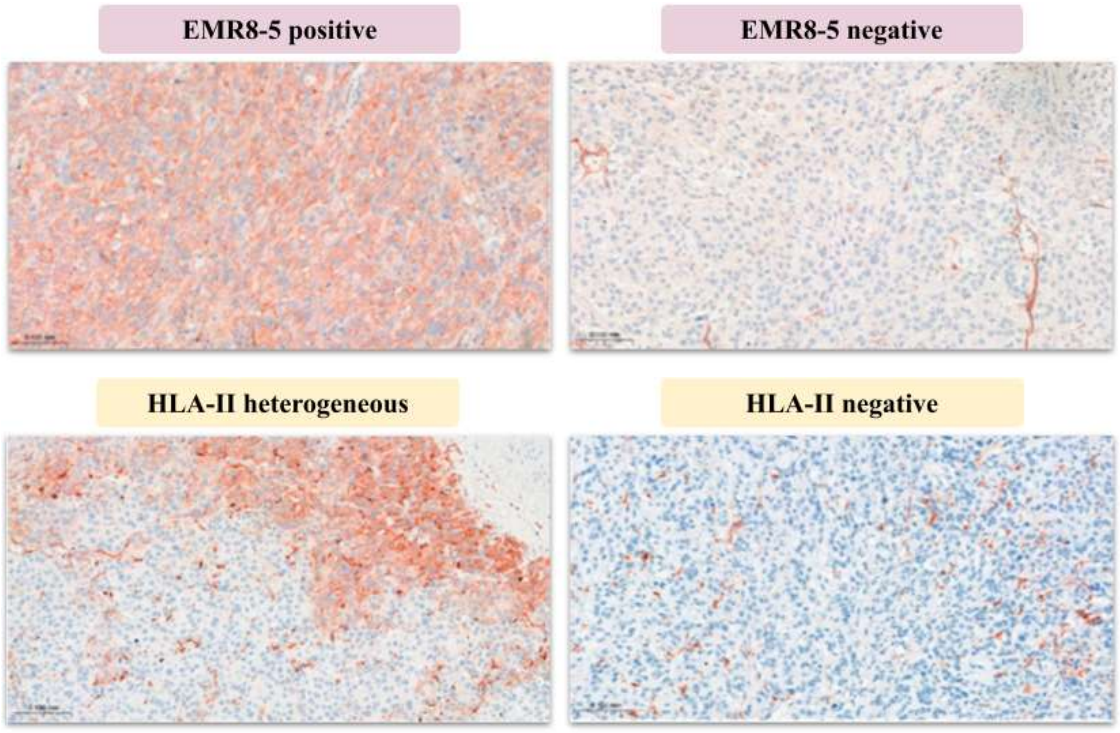



**Figure 8 |** Immunohistochemical staining of melanoma metastases with HLA class I and class II specific antibodies. Scale bars: 100 μm.

Considering all metastases irrespective of their source (patients, location), tumor cell HLA-I positivity levels determined by the four different antibodies (HCA2, HC10, NAMB-1, EMR8-5) showed a strong positive correlation, with the lowest correlation coefficient (0.6812) in the case of HCA2/HC10 and highest (0.8236) in the case of NAMB-1/EMR8-5 (all p values < 0.0001), while HLA-II staining showed a weaker correlation with HLA-I positivity (correlation coefficients 0.2182–0.2944). HLA class I expression scores were the highest in the case of the EMR8-5 pan-HLA-I antibody (mean ± SD, 1.4 ± 0.7) and the lowest in the case of HC10 (1.1 ± 0.8). Melanoma cells in metastases of 20 patients (50%) did not express HLA class II, while mean tumor cell staining higher than 10% was observed in 11 patients (27.5%). The tumor samples of eight patients showed 3–4% HLA-II staining, mainly in tumor cells near the inflammatory cells at the margin of metastases, consistent with locally induced HLA expression.

Among the responders, the proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders (17/28 vs. 2/12, p=0.0158). Similarly, a significant difference was found in the case of two anti-HLA-I

antibodies (HC10 and EMR8-5), with fewer cases showing decreased expression in responders (Table 3). A combined score of HLA-I and -II expression was introduced (being positive in the case of high HLA class I and/or HLA class II expression), which proved very effective in predicting treatment response (p=0.0019) (Table 3).

Table 4 presents individual patients' data and a heatmap showing all markers (HLA expression), demonstrating an overall higher HLA expression in responders.

**Table 3**| Relationship of treatment response with the proportion of patients with high tumor cell HLA expression in metastases

| | **Responder (PR,CR) n=28** | **Non-responder (SD,PD) n=12** | ***p* value[a]** |
|---|---|---|---|
| **HLA class I; Ab clone (cutoff score)** | | | |
| HCA2[b] (≥1.3) | 16/27 (59%) | 5/12 (42%) | 0.4877 |
| HC10 (≥0.6) | 24/28 (86%) | 6/12 (50%) | **0.0411** |
| NAMB-1 (≥1.7) | 14/28 (50%) | 3/12 (25%) | 0.1788 |
| EMR8-5 (≥1.4) | 20/28 (71%) | 4/12 (33%) | **0.0367** |
| **HLA class II (cutoff ≥ 3%)** | 17/28 (61%) | 2/12 (17%) | **0.0158** |
| **HLA I/II score[c] (cutoff ≥ 3)** | 24/28 (86%) | 4/12 (33%) | **0.0019** |

[a]Fisher's exact test. [b]Staining with HCA2 could not be evaluated in one patient because of negativity of normal cells. [c]Combined score of high expression with the anti-HLA-I and/or the anti-HLA-II antibodies. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

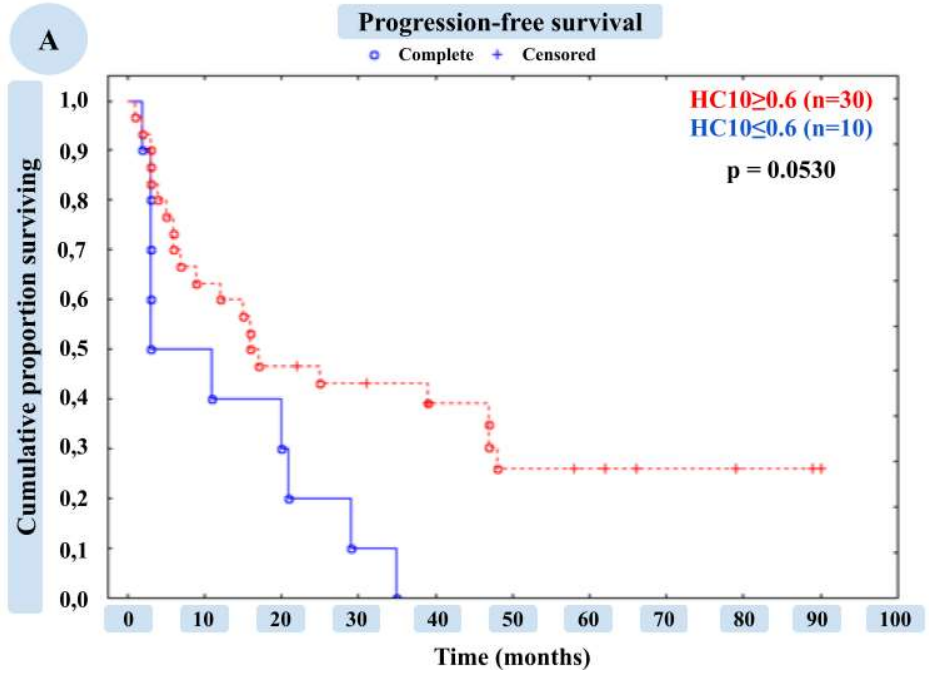
**Table 4**| Clinicopathological data of individual patients and heatmap of immunohistochemistry results

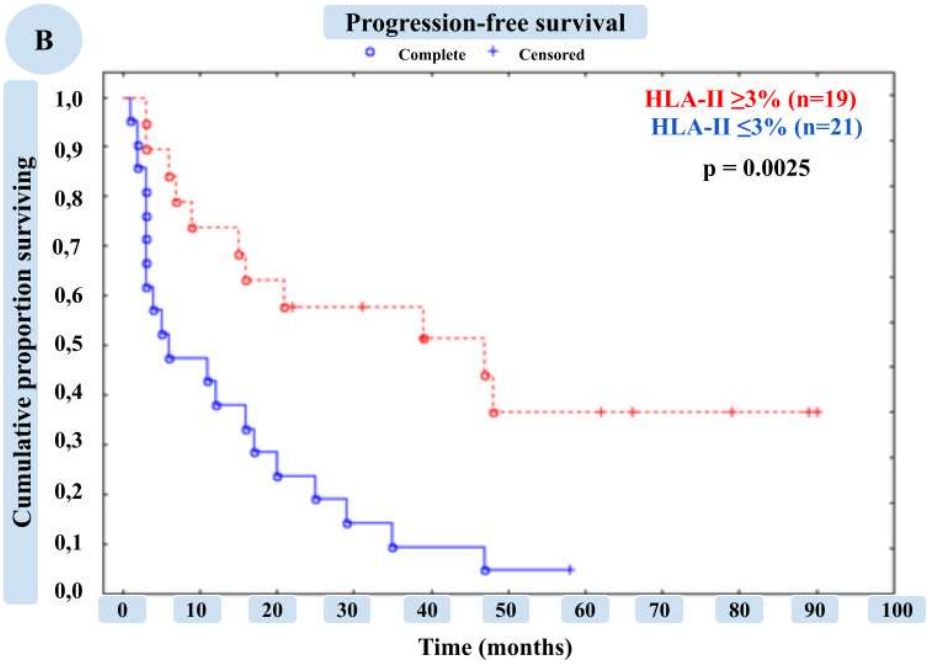
| Patient no. | Gender | Age | ECOG | Line | BRAF | LDH | Anti-PD-1 drug | BORR | PFS(mo) | Progr | OS(mo) | Death | HC10 | HCA2 | B2M | EMR | HLAII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Resp1 | Female | 49 | 0 | 4 | 2 | 0 | Pembrolizumab | CR | 16 | 1 | 68 | 0 | 1,0 | 1,0 | 1,0 | 1,5 | 11 |
| Resp2 | Male | 63 | 0 | 1 | 1 | 0 | Nivolumab | CR | 89 | 0 | 90 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 87 |
| Resp3 | Male | 27 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 47 | 1 | 91 | 0 | 2,0 | 1,3 | 1,0 | 2,0 | 3 |
| Resp4 | Male | 32 | 0 | 1 | 2 | 0 | Pembrolizumab | CR | 6 | 1 | 21 | 1 | 2,0 | 1,7 | 2,0 | 2,0 | 0 |
| Resp5 | Male | 65 | 0 | 3 | 1 | 0 | Nivolumab | CR | 47 | 1 | 87 | 0 | 0,7 | 1,3 | 0,5 | 1,5 | 0 |
| Resp6 | Female | 75 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 39 | 1 | 45 | 1 | 1,8 | 1,7 | 1,7 | 1,8 | 33 |
| Resp7 | Female | 82 | 0 | 1 | 2 | 0 | Pembrolizumab | CR | 21 | 1 | 75 | 1 | 0,5 | 1,0 | 1,0 | 1,0 | 4 |
| Resp8 | Male | 47 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 62 | 0 | 63 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 4 |
| Resp9 | Male | 74 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 39 | 0 | 40 | 0 | 1,9 | 2,0 | 1,9 | 1,9 | 3 |
| Resp10 | Male | 87 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 25 | 1 | 39 | 1 | 1,0 | 0,3 | 1,1 | 1,1 | 0 |
| Resp11 | Male | 54 | 0 | 2 | 2 | 1 | Pembrolizumab | CR | 58 | 0 | 60 | 0 | 2,0 | 1,8 | 2,0 | 2,0 | 0 |
| Resp12 | Female | 78 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 15 | 1 | 15 | 1 | 1,0 |  | 2,0 | 2,0 | 3 |
| Resp13 | Male | 40 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 22 | 0 | 22 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 54 |
| Resp14 | Female | 66 | 0 | 1 | 1 | 0 | Nivolumab | CR | 29 | 1 | 43 | 0 | 0,2 | 0,3 | 0,0 | 0,4 | 0 |
| Resp15 | Male | 67 | 0 | 2 | 1 | 0 | Nivolumab | PR | 17 | 1 | 24 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 0 |
| Resp16 | Female | 56 | 1 | 1 | 1 | 1 | Nivolumab | PR | 90 | 0 | 90 | 0 | 1,0 | 1,0 | 1,0 | 1,0 | 14 |
| Resp17 | Male | 61 | 0 | 2 | 2 | 1 | Nivolumab | PR | 79 | 0 | 79 | 0 | 0,7 | 1,6 | 0,9 | 1,4 | 3 |
| Resp18 | Male | 80 | 0 | 1 | 1 | 0 | Nivolumab | PR | 16 | 1 | 49 | 1 | 1,5 | 1,5 | 1,7 | 2,0 | 0 |
| Resp19 | Male | 60 | 0 | 2 | 2 | 0 | Pembrolizumab | PR | 66 | 0 | 68 | 0 | 0,6 | 1,0 | 1,0 | 1,2 | 32 |
| Resp20 | Male | 59 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 35 | 1 | 66 | 1 | 0,0 | 0,7 | 1,0 | 1,0 | 0 |
| Resp21 | Female | 44 | 0 | 2 | 1 | 0 | Pembrolizumab | PR | 4 | 1 | 13 | 1 | 1,0 | 0,5 | 2,0 | 2,0 | 0 |
| Resp22 | Male | 54 | 0 | 1 | 1 | 1 | Pembrolizumab | PR | 12 | 1 | 15 | 1 | 0,9 | 2,0 | 1,5 | 1,9 | 0 |
| Resp23 | Female | 68 | 0 | 1 | 1 | 0 | Nivolumab | PR | 9 | 1 | 38 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 4 |
| Resp24 | Male | 64 | 0 | 1 | 1 | 0 | Nivolumab | PR | 20 | 1 | 47 | 1 | 0,0 | 0,5 | 0,5 | 0,0 | 0 |
| Resp25 | Female | 64 | 0 | 2 | 2 | 0 | Pembrolizumab | PR | 48 | 1 | 68 | 1 | 1,2 | 2,0 | 1,8 | 1,8 | 33 |
| Resp26 | Female | 52 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 6 | 1 | 34 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 4 |
| Resp27 | Male | 75 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 31 | 0 | 33 | 0 | 1,2 | 0,7 | 1,2 | 1,0 | 23 |
| Resp28 | Female | 58 | 0 | 1 | 2 | 1 | Pembrolizumab | PR | 7 | 1 | 21 | 1 | 1,0 | 1,0 | 1,5 | 1,5 | 24 |
| Nonresp1 | Male | 69 | 1 | 1 | 1 | 1 | Nivolumab | SD | 5 | 1 | 9 | 1 | 1,0 | 0,5 | 0,0 | 1,0 | 0 |
| Nonresp2 | Male | 42 | 0 | 2 | 2 | 1 | Pembrolizumab | SD | 11 | 1 | 51 | 1 | 0,5 | 1,1 | 2,0 | 1,2 | 0 |
| Nonresp3 | Male | 41 | 0 | 1 | 1 | 1 | Pembrolizumab | SD | 3 | 1 | 62 | 0 | 0,0 | 1,0 | 0,5 | 0,5 | 0 |
| Nonresp4 | Male | 53 | 1 | 1 | 1 | 1 | Pembrolizumab | SD | 3 | 1 | 46 | 0 | 0,0 | 1,2 | 1,2 | 1,0 | 0 |
| Nonresp5 | Female | 53 | 1 | 5 | 1 | 1 | Nivolumab | PD | 3 | 1 | 7 | 1 | 0,1 | 0,3 | 0,0 | 0,4 | 0 |
| Nonresp6 | Male | 34 | 0 | 2 | 2 | 1 | Nivolumab | PD | 2 | 1 | 4 | 1 | 2,0 | 2,0 | 1,6 | 2,0 | 0 |
| Nonresp7 | Male | 65 | 0 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 10 | 1 | 0,5 | 1,5 | 1,0 | 1,0 | 0 |
| Nonresp8 | Male | 45 | 0 | 1 | 1 | 1 | Nivolumab | PD | 2 | 1 | 5 | 1 | 0,0 | 0,0 | 0,0 | 0,0 | 0 |
| Nonresp9 | Male | 71 | 0 | 2 | 1 | 0 | Nivolumab | PD | 1 | 1 | 2 | 1 | 1,0 | 2,0 | 1,0 | 2,0 | 0 |
| Nonresp10 | Male | 75 | 1 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 15 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 97 |
| Nonresp11 | Female | 77 | 0 | 1 | 1 | 1 | Pembrolizumab | PD | 3 | 1 | 4 | 1 | 2,0 | 1,7 | 2,0 | 2,0 | 12 |
| Nonresp12 | Male | 40 | 0 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 11 | 1 | 1,2 | 1,2 | 1,5 | 1,1 | 1 |

Red/green color of the heatmap label high/low expression.

Resp: responder, Nonresp: nonresponder, ECOG: Eastern Cooperative Oncology Group, LDH: lactate dehydrogenase (0 vs. 1: lower vs. higher than the upper limit of normal), BORR: best overall response, PFS(mo): progression-free survival (months), Progr: progression, OS(mo): overall survival (months).

Kaplan–Meier survival analysis demonstrated a near-significant (p = 0.0530) association of PFS with HLA class I expression detected by the HC10 antibody (Figure 9A) and a highly significant association (p = 0.0025) with HLA class II expression (Figure 9B). The combined score of HLA class I and class II expression was also significantly linked to PFS (p = 0.0166; Figure 9C). Overall survival analysis demonstrated significant association only with HLA class II expression (p=0.0126; Figure 9D).



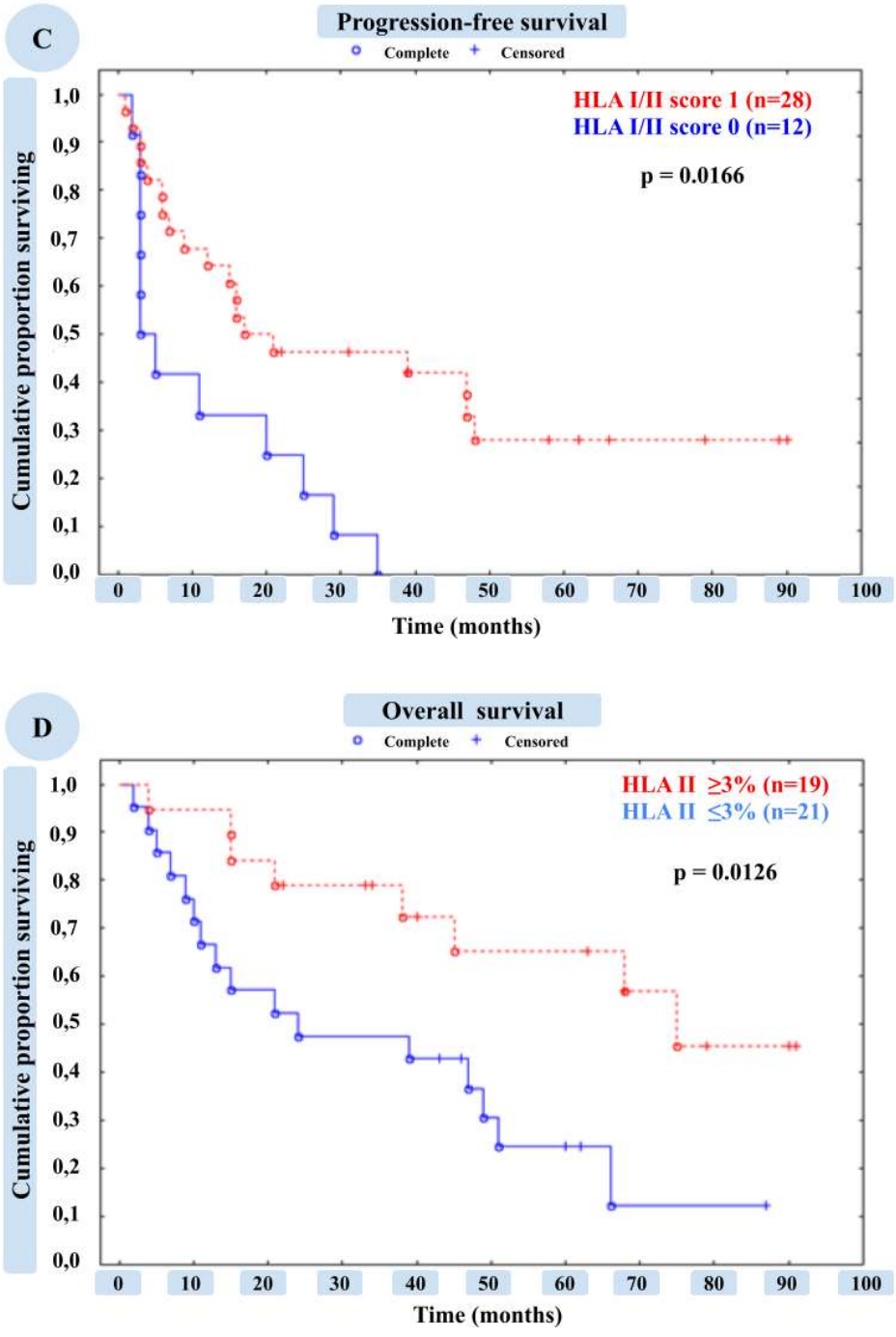



**Figure 9 |** Kaplan–Meier curves of progression-free survival for melanoma patients subdivided according to staining with HLA class I-specific antibody HC10 (A), tumor cell HLA-II expression (B), HLA I/II score (C), overall survival according to tumor cell HLA class II expression (D).

## Discussion

Although ICI therapy has revolutionized the treatment of many cancer types, the majority of patients fail to achieve durable responses, and continued efforts to unravel the mechanisms of immunotherapy resistance are necessary to allow the optimization of therapeutic strategies for patients. In addition, PD-L1 determination is not a satisfactory biomarker in many cases in terms of the expected outcome of therapy, but so far its use has spread widely in several different tumor types, but we know that PD-L1 negative tumors often respond to ICI therapy too.

In our studies, we focused on investigating local immunological parameters, i.e., tumor cell HLA expression and immune cell infiltration, as potential resistance mechanisms and predictive biomarkers of the efficacy of ICI treatment in metastatic melanoma patients.

As antigen presentation by MHC-I molecules on target cells is essential for recognition and killing by CD8+ cytotoxic T lymphocytes, it is a logical assumption that MHC-I expression by tumor cells would be needed for the efficiency of T cell-based immunotherapies such as ICIs. Surprisingly, however, the few earlier studies aiming at demonstrating the predictive role of tumor cell HLA class I protein expression in patients treated with PD-1 inhibitors could not prove an association with therapy outcomes in melanoma (56), although it was found to be predictive in the case of CTLA-4 blocking with ipilimumab (54). On the other hand, HLA-II expression by tumor cells predicted anti-PD-1/PD-L1 therapy efficacy in melanoma and breast cancer, while no such association was found with ipilimumab efficacy in melanoma patients (55, 71).

For these reasons, our research first investigated whether loss of HLA-I expression could be the underlying cause of resistance to ICI therapy in melanoma patients. Subsequently, in the second half of our research, we examined whether HLA-I and HLA-II expression can be used as predictive biomarkers in patients receiving PD-1 inhibitor therapy.

### Study I

In the first study, we analyzed HLA class I tumor cell expression as well as CD8+ T cell and NK cell infiltration in longitudinal tumor samples from a subset of patients with available pre-treatment and post-treatment surgically removed metastases. Analyses

of longitudinal tumor samples from different stages of treatment are necessary for better understanding of the mechanisms of response or resistance to this type of therapy (50). Most such studies performed so far have focused mainly on characterization of early on-treatment tumor biopsies, yielding important information regarding the biological effects of ICI therapies as well as predictive biomarkers (72, 73) while few studies aimed at investigating tumors progressing after or on ICI therapy, especially in case of CTLA-4 inhibitors (74).

To the best of our knowledge, ours is the first study interrogating HLA class I expression longitudinally in tumor samples of patients treated with ipilimumab. We found decreased tumor cell expression in the majority of progressing metastases of all nonresponding patients; this decrease was most marked in the case of patients with the worst prognosis, although a statistical analysis of the correlation with survival could not be performed because of the limited number of patients tested. Nevertheless, these results support the hypothesis of immunoediting in patients receiving ipilimumab treatment, resulting in HLA class I loss and in tumor progression. This process has been described in the case of acquired resistance to immunotherapy, but a low level of antitumor immune activity may be present even in clinically nonresponding patients, which could shape the immunogenicity of the progressing tumors. Unfortunately, we had only one responding patient with residual (minimally progressing) metastases: therefore solid conclusions could not be drawn from our study. Nonetheless, it is worth mentioning that HLA class I expression in both pre-treatment and post-treatment tumors was consistently high in this patient, implicating lack of immunoediting, at least regarding HLA class I expression.

We also examined potential changes in infiltration level of two types of immune effector cells, CD8+ T lymphocytes and NK cells, both of which were found associated with clinical response to ipilimumab in the previous study of the research group on pre-treatment metastatic samples (65). The density of CD8+ T cells showed considerable variability among metastases, even in the same patient, and no consistent difference could be observed between pre-treatment and post-treatment samples. Similarly, no significant pre-treatment/post-treatment change could be found in the case of NK cells; the latter were not detectable or present in only a low number in most of the metastases examined, in agreement with the information in the literature (75, 76). Investigations on longitudinal samples from melanoma patients receiving anti-PD-1 mAbs found elevated infiltration

level of CD8+ T cells in on-treatment samples of responding patients (73, 77), while few and uncertain data are available on progressing cases (78). In a recent study higher density of NK cells was observed in pre-treatment and early during treatment tumor samples of responders compared to nonresponders (75). Few reports have been published on longitudinal studies in melanoma patients treated with ipilimumab. A study on advanced melanoma patients demonstrated increase in tumor-infiltrating lymphocytes in early on-treatment biopsies of patients benefiting from the therapy, but no significant change in the number of CD8+ T cells (72).

We recognize the inherent limitations of our study caused by its retrospective nature and also by the limited number of cases with available pre-treatment and post-treatment surgical samples. However, there are few reports of longitudinal studies on local immunological features of patients receiving immune checkpoint inhibitor therapy, especially in the case of ipilimumab, and most of them encompass a relatively small sample size. The findings of our pilot study will require validation in future prospective studies involving larger patient cohorts enabling more complex statistical analysis.

A strength of our analysis, on the other hand, is that it was performed on whole sections from surgical samples; therefore the results we have presented are expected to be more reliable than those generated by the analysis of biopsies, given the known heterogeneous distribution within tumors of both HLA antigens and immune cells (79, 80).

**Study II**

In our second study on stage IV melanoma patients, a higher level of tumor cell HLA class I expression was detected by two of the four antibodies used: HC10, recognizing mainly HLA-B and -C antigens and the pan-HLA class I EMR8-5; this result is similar to our findings in patients receiving ipilimumab therapy (55). The discrepancy regarding the predictive role of melanoma cell HLA class I expression between our results and those obtained in previous studies (54, 56) may partly be due to methodological differences, for example, using biopsies vs. whole sections; moreover, in one of the previous studies, only HLA-A expression was tested while HLA-B and -C were not (56). On the other hand, in our cohort, HLA class II expression seemed to have a more significant impact on the treatment outcome, which is in line with the results of previous

investigations on melanoma patients receiving anti-PD-1 treatment and different from the findings of studies on ipilimumab therapy (54, 55).

The different mechanisms of action of anti-CTLA-4 and anti-PD-1 agents may explain the observed dissimilarities in the associations of HLA class I vs. class II tumor cell expression with therapy outcomes; however, the exact mechanisms explaining this divergence are unclear at present. An additional potential explanation for the stronger association of HLA class II expression with treatment response is that it is highly inducible by cytokines such as IFN-γ, which is mainly produced by T lymphocytes. Consequently, HLA-II expression generally shows a strong correlation with the density of T cells (81, 82), which is often reported as a factor predicting immunotherapy response; thus, it is possible that the association between HLA-II expression and treatment response results, at least in part, indirectly from the above correlations.

In the present study, the associations with either clinical response or survival did not appear to depend on the percentage of HLA-II-positive tumor cells (comparing cases with 3–4% vs. >10% positivity; see Table 4), which may also support the hypothesis of indirect effect. Another alternative mechanism that may be claimed to be in the background of the impact of HLA-II expression in immunotherapy efficiency is the possibility of the activation of helper and cytotoxic CD4+ T lymphocytes by tumor antigens presented by these molecules (83).

MHC-II molecules can bind an even greater diversity of peptides than MHC-I, and a study on 5942 tumors demonstrated that mutated peptides that poorly bound to MHC-II were positively selected during tumorigenesis, which had a stronger effect compared to selective pressure against MHC-I-restricted neoantigens (84, 85). In a clinical trial of personalized neoantigen vaccine plus anti-PD-1 therapy, the successful induction and cytotoxic potential of neoantigenspecific CD4+ and CD8+ T cells was demonstrated (86). Furthermore, a study analyzing infused TIL products in melanoma patients receiving adoptive cell therapy revealed that neoantigen-specific T cell clones were predominantly CD4+ and displayed cytotoxicity (87).

With regard to the seemingly lower influence of pretreatment tumor cell HLA class I expression (compared to HLA-II expression) on the efficacy of PD-1 blocking agents observed in our and others' studies, it should be mentioned that in our cohort, there were very few patients with completely or almost completely negative tumor cell staining

with the four anti-HLA-I antibodies used, and it is possible that a low level of expression is sufficient for T cell recognition is some cases. Furthermore, the ICI treatment was reported to result in immune activation in the TME, for example, enhanced CD8+ T cell infiltration (88), which could induce HLA class I expression on tumor cells in response to increased lymphocyte cytokine production. The potential changes in TME could not be taken into account in our analysis, which evaluated only pretreatment samples. Nevertheless, we could detect an additional value of determining HLA class I expression in the pretreatment tumor samples since the combined score of HLA-I and HLA-II expression proved a more robust predictor of clinical response than HLA-II expression alone. The same results could be obtained when using a simplified score consisting of the combination of HLA-II expression with HLA-I expression based on labeling by the pan-HLA class I antibody EMR8-5 (instead of on labeling by all four antibodies used), which would provide a biomarker that could be used more easily in routine clinical practice.

Besides tumor cell MHC expression, we also investigated the predictive potential of a panel of immune cell markers and found a significant association with response to anti-PD-1 therapy in the case of eight of the ten immune cell types studied. The densities of most of the studied immune cell types strongly correlated with each other and they frequently showed coordinate presence, which occurred more frequently in the responders. A detailed presentation of these results will be carried out by another member of our research group in his PhD thesis.

The analysis of progression-free survival demonstrated a tendency of better PFS in cases with high HLA class I expression detected by the HC10 antibody. However, significantly longer PFS was demonstrated in cases with high tumor cell expression of HLA class II, high HLA I/II scores. Overall survival, on the other hand, showed a significant association only with HLA-II expression, which was also the factor most significantly influencing PFS. The less prominent associations of the examined factors with OS compared to the clinical response and PFS may be due to the fact that after progression on anti-PD-1 therapy, other treatment modalities were applied in most patients, so their overall survival was not only influenced by the effectivity of PD-1 blocking therapy.

While our study identified potential biomarkers of responses to PD-1 blocking therapy, we recognize its inherent limitations, mainly caused by its retrospective nature.

Moreover, the number of cases included in the analysis was constrained by the availability of sufficient surgical samples and the selection criteria we used in an attempt to decrease patient and sample variability (e.g., including only stage IV melanoma patients, whole sections of surgical samples, and only lymph node and skin/s.c. metastases). On the other hand, we believe that the applied selection criteria enhance the reliability of our results compared to studies performed on samples of unspecified or very heterogeneous locations and studies with a wide range of time intervals between sample acquisition and the implementation of immunotherapy. Also, the use of whole sections vs. small biopsies (or tissue microarrays) could reduce the impact of intratumoral heterogeneity, which is important in the case of immune markers that are often highly heterogeneously distributed within the tissue.

A further limitation of our study is that it included only pretreatment tumor samples, intending to identify potential predictive markers. However, the tumor microenvironment is dynamically changing during progression and therapies, and in order to explore the reasons for acquired resistance to immunotherapy, longitudinal analyses would be beneficial.

Based on the results of our research, we can conclude that our initial assumptions were correct, according to which tumor HLA expression plays a role in the development of resistance to ICI therapy since we found HLA-I downregulation in the progressing metastases of non-responding patients treated with ipilimumab therapy.

The second half of the study confirmed our hypothesis that HLA expression could be a potential predictive biomarker in melanoma patients receiving PD-1 inhibitor therapy. For easier clinical application, we also created a combined scoring system that showed a strong correlation with progression-free survival.

Given the limitations of the two studies, we plan to validate the obtained results in a larger number of cases within the framework of a prospective, longitudinal study in the future. The summary of our two researches is illustrated in the figure below (Figure 10).

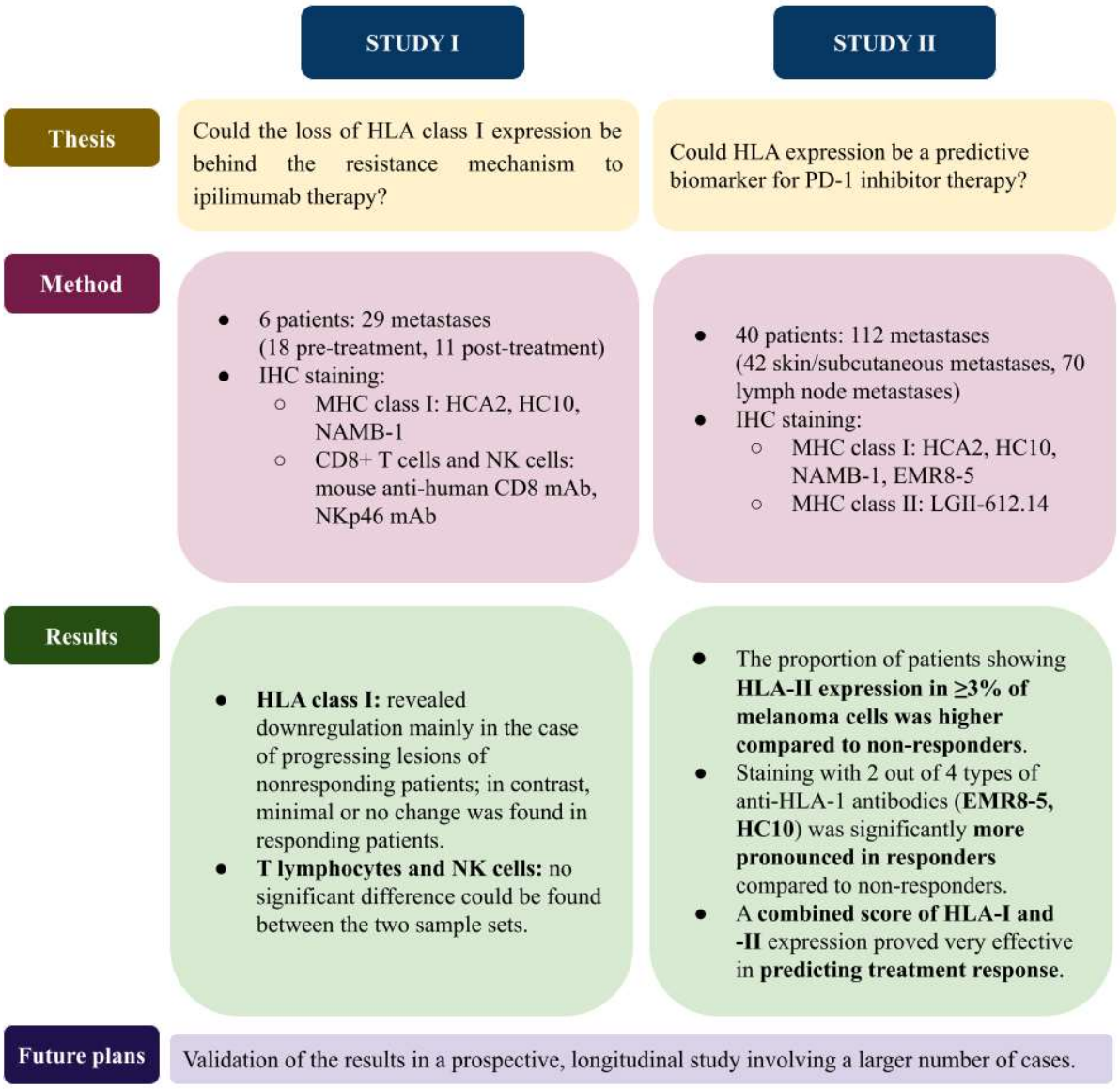



**Figure 10 |** Summary of our research

## Conclusions

### Study I

1. In conclusion, we found a decreased HLA class I expression level by malignant cells in post-treatment progressing metastases of melanoma patients receiving ipilimumab therapy compared to pre-treatment metastatic samples. This finding was a consistent feature in our cohort of patients with progressing tumors, but was not observed in the residual metastases of a responding patient.
2. The extent of infiltration of CD8+ T cells in pretreatment vs. post-treatment tumors showed considerable intertumor variability and did not exhibit any consistent change between pre- and post-treatment time points.
3. We detected a very low number of NK cells infiltrating both pre-treatment and post-treatment tumors; furthermore, no significant difference could be found between the two sample sets.

### Study II

1. Among the responders, the proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders.
2. Similarly, a significant difference was found in the case of two anti-HLA-I antibodies (HC10 and EMR8-5), with fewer cases showing decreased expression in responders.
3. A combined score of HLA-I and -II expression was introduced, which proved very effective in predicting treatment response.
4. Survival analysis demonstrated a significant association of PFS with HLA-II expression and with the combined score of HLA-I/II expression. Overall survival analysis demonstrated significant association only with HLA-II expression.

Our results support earlier reports on the predictive value of tumor cell HLA-II expression and suggest the potential additive value of the HLA-I expression level.
Further work is warranted to validate these findings in larger patient cohorts. Accumulating evidence on immunologic changes observed in longitudinal studies of patients receiving immunotherapy will contribute to an improved understanding of the molecular mechanisms underlying resistance to such therapies and may help to find further predictive biomarkers for ICI therapy.

## Summary

The introduction of ICI therapy has changed the treatment protocol for melanoma, providing patients with a significant survival advantage compared to previous treatment options. Since only a part of the patients benefit from the therapy, and the development of resistance mechanisms can also be expected in them after a while, it has become necessary to develop biomarkers that can be sufficiently predictive of ICI therapy and explain the background of the resistance that has developed.

In our studies, we focused on investigating local immunological parameters, i.e., tumor cell HLA expression and immune cell infiltration, as potential resistance mechanisms to ipilimumab therapy and as predictive biomarkers of the efficacy of PD-1 inhibitor treatment in metastatic melanoma patients.

In our first study we examined 29 metastatic melanoma samples (18 pre-treatment, 11 post-treatment) from six patients treated with ipilimumab. We used immunohistochemical staining to determine HLA class I expression level and the intratumoral infiltration of CD8+ T lymphocytes and NK cells. We revealed HLA-I downregulation mainly in the case of progressing lesions of nonresponding patients; in contrast, minimal or no change was found in responding patients. The infiltration level of CD8+ T lymphocytes and NK cells showed no consistent change between pre- and post-treatment samples.

In our second study we analyzed samples of 112 skin, subcutaneous and lymph node melanoma metastases from 40 patients treated with nivolumab or pembrolizumab. We determined with immunohistochemistry the HLA class I and class II expression level. The proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders and two anti-HLA-I antibodies (HC10 and EMR8-5) showed similar results. A combined score of HLA-I and -II expression proved very effective in predicting treatment response.

Our future plans are to validate a prospective study with longitudinal sample analyses in a larger patient cohorts treated with ICI therapy.

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**Bibliography of the candidate's publications**

**Publications related to the thesis:**

***1.***

*Hegyi, Barbara; Csikó, Kristóf György; Balatoni, Tímea; Fröhlich, Georgina; Bőcs, Katalin; Tóth, Erika; Mohos, Anita; Neumark, Anna Rebeka; Menyhárt, Csenge Dorottya; Ferrone, Soldano; Ladányi, Andrea*

Tumor-Infiltrating Immune Cells and HLA Expression as Potential Biomarkers Predicting Response to PD-1 Inhibitor Therapy in Stage IV Melanoma Patients

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**2.**

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HLA Class I Downregulation in Progressing Metastases of Melanoma Patients Treated With Ipilimumab.

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**Publications not related to the thesis:**

**3.**

*Svajda, Laura; Cserepes, Mihály; Hegyi, Barbara; Niczky, Theodora; Tóvári, József*

Immunmoduláció a tumor mikrokörnyezetében: a tumorhipoxia és a PD-1/PD-L1 együttes gátlásának terápiás lehetőségei

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**4.**

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# HLA Class I Downregulation in Progressing Metastases of Melanoma Patients Treated With Ipilimumab

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Characterization of the molecular mechanisms underlying antitumor immune responses and immune escape mechanisms has resulted in the development of more effective immunotherapeutic strategies, including immune checkpoint inhibitor (ICI) therapy. ICIs can induce durable responses in patients with advanced cancer in a wide range of cancer types, however, the majority of the patients fail to respond to this therapy or develop resistance in the course of the treatment. Information about the molecular mechanisms underlying primary and acquired resistance is limited. Although HLA class I molecules are crucial in the recognition of tumor antigens by cytotoxic T lymphocytes, only a few studies have investigated the role of their expression level on malignant cells in ICI resistance. To address this topic, utilizing immunohistochemical staining with monoclonal antibodies (mAbs) we analyzed HLA class I expression level in pre-treatment and post-treatment tumor samples from melanoma patients treated with ipilimumab. Twenty-nine metastases removed from six patients were available for the study, including 18 pre-treatment and 11 post-treatment lesions. Compared to metastases excised before ipilimumab therapy, post-treatment lesions displayed a significantly lower HLA class I expression level on melanoma cells; HLA class I downregulation was most marked in progressing metastases from nonresponding patients. We also evaluated the level of infiltration by $CD8^+$ T cells and NK cells but did not find consistent changes between pre- and post-treatment samples. Our results indicate the potential role of HLA class I downregulation as a mechanism of ICI resistance.

**Keywords: immunotherapy, melanoma, ipilimumab, HLA class I expression, longitudinal study**

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## INTRODUCTION

Immune checkpoint inhibitor (ICI)-based therapy has brought major breakthrough in cancer treatment, becoming the mainstream of treatment for many cancer types. The first such agent was the anti-CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) monoclonal antibody ipilimumab, which was approved for treatment of patients with advanced melanoma in 2011 [1, 2], later followed by antibodies blocking PD-1 (programmed death receptor 1) or its ligand, PD-L1

(programmed death ligand 1). These monoclonal antibodies have induced impressive clinical responses in a small proportion of patients in a broad spectrum of cancer types. However, the majority of patients do not respond or develop resistance to these immunotherapeutic agents. The mechanisms of primary and acquired resistance are poorly understood. Many potential biomarkers have been proposed that could predict the efficacy of ICI therapies. They include, among others, PD-L1 expression by tumors when PD-1- or PD-L1-specific mAbs are used, tumor mutational burden (TMB), neoantigen load, microsatellite instability, tumor infiltration by immune cells and immune-related gene expression in tumors [3, 4]. Moreover, many potential mechanisms underlying acquired resistance to ICI-based therapy have been identified [3–5]. They include neoantigen loss [6], loss of PTEN expression and activation of β-catenin [7], mutations in JAK1/2 leading to defects in the IFN signaling pathway, mutations in beta-2 microglobulin (B2M), the light chain of HLA class I antigens, resulting in defective HLA class I antigen presentation [8–10], or upregulation of other immune checkpoints such as TIM-3, LAG-3 or VISTA [7, 9]. The same mechanisms have also been implicated in primary resistance to ICI-based therapy [3, 4, 8, 10, 11].

The efficacy of ICI-based therapy depends on the recognition of tumor antigens by cognate cytotoxic T lymphocytes in the context of human leukocyte antigen (HLA) class I molecules. The key role played by HLA class I molecules may account for the described associations of some of their characteristics with response to checkpoint blockade-based therapy. They include the association of maximal heterozygosity of HLA-I loci as well as high evolutionary divergence of HLA class I genotype with improved survival following ICI-based therapy [12, 13], and the association of high degree of HLA-I promiscuity with reduced survival and lower response rate in patients receiving ICIs [14], in addition to the mentioned primary or acquired resistance to anti-PD-1 mAb-based therapy in patients with structural mutations or loss of heterozygosity (LOH) of B2M [8–10]. The frequency of defects in HLA class I antigen processing machinery (APM) component expression and/or function caused by structural mutations is low [15, 16] and therefore has limited clinical relevance. In contrast, the frequency of defects in HLA class I APM component expression and/or function caused by epigenetic mechanisms and/or transcription dysregulation is high in most, if not all cancer types analyzed [15–17]. Nevertheless only a few studies have assessed the value of HLA class I expression level as a biomarker to predict the clinical responses to ICI-based therapy and have found an association between these two parameters [18, 19]. Furthermore, low gene or protein expression of the HLA class I APM components has been described in progressing lesions in some patients with melanoma, lung cancer, or Merkel cell carcinoma treated with ICI-based therapy [7, 9, 10, 20]. These results have been mainly obtained in patients treated with anti-PD-1 mAb; to the best of our knowledge, no information is available about melanoma patients treated with ipilimumab. In a recent study we have shown that tumor cell HLA class I expression level in pre-treatment samples of melanoma patients is a biomarker of clinical response to ipilimumab therapy and of patients' survival [19]. To explore potential changes in HLA-I expression level in ipilimumab-treated patients, in the present investigation we have assessed HLA class I expression level on melanoma cells in pre- and post-treatment metastases removed from patients treated with ipilimumab. Since effective tumor antigen recognition relies on the interaction between $CD8^+$ cytotoxic T lymphocytes and HLA class I molecules while HLA-I negative tumors may be sensitive to killing by natural killer (NK) cells, we also examined infiltration of pre- and post-treatment tumor samples by $CD8^+$ T cells and NK cells.

# MATERIALS AND METHODS

## Tumor Samples

We obtained archived paraffin blocks of sequential (pre- and post-treatment) tissue samples of patients with metastatic melanoma treated with ipilimumab between 2010 and 2015. Sample collection was restricted to metastases surgically removed within a 2 years range before or after ipilimumab treatment; 29 metastases of six patients were available for the study. The clinical characteristics of the patients are shown in **Table 1**. TNM classifications and stage grouping criteria were based on the 7th Edition of AJCC Staging System. Five of the six patients received systemic treatment before ipilimumab therapy; all of them had chemotherapy while two also received radiotherapy, and one patient (Pt3) had already received ipilimumab therapy 32 months before the ipilimumab reinduction treatment evaluated in the present study. Responses to therapy were evaluated based on immune-related response criteria (irRC) [21]. One patient (Pt1) was scored as complete response (CR) with a few residual cutaneous papules, which showed minimal progression 11 months following initiation of ipilimumab therapy and were excised. Pt2 achieved stable disease (SD) lasting for 10 months, while Pt 3 showed short-term SD lasting for 4 months; the other three patients exhibited progressive disease (PD). Pt1 and Pt2 were classified as responders while the other four patients as nonresponders in the analysis. Progression-free survival (PFS) and overall survival (OS) were calculated from the commencement of ipilimumab treatment till the last follow-up, tumor progression or death, respectively. Altogether 29 metastases were studied, 18 pre-treatment and 11 post-treatment surgical samples (**Table 1**). Of the post-treatment samples, three were residual metastases from Pt1 while the other eight were progressing lesions.

## Monoclonal Antibodies

The mouse monoclonal antibody (mAb) HCA2, recognizing B2M-free HLA-A (excluding -A24), -B7301, and -G heavy chains, the mAb HC10, which recognizes B2M-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73), and HLA-C heavy chains and the B2M-specific NAMB-1 were developed and characterized as described [22, 23]. The mouse anti-human CD8 mAb and the mouse anti-human NKp46 mAb were purchased from Dako

**TABLE 1 |** Patient and sample characteristics.

| | Age (years) | Gender | Stage | ECOG Status | BRAF Status | BOR | PFS (months) | OS (months) | Pre samples analyzed | Post samples analyzed |
|---|---|---|---|---|---|---|---|---|---|---|
| Pt1 | 52 | Female | III N3c | 0 | mut | CR | 11 | 67+ | 2 (cut.) | 3 (cut./sc.—residual) |
| Pt2 | 51 | Female | IV M1c | 0 | mut | SD | 10 | 43 | 1 (sc.) | 1 (sc.—progression) |
| Pt3 | 73 | Male | IV M1a | 0 | wt | SD | 4 | 42 | 4 (LN, cut./sc.) | 2 (LN, sc.—progression) |
| Pt4 | 53 | Female | IV M1b | 0 | mut | PD | 4 | 29 | 3 (LN, sc.) | 3 (sc.—progression) |
| Pt5 | 59 | Male | IV M1c | 1 | wt | PD | 3 | 9 | 1 (sc.) | 1 (cut.—progression) |
| Pt6 | 57 | Female | IV M1c | 0 | mut | PD | 3 | 8 | 7 (LN, breast) | 1 (LN—progression) |

*ECOG, Eastern Cooperative Oncology Group; mut, mutant; wt, wild type; BOR, best overall response; CR, complete response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival; Pre, pre-treatment; Post, post-treatment; cut., cutaneous; sc., subcutaneous; LN, lymph node.*

(Glostrup, Denmark) and from R&D Systems (Abingdon, United Kingdom), respectively.

## Immunohistochemical Staining of Tumor Tissue Sections

Immunohistochemical staining of tissue sections of formalin-fixed, paraffin-embedded tumor samples was performed as described earlier [19, 24]. Briefly, deparaffinated sections were treated with 3% $H_2O_2$ in methanol to block endogenous peroxidases, then antigen retrieval was performed by heating at 98°C for 40 min in citrate buffer (pH 6.0), followed by incubation with protein blocking solution (Protein Block, Serum-Free, Dako) for 10 min at room temperature, and incubation with the primary antibodies overnight at 4°C. For staining detection the SS™ One-Step Polymer-HRP IHC Detection System (BioGenex, Fremont, CA) and 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA) were used followed by counterstaining with hematoxylin. In the case of labeling with anti-HLA class I mAbs, the percentage of the area displaying stained melanoma cells was determined in the metastases. Intratumoral density of $CD8^+$ and $NKp46^+$ lymphocytes was assessed as described earlier [24]; briefly, the number of labeled cells was counted within the metastases in at least 10 (median: 35, range: 10–120) randomly chosen fields per sample, using a graticule of 10 × 10 squares designating an area of 0.0625 $mm^2$ at ×400 magnification. For patients with more than one metastasis available the average values were also calculated for each marker, separately for pre- and post-treatment samples. The statistical significance of the differences between pre- and post-treatment samples was determined using the Mann-Whitney U test.

## Computerized Analysis of the Staining Intensity by Anti-HLA Class I Antibodies

The immunohistochemistry slides were acquired in TissueFaxs brightfield (Tissuegnostics, Vienna, Austria) system with a ×40 magnification dry lens coupled onto a Zeiss Axio Imager Z2 Microscope (Jena, Germany) and an eight slide automatic stage (Märzenhäuser, Wetzlar, Germany) using a Pixelink camera (Pixelink, Rochester, NY, United States). Regions of interest containing metastases without obvious artifacts were selected (**Supplementary Figure S1**) and analyzed using the HistoQuest (TissueGnostics) image cytometry software. "Cells" were identified on the basis of the hematoxylin stained nuclei and the immunohistochemical reaction was identified by ring mask (**Supplementary Figure S2**) [25]. The cell nuclei area was used to distinguish among cell populations (**Supplementary Figure S3**). The staining signal was quantified using a single-reference-shade color deconvolution algorithm [26]. Quantifications were confirmed visually by the backward connection function of the HistoQuest program (**Supplementary Figures S3, S4**).

# RESULTS

Utilizing IHC staining with mAbs we analyzed the expression of HLA class I subunits in sequential metastasis samples from six melanoma patients treated with ipilimumab; the samples analyzed included 18 pre- and 11 post-treatment surgically excised metastases (**Table 1**). Comparing pre-treatment and post-treatment samples of all patients evaluated together, the expression of HLA class I subunits, as measured by the % of stained melanoma cells, was significantly lower in post-treatment metastases compared to pre-treatment ones (**Figures 1**, **2**). The medians and ranges of the percentage values of melanoma cells stained by HLA-A heavy chain-specific mAb HCA-2, by HLA-B,C heavy chain-specific mAb HC10 and by anti-B2M mAb NAMB-1 were 94.0 (5.1–100), 91.0 (4.5–100) and 90.5 (62.2–100) in the pre-treatment metastases, and 63.5 (0–83.6), 25.0 (0–84.2) and 57.6 (0–93.1) in the post-treatment metastases, respectively. The percentage of melanoma cells stained by all three mAbs tested was higher than 80 in the majority of the 18 pre-treatment metastases analyzed, compared to only 1 of the 11 post-treatment metastases. In agreement with our previous results [19], metastases with a heterogenous staining pattern displayed higher labeling at the margin of the tumors in the proximity of inflammatory cells, consistent with locally induced expression. Percentages of staining with the three antibodies were fairly consistent in the majority of cases, with discrepancies larger than 30% in only 7 of the 29 metastases. Furthermore, comparing tumor cell staining of different lesions with the same antibody, we detected a moderate level of intrapatient heterogeneity in most patients in the case of pre-treatment

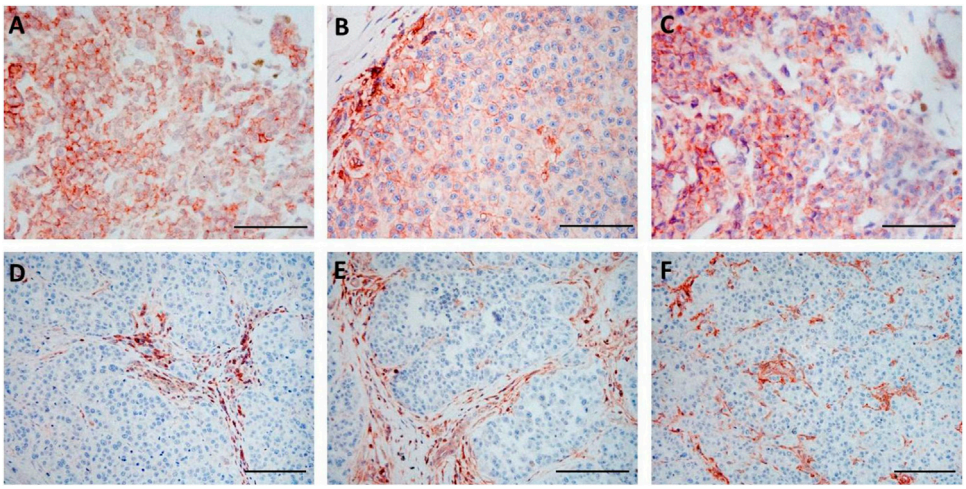


**FIGURE 1 |** Immunohistochemical staining of pre-treatment **(A–C)** and post-treatment **(D–F)** samples from the same patient (Pt3) with HLA-A heavy chain-specific mAb HCA2 **(A,D)**, HLA-B,C heavy chain-specific mAb HC10 **(B,E)** and B2M-specific mAb NAMB-1 **(C,F)** (3-amino-ethylcarbazole, red). Scale bars: 100 μm.

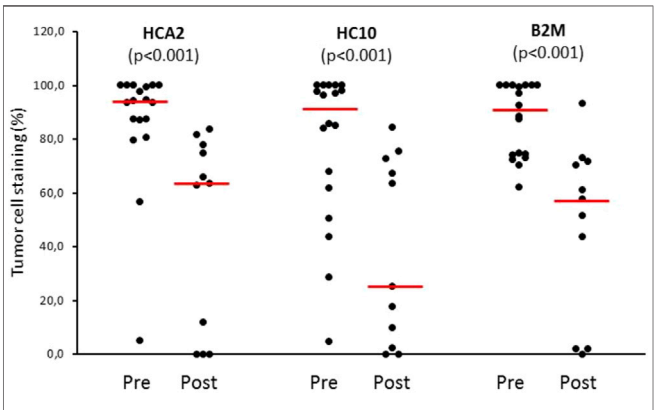


**FIGURE 2 |** HLA class I expression of melanoma cells (% of stained area) in pre-treatment (Pre, $n$ = 18) and post-treatment (Post, $n$ = 11) metastases from ipilimumab-treated patients. Circles: percentage values of individual samples; horizontal line: median.

metastases and in two of the three patients with more than one post-treatment lesions (**Supplementary Table S1**).

Comparison of the HLA class I subunit expression levels in pre- and post-treatment metastases removed from each individual patient revealed HLA-I downregulation mainly in the case of progressing lesions of nonresponding patients; in contrast, minimal or no change was found in responding patients (Pt1 and Pt2). Interestingly, in Pt1 exhibiting the best overall response and long-term survival, the baseline HLA class I subunit expression was high in the pre-treatment metastases and remained high in the post-treatment (residual) metastases (**Figures 3A–C**, **Supplementary Table S1**). In contrast, HLA class I subunit downregulation was maximal in the metastases from Pt5 and Pt6 exhibiting the shortest PFS and OS (**Figure 3D**, **Supplementary Table S1**). Results of quantitative evaluation of staining intensity in representative pre- and post-treatment samples of nonresponding patients with progressing lesions are in agreement with this finding (**Figure 3E**).

Since the efficacy of immune checkpoint inhibitors depends on the recognition of tumor antigen derived peptides by cytotoxic T lymphocytes in the context of HLA class I proteins, we also examined the extent of infiltration of $CD8^+$ T cells in pre-treatment vs. post-treatment tumors. The infiltration showed considerable intertumor variability and did not exhibit any consistent change between pre- and post-treatment time points (**Supplementary Figure S5**). Furthermore, the extent of NK cell infiltration was also tested because these cells are known to recognize HLA class I negative cells so their activity could possibly complement that of $CD8^+$ T lymphocytes. Using NKp46 as a NK cell marker, we detected a very low number of NK cells infiltrating both pre-treatment and post-treatment tumors; furthermore, no significant difference could be found between the two sample sets (**Supplementary Figure S5**).

## DISCUSSION

Many currently used antitumor immunotherapeutic modalities rely on T cell recognition of tumor antigens, in which presentation by HLA class I antigens has a crucial role. It is also an important factor in spontaneous (not therapy-induced) immunity against tumors, which is reflected by the reported association of defects of HLA class I APM component expression with immune escape, disease progression and poor prognosis in several tumor types [16, 17, 27]. Moreover, B2M aberrations have been implicated as resistance mechanisms in patients treated with different T-cell based immunotherapies [28–30] or with immune checkpoint inhibitors [8–10]. However, genomic loss of B2M occurs infrequently, and there is little information about the role of other possible causes of decreased B2M and HLA class I expression in unresponsiveness or acquired resistance to ICIs.

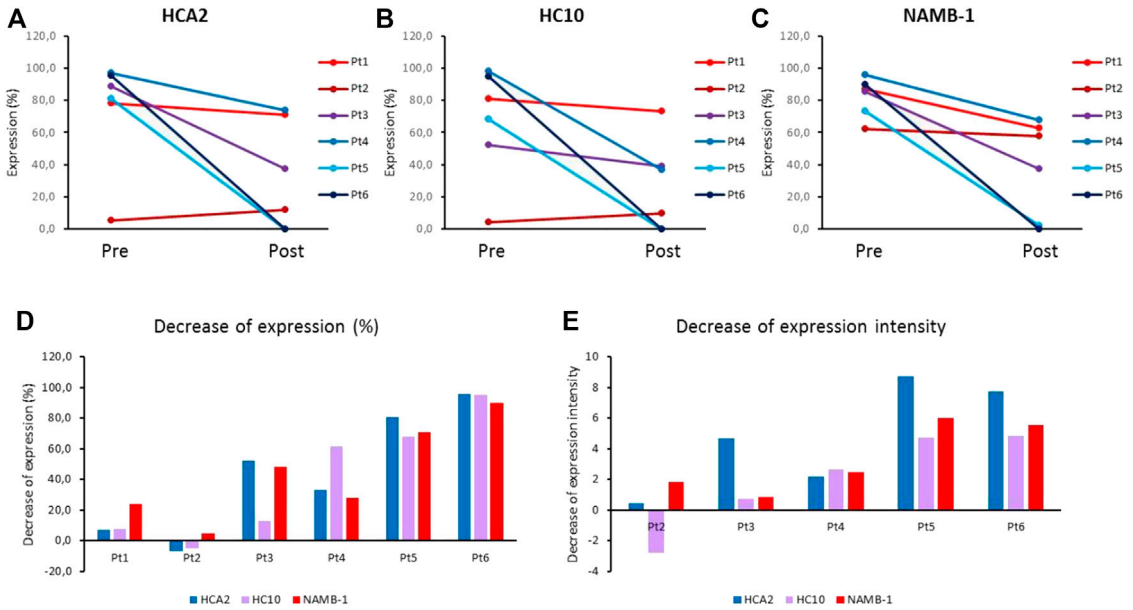


**FIGURE 3 | (A–C)** Average HLA class I expression of melanoma cells (% of stained area) in pre-treatment (Pre) and post-treatment (Post) metastases from ipilimumab-treated patients, labeled by HLA class I subunit-specific mAbs HCA2 **(A)**, HC10 **(B)** and NAMB-1 **(C)**. **(D,E)** Decrease of mean expression (% of stained area) **(D)** and of staining intensity **(E)** in post-treatment metastases as compared to autologous pre-treatment metastases from ipilimumab-treated patients, stained by HLA class I subunit-specific mAbs.

Results of studies on associations of gene alterations or loss of HLA class I APM components with response to ICIs are equivocal. While mutations or LOH of B2M were described in some patients exhibiting primary or acquired resistance to PD-1 inhibitors [8–10], other recent studies did not find an association between LOH in B2M or HLA class I loci and response to anti-PD-1/PD-L1 agents [31, 32]. Moreover, pre-treatment HLA class I gene expression or mutational status did not differ in responders vs. nonresponders to ipilimumab [33]. On the other hand, an APM score composed of eight APM-associated genes (including B2M) predicted response to anti-PD-1/PD-L1 agents [34]. Furthermore, HLA genes were found to be upregulated in on-therapy samples of responders, but downregulated in nonresponders in melanoma patients treated with anti-PD-1 therapy [35].

Few studies have examined protein expression of HLA class I molecules in patients receiving ICI therapy. A study on metastatic melanoma patients treated with ICI therapies [10] found decreased B2M and/or HLA class I expression in some patients harboring B2M gene alterations and showing primary or acquired ICI resistance. Another report on metastatic melanoma [7] described downregulation of HLA-A expression in biopsies of progressing lesions compared to pre-treatment ones in 4 of 18 melanoma patients receiving ICI therapy [7]. However, no significant alterations in gene or protein expression of HLA class I were found in progressing tumors in six patients with different types of carcinomas [36]. A recent study [37] demonstrated low HLA class I expression in 40% of pre-treatment and 31% of progression melanoma tumors, and no association with response to PD-1 inhibition. Similarly, no association between HLA class I expression and response to anti-PD-1 therapy was found in melanoma patients in another study, although it proved predictive of response to anti-CTLA-4 treatment [18]. The results of our previous study corroborated the role of HLA class I expression in influencing the efficacy of ipilimumab [19].

In the present work, we analyzed HLA class I tumor cell expression as well as $CD8^+$ T cell and NK cell infiltration in longitudinal tumor samples from a subset of patients with available pre-treatment and post-treatment surgically removed metastases. Analyses of longitudinal tumor samples from different stages of treatment are necessary for better understanding of the mechanisms of response or resistance to this type of therapy [3]. Most such studies performed so far have focused mainly on characterization of early on-treatment tumor biopsies, yielding important information regarding the biological effects of ICI therapies as well as predictive biomarkers [35, 38–42] while few studies aimed at investigating tumors progressing after or on ICI therapy, especially in case of CTLA-4 inhibitors [43]. To the best of our knowledge, ours is the first study interrogating HLA class I expression longitudinally in tumor samples of patients treated with ipilimumab. We found decreased tumor cell expression in the majority of progressing metastases of all nonresponding patients; this decrease was most marked in the case of patients with the worst prognosis, although a statistical analysis of the correlation with survival could not be performed because of the limited number of patients tested. Nevertheless, these results support the hypothesis of immunoediting in patients receiving ipilimumab treatment, resulting in HLA class I loss and in tumor progression. This process has been described in the case of acquired resistance to immunotherapy, but a low level of antitumor immune activity may be present even in clinically nonresponding patients, which could

shape the immunogenicity of the progressing tumors. Unfortunately, we had only one responding patient with residual (minimally progressing) metastases: therefore solid conclusions could not be drawn from our study. Nonetheless, it is worth mentioning that HLA class I expression in both pre-treatment and post-treatment tumors was consistently high in this patient, implicating lack of immunoediting, at least regarding HLA class I expression.

We also examined potential changes in infiltration level of two types of immune effector cells, $CD8^+$ T lymphocytes and NK cells, both of which were found associated with clinical response to ipilimumab in our previous study on pre-treatment metastatic samples [24]. The density of $CD8^+$ T cells showed considerable variability among metastases, even in the same patient, and no consistent difference could be observed between pre-treatment and post-treatment samples. Similarly, no significant pre-treatment/post-treatment change could be found in the case of NK cells; the latter were not detectable or present in only a low number in most of the metastases examined, in agreement with the information in the literature [24, 44, 45]. Investigations on longitudinal samples from melanoma patients receiving anti-PD-1 mAbs found elevated infiltration level of $CD8^+$ T cells in on-treatment samples of responding patients [35, 40, 41, 46], while few and uncertain data are available on progressing cases [41]. In a recent study higher density of NK cells was observed in pre-treatment and early during treatment tumor samples of responders compared to nonresponders [45]. Few reports have been published on longitudinal studies in melanoma patients treated with ipilimumab. A study on advanced melanoma patients demonstrated increase in tumor-infiltrating lymphocytes in early on-treatment biopsies of patients benefiting from the therapy, but no significant change in the number of $CD8^+$ T cells [38].

We recognize the inherent limitations of our study caused by its retrospective nature and also by the limited number of cases with available pre-treatment and post-treatment surgical samples. However, there are few reports of longitudinal studies on local immunological features of patients receiving immune checkpoint inhibitor therapy, especially in the case of ipilimumab, and most of them encompass a relatively small sample size. The findings of our pilot study will require validation in future prospective studies involving larger patient cohorts enabling more complex statistical analysis. A strength of our analysis, on the other hand, is that it was performed on whole sections from surgical samples; therefore the results we have presented are expected to be more reliable than those generated by the analysis of biopsies, given the known heterogeneous distribution within tumors of both HLA antigens and immune cells [47, 48].

In conclusion, we found a decreased HLA class I expression level by malignant cells in post-treatment progressing metastases of melanoma patients receiving ipilimumab therapy compared to pre-treatment metastatic samples. This finding was a consistent feature in our cohort of patients with progressing tumors, but was not observed in the residual metastases of a responding patient. Further work is warranted to validate these findings in larger patient cohorts, as well as to explore whether HLA class I loss represents a common mechanism of primary and acquired resistance to immune checkpoint inhibitors as well as other T-cell based immunotherapeutic modalities in melanoma and other cancer types. Accumulating evidence on immunologic changes observed in longitudinal studies of patients receiving immunotherapy will contribute to an improved understanding of the molecular mechanisms underlying resistance to such therapies and may help to find appropriate strategies to overcome them [3–5].

## ETHICS STATEMENT

The study followed the Declaration of Helsinki and was approved by the Scientific and Ethical Committee of Medical Research Council, Hungary (2506-3/2017/EKU, 12120-1/2019/EKU). Informed consents from patients were not required by the board in case of retrospective studies where it is not possible to obtain consents from the majority of patients as in this case where most patients were deceased at the time of the study.

## AUTHOR CONTRIBUTIONS

Study conception and design: AL. Sample acquisition and patient data management: TB and GL. Immunohistochemistry evaluation: AL, BH, and TB. Computerized image analysis: RR, CW, and JD. Supervision: SF. Manuscript writing and reviewing: AL, JD, and SF.

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## CONFLICT OF INTEREST

AL is an assistant chief editor for Pathology and Oncology Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.por-journal.com/articles/10.3389/pore.2022.1610297/full#supplementary-material

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# Tumor-Infiltrating Immune Cells and HLA Expression as Potential Biomarkers Predicting Response to PD-1 Inhibitor Therapy in Stage IV Melanoma Patients

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**Abstract:** PD-1 inhibitors are known to be effective in melanoma; however, a considerable proportion of patients fail to respond to therapy, necessitating the identification of predictive markers. We examined the predictive value of tumor cell HLA class I and II expression and immune cell infiltration in melanoma patients treated with PD-1 inhibitors. Pretreatment surgical samples from 40 stage IV melanoma patients were studied immunohistochemically for melanoma cell expression of HLA class I molecules (using four antibody clones with different specificities), HLA-II, and immune cell infiltration (using a panel of 10 markers). Among the responders, the ratio of patients showing melanoma cell HLA-II expression was higher compared to non-responders ($p = 0.0158$), and similar results were obtained in the case of two anti-HLA-I antibodies. A combined score of HLA-I/II expression also predicted treatment response ($p = 0.0019$). Intratumoral infiltration was stronger in the responders for most immune cell types. Progression-free survival showed an association with HLA-II expression, the combined HLA score, and the density of immune cells expressing CD134 and PD-1, while overall survival was significantly associated only with HLA class II expression. Our findings corroborate previous results indicating the importance of immune cell infiltration and tumor cell HLA-II expression in the efficacy of PD-1 inhibitor treatment in a "real world" patient cohort and suggest the potential predictive role of HLA class I expression.

**Keywords:** melanoma; immunotherapy; PD-1 inhibitors; biomarker; tumor-infiltrating immune cells; HLA class I and II expression

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## 1. Introduction

Immune checkpoint inhibitors (ICIs), especially antibodies targeting the PD-1/PD-L1 pathway, have transformed cancer treatment, with documented favorable effects in a variety of tumor types, even in advanced stages. However, a considerable proportion of patients (the size of which varies depending on the tumor type) fail to respond to therapy or develop resistance after the initial response. The factors causing either primary or acquired resistance are only partly elucidated, and it is of primary importance to search for biomarkers that could predict the likelihood of therapeutic effects [1–3].

Many potential biomarkers have been proposed that could predict the efficacy of ICI therapies. They include, among others, PD-L1 expression in the case of PD-1/PD-L1 targeted agents, tumor mutational burden (TMB), neoantigen load, microsatellite instability, antigen presentation deficiencies, tumor infiltration by immune cells, and immune-related gene expression in tumors [1–7]. However, few of the above biomarkers have been validated, and most of them were investigated only in one or a few studies. Although malignant melanoma is one of the tumor types with the best response rates to ICIs, more than half of stage IV patients receiving anti-PD-1 therapy still experience treatment resistance [8–10]. There is no approved predictive biomarker for PD-1 blocking therapy in metastatic melanoma patients. PD-L1 expression of tumor cells and/or tumor-infiltrating immune cells, which is a prerequisite of anti-PD-1/PD-L1 treatment for several cancer types, is not a clinically validated biomarker in melanoma; it was found to be associated with clinical response or post-therapy survival in most but not all studies [4,11–14]. Among other components of the tumor immune microenvironment (TIME), infiltration by $CD8^+$ T lymphocytes was consistently found to be predictive of response while results concerning $CD4^+$ T cells are controversial [4,13,15–18]. Other immune cell subset markers (e.g., CD16, CD45RO) and immune checkpoints such as PD-1 and CTLA-4 have also been found to be associated with the treatment outcomes in some studies [4,13,18,19].

Since antigen presentation by MHC class I molecules is the prerequisite of tumor cell recognition by cytotoxic T lymphocytes, considered to be major players in antitumor immune responses, it could be logically expected that MHC-I expression by tumor cells would be required for the efficacy of T cell-based immunotherapies, including ICIs. Human cancer cells frequently downregulate the components of HLA class I antigen processing machinery (APM), which was found to be associated with poor prognosis in several tumor types [20]. The mutation or loss of heterozygosity of β2-microglobulin (β2M) was identified as a mechanism of resistance to immunotherapies, including PD-1 inhibitors [7,21]. However, decreased HLA class I expression is most frequently caused by epigenetic or transcriptional mechanisms [20,22]. The potential association of HLA class I protein expression loss with ICI therapy resistance has been explored only to a limited extent [23–25]. Surprisingly, the studies demonstrated the predictive role of tumor cell HLA class I expression in melanoma patients treated with the CTLA-4 inhibitor ipilimumab [24,25] but not in those receiving PD-1 blocking agents [23,24]. Conversely, HLA class II expression on tumor cells proved to be predictive of anti-PD-1 efficacy but not anti-CTLA-4 effects [23–25].

In our previous studies, we proved the role of HLA class I expression and infiltration by several immune cell types (e.g., $CD4^+$ and $CD8^+$ T lymphocytes, regulatory T cells, B lymphocytes, NK cells, $CD68^+$ macrophages, $PD\text{-}1^+$ and $CD134^+$ lymphocytes) in predicting responses to treatment and/or the survival of melanoma patients receiving ipilimumab therapy [25,26]. The aim of the present study was to explore tumor cell HLA class I and class II expression and tumor-infiltrating immune cell types as potential predictive markers in melanoma patients treated with PD-1 inhibitors.

## 2. Materials and Methods

### *2.1. Patients and Tumor Samples*

Archived paraffin blocks of pretreatment surgical samples from 40 stage IV melanoma patients receiving anti-PD-1 therapy (nivolumab 240 mg every two weeks or pembrolizumab 200 mg every 3 weeks, start of treatment in 2015–2022) at the National Institute of On-

cology were selected. Only patients receiving at least 3 cycles of anti-PD-1 therapy were included. Tumor resection samples of lymph node ($n$ = 70) and skin/subcutaneous metastases ($n$ = 42), obtained within 4 years of starting anti-PD-1 therapy (median 10 months, range 1–48), were selected; the study cohort consisted of 40 patients (1–9 examined lesions per patient). The primary site was cutaneous in 38 cases and unknown in 2 cases. Most patients ($n$ = 28) received the anti-PD-1 treatment as first-line therapy. Response assessment was based on immune-related response criteria (irRC) [27]; patients with complete or partial remission as best overall response were considered responders in the evaluation. Patients' follow-up was performed by CT every 3 months and if it was necessary, MRI or PET/CT was also performed. Follow-up time of surviving patients was median 62.5 months (22–91). Progression-free survival (PFS) and overall survival (OS) were defined as the time from commencing anti-PD-1 treatment to disease progression or death or last follow-up, and death or last follow-up, respectively. Patient characteristics are shown in Table 1.

**Table 1.** Patient and sample characteristics.

| | **Responder (CR, PR)** ***n* = 28** | **Non-Responder (SD, PD)** ***n* = 12** | ***p* Value** |
|---|---|---|---|
| Age, years: median (range) | 62 (27–87) | 53 (34–77) | NS [a] |
| Gender | | | |
| female | 11 | 2 | |
| male | 17 | 10 | NS [b] |
| ECOG performance status | | | |
| 0 | 27 | 8 | |
| 1 | 1 | 4 | 0.0223 [b] |
| BRAF mutation status | | | |
| wild type | 20 | 10 | |
| mutant | 8 | 2 | NS [b] |
| LDH level | | | |
| normal | 23 | 4 | |
| >ULN | 5 | 8 | 0.0075 [b] |
| Anti-PD-1 drug | | | |
| nivolumab | 9 | 8 | |
| pembrolizumab | 19 | 4 | NS [b] |
| Line of therapy | | | |
| 1 | 20 | 8 | |
| 2–5 | 8 | 4 | NS [b] |
| PFS, months: median (range) | 27 (4–90+) | 3 (1–11) | 0.0000 [a] |
| OS, months: median (range) | 46 (13–91+) | 9 (4–62+) | 0.0002 [a] |

[a] Mann–Whitney test, [b] Fisher's exact test. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, ECOG: Eastern Cooperative Oncology Group, BRAF: B-Raf proto-oncogene, LDH: lactate dehydrogenase, PFS: progression-free survival, OS: overall survival, ULN: upper limit of normal, NS: not significant.

### *2.2. Immunohistochemical Staining*

Immunohistochemical staining of tissue sections of formalin-fixed, paraffin-embedded tumor samples was performed utilizing standard methodology as described earlier [25,26]. Briefly, after deparaffination, sections were treated with 3% $H_2O_2$ in methanol to block endogenous peroxidases, followed by antigen retrieval by heating at 98 °C for 40 min in citrate buffer (pH 6.0). After incubation with protein blocking solution (Protein Block, Serum-Free, Dako, Glostrup, Denmark) for 10 min at room temperature, primary antibodies were applied overnight at 4 °C. For the detection of immune cell markers, the following monoclonal antibodies were used: CD8 (C8/144B; Dako), CD45R0 (UCHL1; Dako), CD20y (L26; Dako), FOXP3 (236/E7; eBioscience, San Diego, CA, USA), NKp46/NCR1 (195314;

R&D Systems, Minneapolis, MN, USA), CD103 (EPR4166(2); Abcam, Cambridge, UK), CD134 (Ber-ACT35; BioLegend, San Diego, CA, USA), CD137 (BBK-2; Santa Cruz Biotechnology, Dallas, TX, USA), PD-1 (NAT-105; Bio SB, Santa Barbara, CA, USA), PD-L1 (73-10; Abcam). For detecting MHC class I molecules, HCA2 (Origene, Rockville, MD, USA), recognizing β2M-free HLA-A (excluding -A24), -B7301, and -G heavy chains; HC10 (Origene), recognizing β2M-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73) and HLA-C heavy chains; the β2-microglobulin (β2M) specific monoclonal antibody NAMB-1; and the anti-pan HLA class I EMR8-5 (Abcam) were utilized; HLA-DR,DQ,DP was detected using the monoclonal antibody LGII-612.14. Staining was detected using the EnVision+ System HRP Labeled Polymer Anti-mouse reagent (Dako) and the MACH2 Rabbit HRP-Polymer (Biocare Medical, Pacheco, CA, USA) for mouse and rabbit primary antibodies, respectively, followed by staining with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA, USA) and hematoxylin counterstaining.

### *2.3. Evaluation of the Immune Reactions*

Evaluation of immunohistochemical staining was performed independently by two researchers who were blinded to the clinical information, as described earlier [25,26], using light microscope equipped with an eyepiece graticule, and the mean value of their separate counts was used for the analysis. For immune cell evaluation, labeled cells within the metastases were counted in at least 10 randomly chosen fields per section, using the graticule of 10 × 10 squares designating an area of 0.0625 $mm^2$ at 400× magnification. PD-L1 expression by immune cells was evaluated similarly to the other immune cell markers, while its expression by tumor cells (tumor proportion score, TPS) was also registered. HLA class I staining was scored as 0, 1, and 2 when the percentage of stained melanoma cells was <25%, 25–75%, and >75%, respectively, based on the criteria established by the 12th International Histocompatibility Workshop (1996). Expression on normal cells in the samples (e.g., immune cells, cells of the vasculature) served as positive control. In the case of HLA-DR,DQ,DP, the percentage of the area displaying positive tumor cell staining was determined in the metastases. We also calculated a combined score of HLA class I and class II expression (HLA I/II score) based on the number of anti-HLA-I antibodies showing higher positivity than the cutoff level, combined with HLA-II positivity in a given sample (score of 1 in the case of high labeling with at least 3 anti-HLA-I antibodies, and/or HLA-II expression $\geq$3%; score of 0 when neither of the above criteria are met). For patients with more than one metastasis available, the average scores were calculated for each marker. Cutoff levels were set up based on the median of the given variable in the whole patient cohort, with modifications for better discriminating power in some cases, and the proportion of patients with a mean cell density/HLA expression score higher than the cutoff level was also determined for all markers.

### *2.4. Statistical Analysis*

For the statistical analysis of differences in cell densities and HLA expression levels between different patient groups, we applied the Mann–Whitney U test, while Fisher's exact test was used to compare the proportions of cases in different groups. Correlations were analyzed by Pearson's nonparametric correlation method. Survival analysis was carried out using the Kaplan–Meier method and log-rank test. Differences were considered significant in the case of $p$ values $\leq 0.05$. Statistics were calculated using the Statistica software version 12.5 (StatSoft, Tulsa, OK, USA).

## 3. Results

Of the 40 stage IV melanoma patients involved in this study, 28 exhibited a complete ($n$ = 14) or partial response ($n$ = 14) (Table 1). Among clinical parameters, the ECOG performance status and pretreatment LDH level showed an association with the response. Pretreatment surgical samples of lymph node and cutaneous/subcutaneous melanoma

metastases were immunohistochemically analyzed for HLA expression (Figure 1a), evaluating it using scores between 0 and 2 for HLA class I and determining the percentage of positive tumor area in the case of HLA class II. Considering all metastases, tumor cell HLA-I positivity levels determined by the four different antibodies (HCA2, HC10, NAMB-1, EMR8-5) showed a strong positive correlation, with the lowest correlation coefficient (0.6812) in the case of HCA2/HC10 and highest (0.8236) in the case of NAMB-1/EMR8-5 (all $p$ values < 0.0001), while HLA-II staining showed a weaker correlation with HLA-I positivity (correlation coefficients 0.2182–0.2944). HLA class I expression scores were the highest in the case of the EMR8-5 pan-HLA-I antibody (mean ± SD, 1.4 ± 0.7) and the lowest in the case of HC10 (1.1 ± 0.8). Melanoma cells in metastases of 20 patients (50%) did not express HLA class II, while mean tumor cell staining higher than 10% was observed in 11 patients (27.5%). The tumor samples of eight patients showed 3–4% HLA-II staining, mainly in tumor cells near the inflammatory cells at the margin of metastases, consistent with locally induced HLA expression.

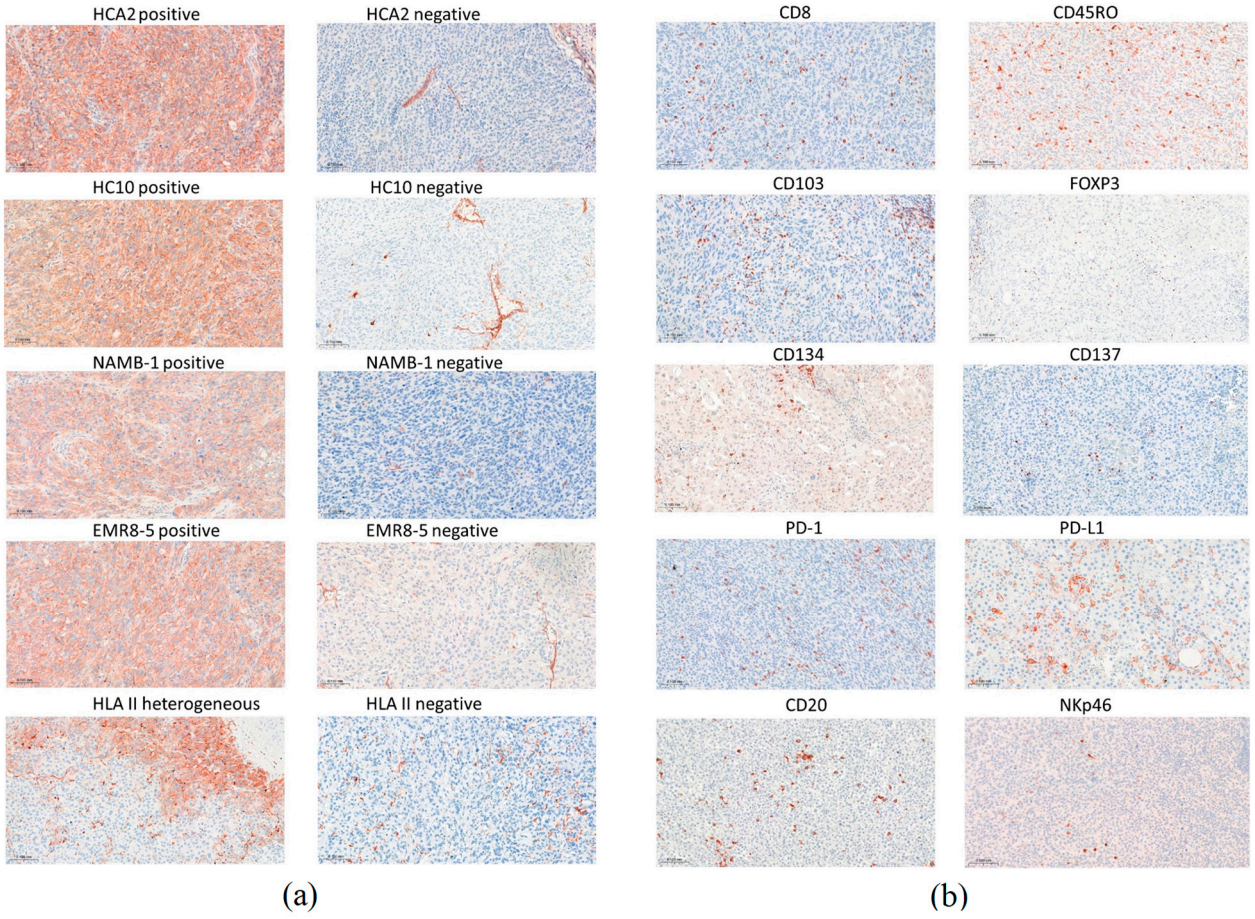


**Figure 1.** Immunohistochemical staining of melanoma metastases with HLA class I and class II-specific antibodies (**a**) and labeling of immune cell markers (**b**) (3-amino-ethylcarbazole, red). Scale bars: 100 μm.

Among the responders, the proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders (17/28 vs. 2/12, $p$ = 0.0158). Similarly, a significant difference was found in the case of two anti-HLA-I antibodies (HC10 and EMR8-5), with fewer cases showing decreased expression in responders (Table 2). A combined score of HLA-I and -II expression was introduced (being positive in the case of high HLA class I and/or HLA class II expression), which proved very effective in predicting treatment response ($p$ = 0.0019) (Table 2).

**Table 2.** Relationship of treatment response with the proportion of patients with high tumor cell HLA expression in metastases.

| | **Responder (CR, PR)** ***n* = 28** | **Non-Responder (SD, PD)** ***n* = 12** | ***p* Value** [a] |
|---|---|---|---|
| HLA class I; Ab clone (cutoff score) | | | |
| HCA2 [b] (≥1.3) | 16/27 (59%) | 5/12 (42%) | 0.4877 |
| HC10 (≥0.6) | 24/28 (86%) | 6/12 (50%) | 0.0411 |
| NAMB-1 (≥1.7) | 14/28 (50%) | 3/12 (25%) | 0.1788 |
| EMR8-5 (≥1.4) | 20/28 (71%) | 4/12 (33%) | 0.0367 |
| HLA class II (cutoff: ≥3%) | 17/28 (61%) | 2/12 (17%) | 0.0158 |
| HLA I/II score [c] (cutoff: ≥3) | 24/28 (86%) | 4/12 (33%) | 0.0019 |

[a] Fisher's exact test. [b] Staining with HCA2 could not be evaluated in one patient because of negativity of normal cells. [c] Combined score of high expression with the anti-HLA-I and/or the anti-HLA-II antibodies. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

We also determined the intratumoral density of immune cell subsets in the melanoma metastases using 10 different markers (CD8, CD45RO, CD20, FOXP3, NKp46, CD103, CD134, CD137, PD-1, PD-L1) (Figure 1b). Among the immune cell types, CD45RO$^+$ T lymphocytes were present in the highest number, followed by CD8$^+$ T lymphocytes, PD-L1$^+$, CD103$^+$, and PD-1$^+$ cells, while NKp46$^+$ NK cells and cells expressing the CD134 and CD137 activation markers were the least numerous (Table 3). Evaluating the infiltration of the labeled cells in relation to immunotherapy response revealed that most of the immune cell subsets were present at higher amounts in the metastases of responding patients compared to non-responders (Table 3, Figure 2). Analyzing the proportion of patients with high mean intratumoral cell density showed higher prevalence in responders in the case of eight of the ten immune cell markers (Table 3). The most significant differences were observed in the case of PD-1 (23/28 vs. 3/12, $p = 0.0010$) and PD-L1 (19/28 vs. 1/12, $p = 0.0012$). Interestingly, PD-L1 expression (≥1%) by tumor cells did not predict treatment response significantly (9/28 of responders, 1/12 of non-responders; $p = 0.2307$), in contrast to its expression by immune cells.

Table S1 presents individual patients' data and a heatmap showing all markers (HLA expression and immune cell density), demonstrating an overall higher HLA expression and immune cell prevalence in responders. A coordinated high expression of at least six of the ten immune cell markers was found in 18 of the 28 responders (64%) and only 1 of the 12 non-responders (8%) ($p = 0.0015$).

**Table 3.** Relationship of treatment response with the density of immune cells infiltrating metastases.

| **Density of Labeled Cells (*n*/mm$^2$), Median (Range)** | | | |
|---|---|---|---|
| **Immune Cell Marker** | **Responder (CR, PR)** ***n* = 28** | **Non-Responder (SD, PD)** ***n* = 12** | ***p* Value** [a] |
| CD8 | 96.0 (12.8–540.8) | 31.2 (1.6–347.2) | 0.0112 |
| CD45RO | 416.8 (64.0–769.6) | 194.4 (83.2–774.4) | 0.1918 |
| CD20 | 18.4 (0.0–195.2) | 7.2 (0.0–27.2) | 0.0093 |
| NKp46 | 3.0 (0.0–16.3) | 1.3 (0.0–10.6) | 0.3132 |
| FOXP3 | 24.0 (4.8–100.8) | 16.0 (6.4–108.8) | 0.1084 |
| CD134 | 4.9 (0.0–68.0) | 2.5 (0.0–10.7) | 0.0390 |
| CD137 | 7.4 (1.1–37.3) | 3.8 (0.2–17.0) | 0.2992 |
| CD103 | 76.0 (17.6–246.4) | 29.6 (1.6–131.2) | 0.0077 |
| PD-1 | 71.2 (4.8–393.6) | 13.6 (0.0–326.4) | 0.0011 |
| PD-L1 | 86.4 (1.6–705.6) | 16.8 (0.0–123.2) | 0.0046 |

**Table 3.** *Cont.*

| Proportion of Cases with High Cell Density (%) | | | |
|---|---|---|---|
| **Immune Cell Marker (Cutoff)** | **Responder (CR, PR)** $n = 28$ | **Non-Responder (SD, PD)** $n = 12$ | ***p* Value** [b] |
| CD8 (≥54 cells/mm$^2$) | 20/28 (71%) | 3/12 (25%) | 0.0130 |
| CD45RO (≥264 cells/mm$^2$) | 18/28 (64%) | 3/12 (25%) | 0.0378 |
| CD20 (≥28 cells/mm$^2$) | 10/28 (36%) | 0/12 (0%) | 0.0188 |
| NKp46 (≥3.4 cells/mm$^2$) | 13/28 (46%) | 3/12 (25%) | 0.2969 |
| FOXP3 (≥20 cells/mm$^2$) | 18/28 (64%) | 3/12 (25%) | 0.0378 |
| CD134 (≥4.1 cells/mm$^2$) | 17/28 (61%) | 2/12 (17%) | 0.0158 |
| CD137 (≥6.7 cells/mm$^2$) | 14/28 (50%) | 3/12 (25%) | 0.1788 |
| CD103 (≥36 cells/mm$^2$) | 21/28 (75%) | 4/12 (33%) | 0.0297 |
| PD-1 (≥20 cells/mm$^2$) | 23/28 (82%) | 3/12 (25%) | 0.0010 |
| PD-L1 (≥54 cells/mm$^2$) | 19/28 (68%) | 1/12 (8%) | 0.0012 |

[a] Mann–Whitney test, [b] Fisher's exact test. CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

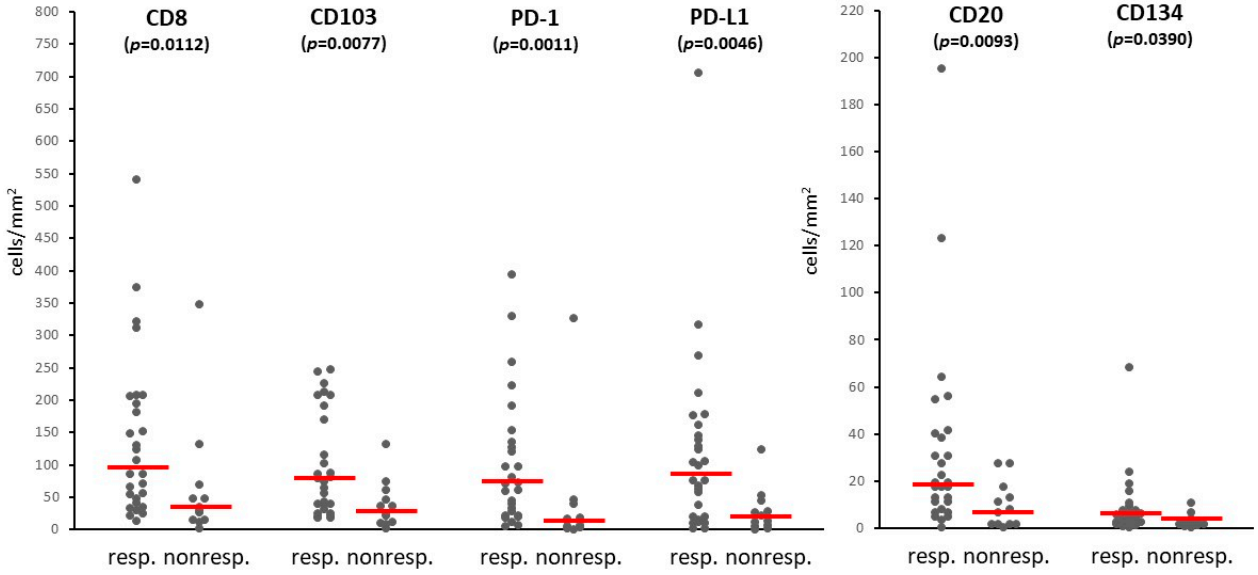


**Figure 2.** Immune cell infiltration in metastases of responder and non-responder patients treated with PD-1 inhibitors (dots: mean density values in samples from individual patients; horizontal line: median).

Kaplan–Meier survival analysis demonstrated a near-significant ($p = 0.0530$) association of PFS with HLA class I expression detected by the HC10 antibody and a highly significant association ($p = 0.0025$) with HLA class II expression (Figure 3a,b). The combined score of HLA class I and class II expression was also significantly linked to PFS ($p = 0.0166$; Figure 3c). Among immune cell markers, a significant survival correlation was demonstrated in the case of CD134 and PD-1, both showing longer PFS in cases with high immune cell density ($p = 0.0318$ and $p = 0.0230$, respectively; Figure 3d,e). Overall survival was significantly associated only with HLA class II expression ($p = 0.0126$; Figure 3f).

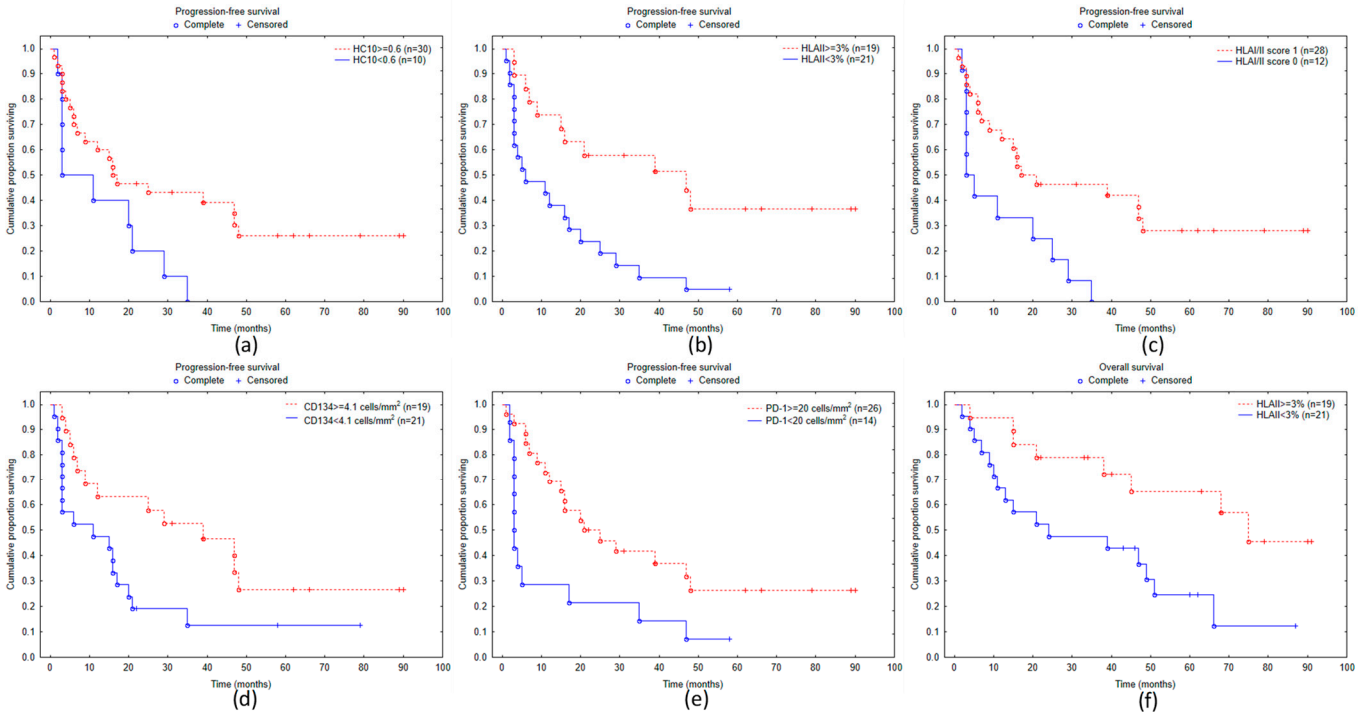


**Figure 3.** Kaplan–Meier curves of progression-free survival for melanoma patients subdivided according to staining with HLA class I-specific antibody HC10 (**a**), tumor cell HLA-II expression (**b**), HLA I/II score (**c**), density of CD134$^+$ (**d**), PD-1$^+$ cells (**e**), overall survival according to tumor cell HLA class II expression (**f**).

## 4. Discussion

Although ICI therapy has revolutionized the treatment of many cancer types, the majority of patients fail to achieve durable responses, and continued efforts to unravel the mechanisms of immunotherapy resistance are necessary to allow the optimization of therapeutic strategies for patients.

In our study, we focused on investigating local immunological parameters, i.e., tumor cell HLA expression and immune cell infiltration, as potential biomarkers of the efficacy of anti-PD-1 treatment in metastatic melanoma patients. As antigen presentation by MHC-I molecules on target cells is essential for recognition and killing by CD8$^+$ cytotoxic T lymphocytes, it is a logical assumption that MHC-I expression by tumor cells would be needed for the efficiency of T cell-based immunotherapies such as ICIs. Surprisingly, however, the few earlier studies aiming at demonstrating the predictive role of tumor cell HLA class I protein expression in patients treated with PD-1 inhibitors could not prove an association with therapy outcomes in melanoma [23,24], although it was found to be predictive in the case of CTLA-4 blocking with ipilimumab [24,25]. On the other hand, HLA-II expression by tumor cells predicted anti-PD-1/PD-L1 therapy efficacy in melanoma and breast cancer, while no such association was found with ipilimumab efficacy in melanoma patients [23–25,28].

In our present study on stage IV melanoma patients, a higher level of tumor cell HLA class I expression was detected by two of the four antibodies used: HC10, recognizing mainly HLA-B and -C antigens and the pan-HLA class I EMR8-5; this result is similar to our findings in patients receiving ipilimumab therapy [25]. The discrepancy regarding the predictive role of melanoma cell HLA class I expression between our results and those obtained in previous studies [23,24] may partly be due to methodological differences, for example, using biopsies vs. whole sections; moreover, in one of the previous studies, only HLA-A expression was tested while HLA-B and -C were not [23]. On the other hand, in our cohort, HLA class II expression seemed to have a more significant impact on the treatment outcome, which is in line with the results of previous investigations on melanoma

patients receiving anti-PD-1 treatment [23,24] and different from the findings of studies on ipilimumab therapy [24,25]. The different mechanisms of action of anti-CTLA-4 and anti-PD-1 agents may explain the observed dissimilarities in the associations of HLA class I vs. class II tumor cell expression with therapy outcomes; however, the exact mechanisms explaining this divergence are unclear at present.

An additional potential explanation for the stronger association of HLA class II expression with treatment response is that it is highly inducible by cytokines such as IFN-γ, which is mainly produced by T lymphocytes. Consequently, HLA-II expression generally shows a strong correlation with the density of T cells [25,29,30], which is often reported as a factor predicting immunotherapy response, which is also supported by our results; thus, it is possible that the association between HLA-II expression and treatment response results, at least in part, indirectly from the above correlations. In the present study, the associations with either clinical response or survival did not appear to depend on the percentage of HLA-II-positive tumor cells (comparing cases with 3–4% vs. >10% positivity; see Table S1), which may also support the hypothesis of indirect effect. Another alternative mechanism that may be claimed to be in the background of the impact of HLA-II expression in immunotherapy efficiency is the possibility of the activation of helper and cytotoxic $CD4^+$ T lymphocytes by tumor antigens presented by these molecules [31]. MHC-II molecules can bind an even greater diversity of peptides than MHC-I, and a study on 5942 tumors demonstrated that mutated peptides that poorly bound to MHC-II were positively selected during tumorigenesis, which had a stronger effect compared to selective pressure against MHC-I-restricted neoantigens [32,33]. In a clinical trial of personalized neoantigen vaccine plus anti-PD-1 therapy, the successful induction and cytotoxic potential of neoantigen-specific $CD4^+$ and $CD8^+$ T cells was demonstrated [34]. Furthermore, a study analyzing infused TIL products in melanoma patients receiving adoptive cell therapy revealed that neoantigen-specific T cell clones were predominantly $CD4^+$ and displayed cytotoxicity [35].

With regard to the seemingly lower influence of pretreatment tumor cell HLA class I expression (compared to HLA-II expression) on the efficacy of PD-1 blocking agents observed in our and others' studies, it should be mentioned that in our cohort, there were very few patients with completely or almost completely negative tumor cell staining with the four anti-HLA-I antibodies used, and it is possible that a low level of expression is sufficient for T cell recognition is some cases. Furthermore, the ICI treatment was reported to result in immune activation in the TME, for example, enhanced $CD8^+$ T cell infiltration [4,19,36], which could induce HLA class I expression on tumor cells in response to increased lymphocyte cytokine production. The potential changes in TME could not be taken into account in our analysis, which evaluated only pretreatment samples. Nevertheless, we could detect an additional value of determining HLA class I expression in the pretreatment tumor samples since the combined score of HLA-I and HLA-II expression proved a more robust predictor of clinical response than HLA-II expression alone. The same results could be obtained when using a simplified score consisting of the combination of HLA-II expression with HLA-I expression based on labeling by the pan-HLA class I antibody EMR8-5 (instead of on labeling by all four antibodies used), which would provide a biomarker that could be used more easily in routine clinical practice.

Besides tumor cell MHC expression, we also investigated the predictive potential of a panel of immune cell markers and found a significant association with response to anti-PD-1 therapy in the case of eight of the ten immune cell types studied: $CD8^+$ and $CD45RO^+$ T lymphocytes, regulatory T cells ($FOXP3^+$), $CD20^+$ B lymphocytes, and cells expressing activation markers/immune checkpoints CD134, PD-1, PD-L1, and CD103, a marker of tissue-resident T cells. Correlations of the prevalence of immune cell subsets with disease outcomes in metastatic melanoma patients after anti-PD-1 therapy have been consistently reported for $CD8^+$ T cells [4,13,15–18] and found in some studies for other immune cell markers, including CD45RO and FOXP3 [13], PD-1, PD-L1 [4,13], and CD103 [37].

To the best of our knowledge, our study is the first that identified the density of $CD134^+$ cells as a factor predicting the response to treatment with anti-PD-1 agents in

metastatic melanoma patients and also showed an association with PFS. CD134 (OX40) is an important costimulatory immune checkpoint molecule, also a T cell activation marker, expressed mainly by $CD4^+$ T cells, also pointing to the potential importance of $CD4^+$ T cell activation upon antigen presentation by HLA-II. It shows transient expression after antigen stimulation, which may be one of the reasons for the relatively low number of $CD134^+$ cells in tumors; furthermore, they generally show a preferential location in peritumoral/stromal areas while, in our study, intratumoral density was evaluated. The prevalence of $CD134^+$ cells was proven to be a prognostic factor in primary melanoma [38] and other cancers [39,40] and a biomarker that could predict the response to ipilimumab therapy [26] in previous studies.

The results regarding $CD20^+$ B lymphocytes are controversial; no correlation with response was found in one study [17], although an association with treatment response was demonstrated in a neoadjuvant trial [41]. The role of B cells in antitumor immune responses is also not clear, with controversial findings regarding their pro- vs. antitumor effects. This discrepancy may be related to the diversity of their functional activities; they can promote tumor growth via multiple mechanisms, e.g., driving chronic inflammation, or immune suppression by regulatory B cells, but may also function as effective antigen-presenting cells promoting antitumor T cell responses [42,43]. Costimulation via the OX40L/OX40 pathway has been described as a mechanism of the B lymphocyte-mediated expansion of $CD4^+$ T cells in a murine model [44]. In this regard, our earlier findings of the colocalization of B cells and $OX40^+$ T lymphocytes in primary melanoma may be of interest, suggesting a possible role of B cells in antigen presentation and costimulation [45].

The densities of most of the studied immune cell types strongly correlated with each other and they frequently showed coordinate presence, which occurred more frequently in the responders. Besides the factors involved in this study, many other elements of the TME may affect the efficacy of immunotherapy, such as macrophages, myeloid-derived suppressor cells, cytokines, and chemokines, among others. According to recent analyses, the application of multiplex immunohistochemistry (IHC)/immunofluorescence techniques yields more accurate predictive markers than studying single factors [18,46]. Multiplex imaging techniques allowing the evaluation of the spatial organization of the tumor microenvironment (TME) are increasingly applied in the search for factors predicting the efficacy of immunotherapy [47,48].

Among the immune cell markers, the most significant association with treatment response was observed in the case of PD-1 and PD-L1; the former showed a correlation with PFS as well. Interestingly, while the intratumoral density of PD-L1-positive immune cells was demonstrated to predict clinical responses to PD-1 blocking therapy in our patient cohort, its expression by melanoma cells did not prove predictive. Evaluating PD-L1 expression by using the CPS (combined positive score) and TPS (tumor proportion score) supported these findings. Previous studies exploring the predictive role of PD-L1 in melanoma patients treated with anti-PD-1 immunotherapy mainly measured its expression by tumor cells, or by tumor and immune cells without distinction [4,11–14], and did not evaluate the impact of immune cell expression separately. In a study applying digital spatial profiling, PD-L1 expression in the $CD68^+$ (macrophage) compartment but not in the tumor compartment proved predictive of immunotherapy effects [49]. In the study by Herbst et al. [50], PD-L1 expression by tumor-infiltrating immune cells, but not by tumor cells showed an association with the response to atezolizumab (anti-PD-L1) treatment in a mixed cohort of different cancer and in the subgroup of NSCLC. Furthermore, according to several studies in mouse models, PD-L1 expression on host myeloid cells, and not on tumor cells, proved essential for PD-L1 pathway-mediated tumor regression [51,52].

The analysis of progression-free survival demonstrated a tendency of better PFS in cases with high HLA class I expression detected by the HC10 antibody. However, significantly longer PFS was demonstrated in cases with high tumor cell expression of HLA class II, high HLA I/II scores, and a high density of $PD\text{-}1^+$ and $CD134^+$ cells. Overall survival, on the other hand, showed a significant association only with HLA-II expression, which

was also the factor most significantly influencing PFS. The less prominent associations of the examined factors with OS compared to the clinical response and PFS may be due to the fact that after progression on anti-PD-1 therapy, other treatment modalities were applied in most patients, so their overall survival was not only influenced by the effectivity of PD-1 blocking therapy.

While our study identified several potential biomarkers of responses to PD-1 blocking therapy, we recognize its inherent limitations, mainly caused by its retrospective nature. Moreover, the number of cases included in the analysis was constrained by the availability of sufficient surgical samples and the selection criteria we used in an attempt to decrease patient and sample variability (e.g., including only stage IV melanoma patients, whole sections of surgical samples, and only lymph node and skin/s.c. metastases). On the other hand, we believe that the applied selection criteria enhance the reliability of our results compared to studies performed on samples of unspecified or very heterogeneous locations and studies with a wide range of time intervals between sample acquisition and the implementation of immunotherapy. Also, the use of whole sections vs. small biopsies (or tissue microarrays) could reduce the impact of intratumoral heterogeneity, which is important in the case of immune markers that are often highly heterogeneously distributed within the tissue.

A further limitation of our study is that it included only pretreatment tumor samples, intending to identify potential predictive markers. However, the tumor microenvironment is dynamically changing during progression and therapies, and in order to explore the reasons for acquired resistance to immunotherapy, longitudinal analyses would be beneficial. In our previous investigation comparing HLA class I expression and immune cell infiltration in pre- and post-treatment tumor samples of melanoma patients receiving ipilimumab therapy, we found HLA-I downregulation in the progressing metastases of non-responding patients [53]. Our future plans include using this approach in a longitudinal study of patients in the present cohort treated with PD-1 inhibitors.

## 5. Conclusions

Summarizing our findings, they corroborate previous results, indicating the importance of tumor infiltration by $CD8^+$ T lymphocytes, $PD\text{-}1^+$ and $PD\text{-}L1^+$ cells, as well as immune cell subsets that are less frequently studied, such as $CD45RO^+$ memory T cells, $FOXP3^+$ regulatory T cells, $CD103^+$ tissue-resident T cells, $CD20^+$ B lymphocytes, in the efficacy of PD-1 blocking therapy. This study also proposes the density of T cells expressing CD134 as a potential new biomarker in a "real world" patient cohort. A coordinated high expression of the immune cell markers was also strongly associated with the treatment response. Furthermore, our results support earlier reports on the predictive value of tumor cell HLA class II expression and suggest the potential additive value of the HLA-I expression level.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/biom14121609/s1, Table S1. Clinicopathological data of individual patients and heatmap of immunohistochemistry results.

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**Informed Consent Statement:** Informed consent from patients was not required by the board in the case of retrospective studies, where it is not possible to obtain consent from the majority of patients as most patients were deceased at the time of the study. In the case of non-interventional, retrospective studies, patients' informed consent is not required. https://njt.hu/jogszabaly/2009-235-20-22 (accessed on 12 December 2024).

**Data Availability Statement:** Data supporting the reported results can be found in the Supplementary Materials or provided by the corresponding author upon reasonable request.

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Table S1. Clinicopathological data of individual patients and heatmap of immunohistochemistry results

| Patient no. | ECOG | Line | BRAF | LDH | Anti-PD-1 drug | BORR | PFS(mo) | Progr | OS(mo) | Death | HC10 | HCA2 | B2M | EMR | HLAII | CD8 | CD45RO | CD20 | FOXP3 | CD134 | CD137 | CD103 | NKp46 | PD-1 | PD-L1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Resp1 | 0 | 4 | 2 | 0 | Pembrolizumab | CR | 16 | 1 | 68 | 0 | 1,0 | 1,0 | 1,0 | 1,5 | 11 | 65,6 | 134,4 | 6,4 | 14,4 | 1,8 | 4,2 | 86,4 | 3,5 | 60,8 | 11,2 |
| Resp2 | 0 | 1 | 1 | 0 | Nivolumab | CR | 89 | 0 | 90 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 87 | 33,6 | 724,8 | 22,4 | 33,6 | 6,7 | 2,7 | 36,8 | 4,2 | 20,8 | 36,8 |
| Resp3 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 47 | 1 | 91 | 0 | 2,0 | 1,3 | 1,0 | 2,0 | 3 | 123,2 | 752,0 | 195,2 | 100,8 | 15,8 | 25,8 | 102,4 | 9,0 | 80,0 | 75,2 |
| Resp4 | 0 | 1 | 2 | 0 | Pembrolizumab | CR | 6 | 1 | 21 | 1 | 2,0 | 1,7 | 2,0 | 2,0 | 0 | 84,8 | 507,2 | 38,4 | 81,6 | 68,0 | 8,6 | 225,6 | 1,1 | 126,4 | 128,0 |
| Resp5 | 0 | 3 | 1 | 0 | Nivolumab | CR | 47 | 1 | 87 | 0 | 0,7 | 1,3 | 0,5 | 1,5 | 0 | 56,0 | 107,2 | 8,0 | 17,6 | 5,3 | 2,6 | 41,6 | 2,1 | 11,2 | 19,2 |
| Resp6 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 39 | 1 | 45 | 1 | 1,8 | 1,7 | 1,7 | 1,8 | 33 | 204,8 | 521,6 | 30,4 | 44,8 | 24,0 | 5,3 | 56,0 | 1,3 | 190,4 | 161,6 |
| Resp7 | 0 | 1 | 2 | 0 | Pembrolizumab | CR | 21 | 1 | 75 | 1 | 0,5 | 1,0 | 1,0 | 1,0 | 4 | 54,4 | 110,4 | 0,0 | 9,6 | 0,0 | 4,0 | 38,4 | 0,0 | 27,2 | 14,4 |
| Resp8 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 62 | 0 | 63 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 4 | 206,4 | 769,6 | 17,6 | 17,6 | 5,9 | 36,6 | 206,4 | 14,4 | 393,6 | 705,6 |
| Resp9 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 39 | 0 | 40 | 0 | 1,9 | 2,0 | 1,9 | 1,9 | 3 | 84,8 | 225,6 | 17,6 | 20,8 | 4,5 | 10,7 | 115,2 | 3,7 | 44,8 | 67,2 |
| Resp10 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 25 | 1 | 39 | 1 | 1,0 | 0,3 | 1,1 | 1,1 | 0 | 147,2 | 425,6 | 54,4 | 41,6 | 5,8 | 15,5 | 73,6 | 9,4 | 96,0 | 268,8 |
| Resp11 | 0 | 2 | 2 | 1 | Pembrolizumab | CR | 58 | 0 | 60 | 0 | 2,0 | 1,8 | 2,0 | 2,0 | 0 | 12,8 | 110,4 | 11,2 | 4,8 | 1,3 | 1,1 | 17,6 | 0,2 | 4,8 | 1,6 |
| Resp12 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 15 | 1 | 15 | 1 | 1,0 |  | 2,0 | 2,0 | 3 | 208,0 | 408,0 | 27,2 | 20,8 | 0,8 | 6,4 | 190,4 | 5,3 | 134,4 | 104,0 |
| Resp13 | 0 | 1 | 1 | 0 | Pembrolizumab | CR | 22 | 0 | 22 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 54 | 540,8 | 681,6 | 40,0 | 48,0 | 1,9 | 9,0 | 243,2 | 7,2 | 329,6 | 211,2 |
| Resp14 | 0 | 1 | 1 | 0 | Nivolumab | CR | 29 | 1 | 43 | 0 | 0,2 | 0,3 | 0,0 | 0,4 | 0 | 193,6 | 257,6 | 41,6 | 43,2 | 9,3 | 8,3 | 38,4 | 1,9 | 72,0 | 315,2 |
| Resp15 | 0 | 2 | 1 | 0 | Nivolumab | PR | 17 | 1 | 24 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 0 | 32,0 | 683,2 | 11,2 | 40,0 | 2,6 | 11,4 | 20,8 | 5,6 | 6,4 | 144,0 |
| Resp16 | 1 | 1 | 1 | 1 | Nivolumab | PR | 90 | 0 | 90 | 0 | 1,0 | 1,0 | 1,0 | 1,0 | 14 | 24,0 | 64,0 | 19,2 | 14,4 | 6,7 | 10,7 | 24,0 | 0,8 | 20,8 | 19,2 |
| Resp17 | 0 | 2 | 2 | 1 | Nivolumab | PR | 79 | 0 | 79 | 0 | 0,7 | 1,6 | 0,9 | 1,4 | 3 | 20,8 | 84,8 | 3,2 | 6,4 | 2,4 | 1,4 | 24,0 | 3,2 | 32,0 | 9,6 |
| Resp18 | 0 | 1 | 1 | 0 | Nivolumab | PR | 16 | 1 | 49 | 1 | 1,5 | 1,5 | 1,7 | 2,0 | 0 | 150,4 | 627,2 | 56,0 | 52,8 | 1,9 | 13,3 | 246,4 | 4,0 | 222,4 | 97,6 |
| Resp19 | 0 | 2 | 2 | 0 | Pembrolizumab | PR | 66 | 0 | 68 | 0 | 0,6 | 1,0 | 1,0 | 1,2 | 32 | 310,4 | 400,0 | 12,8 | 12,8 | 4,3 | 3,2 | 208,0 | 1,6 | 120,0 | 57,6 |
| Resp20 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 35 | 1 | 66 | 1 | 0,0 | 0,7 | 1,0 | 1,0 | 0 | 40,0 | 92,8 | 4,8 | 9,6 | 1,6 | 1,1 | 19,2 | 0,3 | 17,6 | 1,6 |
| Resp21 | 0 | 2 | 1 | 0 | Pembrolizumab | PR | 4 | 1 | 13 | 1 | 1,0 | 0,5 | 2,0 | 2,0 | 0 | 28,8 | 76,8 | 4,8 | 28,8 | 18,7 | 2,4 | 17,6 | 0,0 | 19,2 | 9,6 |
| Resp22 | 0 | 1 | 1 | 1 | Pembrolizumab | PR | 12 | 1 | 15 | 1 | 0,9 | 2,0 | 1,5 | 1,9 | 0 | 70,4 | 441,6 | 30,4 | 20,8 | 10,6 | 4,2 | 30,4 | 0,5 | 38,4 | 105,6 |
| Resp23 | 0 | 1 | 1 | 0 | Nivolumab | PR | 9 | 1 | 38 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 4 | 107,2 | 678,4 | 19,2 | 24,0 | 6,6 | 11,4 | 80,0 | 1,0 | 97,6 | 176,0 |
| Resp24 | 0 | 1 | 1 | 0 | Nivolumab | PR | 20 | 1 | 47 | 1 | 0,0 | 0,5 | 0,5 | 0,0 | 0 | 48,0 | 344,0 | 17,6 | 16,0 | 3,5 | 11,2 | 64,0 | 1,0 | 70,4 | 177,6 |
| Resp25 | 0 | 2 | 2 | 0 | Pembrolizumab | PR | 48 | 1 | 68 | 1 | 1,2 | 2,0 | 1,8 | 1,8 | 33 | 180,8 | 443,2 | 6,4 | 54,4 | 7,7 | 4,2 | 169,6 | 3,7 | 59,2 | 75,2 |
| Resp26 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 6 | 1 | 34 | 0 | 2,0 | 2,0 | 2,0 | 2,0 | 4 | 129,6 | 299,2 | 12,8 | 24,0 | 3,0 | 3,5 | 84,8 | 2,7 | 96,0 | 62,4 |
| Resp27 | 0 | 1 | 1 | 0 | Pembrolizumab | PR | 31 | 0 | 33 | 0 | 1,2 | 0,7 | 1,2 | 1,0 | 23 | 321,6 | 430,4 | 64,0 | 32,0 | 4,3 | 12,5 | 212,8 | 12,6 | 257,6 | 123,2 |
| Resp28 | 0 | 1 | 2 | 1 | Pembrolizumab | PR | 7 | 1 | 21 | 1 | 1,0 | 1,0 | 1,5 | 1,5 | 24 | 374,4 | 616,0 | 123,2 | 44,8 | 7,7 | 37,3 | 78,4 | 16,3 | 152,0 | 137,6 |
| Nonresp1 | 1 | 1 | 1 | 1 | Nivolumab | SD | 5 | 1 | 9 | 1 | 1,0 | 0,5 | 0,0 | 1,0 | 0 | 48,0 | 584,0 | 17,6 | 36,8 | 10,7 | 15,5 | 73,6 | 2,9 | 17,6 | 52,8 |
| Nonresp2 | 0 | 2 | 2 | 1 | Pembrolizumab | SD | 11 | 1 | 51 | 1 | 0,5 | 1,1 | 2,0 | 1,2 | 0 | 48,0 | 86,4 | 1,6 | 17,6 | 1,4 | 1,8 | 35,2 | 0,0 | 38,4 | 11,2 |
| Nonresp3 | 0 | 1 | 1 | 1 | Pembrolizumab | SD | 3 | 1 | 62 | 0 | 0,0 | 1,0 | 0,5 | 0,5 | 0 | 33,6 | 387,2 | 0,0 | 6,4 | 2,9 | 2,6 | 20,8 | 0,0 | 16,0 | 25,6 |
| Nonresp4 | 1 | 1 | 1 | 1 | Pembrolizumab | SD | 3 | 1 | 46 | 0 | 0,0 | 1,2 | 1,2 | 1,0 | 0 | 28,8 | 158,4 | 12,8 | 19,2 | 3,2 | 1,8 | 9,6 | 1,6 | 12,8 | 27,2 |
| Nonresp5 | 1 | 5 | 1 | 1 | Nivolumab | PD | 3 | 1 | 7 | 1 | 0,1 | 0,3 | 0,0 | 0,4 | 0 | 25,6 | 83,2 | 6,4 | 11,2 | 4,0 | 6,6 | 35,2 | 6,6 | 14,4 | 12,8 |
| Nonresp6 | 0 | 2 | 2 | 1 | Nivolumab | PD | 2 | 1 | 4 | 1 | 2,0 | 2,0 | 1,6 | 2,0 | 0 | 11,2 | 137,6 | 1,6 | 6,4 | 1,8 | 4,8 | 1,6 | 6,6 | 6,4 | 43,2 |
| Nonresp7 | 0 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 10 | 1 | 0,5 | 1,5 | 1,0 | 1,0 | 0 | 1,6 | 206,4 | 1,6 | 16,0 | 0,0 | 4,2 | 11,2 | 0,5 | 1,6 | 0,0 |
| Nonresp8 | 0 | 1 | 1 | 1 | Nivolumab | PD | 2 | 1 | 5 | 1 | 0,0 | 0,0 | 0,0 | 0,0 | 0 | 14,4 | 120,0 | 1,6 | 12,8 | 2,2 | 3,5 | 8,0 | 3,2 | 3,2 | 4,8 |
| Nonresp9 | 0 | 2 | 1 | 0 | Nivolumab | PD | 1 | 1 | 2 | 1 | 1,0 | 2,0 | 1,0 | 2,0 | 0 | 131,2 | 252,8 | 8,0 | 41,6 | 0,8 | 17,0 | 60,8 | 1,0 | 46,4 | 20,8 |
| Nonresp10 | 1 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 15 | 1 | 2,0 | 2,0 | 2,0 | 2,0 | 97 | 347,2 | 774,4 | 27,2 | 108,8 | 6,4 | 16,0 | 131,2 | 10,6 | 326,4 | 123,2 |
| Nonresp11 | 0 | 1 | 1 | 1 | Pembrolizumab | PD | 3 | 1 | 4 | 1 | 2,0 | 1,7 | 2,0 | 2,0 | 12 | 14,4 | 182,4 | 11,2 | 8,0 | 1,1 | 0,2 | 46,4 | 1,0 | 4,8 | 1,6 |
| Nonresp12 | 0 | 1 | 1 | 0 | Nivolumab | PD | 3 | 1 | 11 | 1 | 1,2 | 1,2 | 1,5 | 1,1 | 1 | 68,8 | 262,4 | 27,2 | 16,0 | 2,7 | 1,8 | 24,0 | 0,5 | 0,0 | 6,4 |

Red/green color of the heatmap label high/low expression or high/low immune cell density.
Resp: responder, Nonresp: nonresponder, ECOG: Eastern Cooperative Oncology Group, LDH: lactate dehydrogenase (0 vs. 1: lower vs. higher than the upper limit of normal), BORR: best overall response, PFS(mo): progression-free survival (months), Progr: progression, OS(mo): overall survival(months)