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DIFARMA

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PhD Thesis in

Role of CD73 in patients with advanced melanoma treated with anti-PD-1 agents

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“A hill whose height remained unknown was an insult to the intelligence and made him uneasy. Without continually establishing one´s own position, how could one move forward? A riddle, no matter how small, could not be left by the side of the road”.

D. Kehlmann, Measuring the world

Abstract

The treatment of metastatic melanoma was revolutionized by the approval of inhibitors of immune-checkpoints, including antibodies targeting the programmed cell death protein 1 (PD-1), such as nivolumab and pembrolizumab. Anti PD-1 agents improved the survival of patients with advanced melanoma; however, a great percentage of patients do not benefit from treatment with these drugs and understanding the mechanisms influencing the response to anti PD-1 agents is an urgent need.

Extracellular adenosine is a potent anti-inflammatory mediator able to impair anti-tumor immune response. The adenosine pathway has been indicated as one of the mechanisms causing immune suppression and resistance to immune-checkpoint inhibitors. The main enzyme responsible for extracellular adenosine production is the ectonucleotidase CD73, which hydrolyzes AMP into adenosine and inorganic phosphate.

CD73 is anchored to the membrane of many cell types, and its expression is upregulated in several human cancers. This ectonucleotidase is also found on the membrane of exosomes, extracellular vesicles (30-150 nm) that are involved in cell-to-cell communication and are produced by almost all cell types, including cancer cells and immune cells. CD73 can be cleaved from the cell membrane and the soluble form is free to circulate in biological fluids.

The main goal of this PhD project was to investigate the potential of all the forms of CD73 as predictive factors of response in patients with advanced melanoma receiving anti- PD-1 agents (nivolumab, pembrolizumab) alone or in combination with anti CTLA-4 (ipilimumab). The project was divided in four parts, each one focused on a different form of CD73: the cell-bound form expressed by circulating lymphocytes, the soluble and the exosomal form in serum and the cell-bound form within the melanoma lesion.

For the first part of the project, I analyzed the frequency of CD8+ lymphocytes phenotypes in a cohort of 100 patients with melanoma, using blood samples collected prior to the start of treatment (baseline) with nivolumab. High frequency of baseline circulating CD8+PD-1+CD73+ lymphocytes resulted associated with worse survival and no clinical benefits to nivolumab treatment.

In the second part, I characterized the expression and the activity of soluble CD73, in a retrospective study involving 546 melanoma patients, treated with nivolumab or pembrolizumab, or nivolumab plus ipilimumab. Both the activity and the expression of CD73 resulted higher in patients than in healthy donors, but the ROC curve analysis revealed that the enzymatic activity better stratifies patients from healthy donors. High CD73 activity resulted associated with no-response to therapy with anti-PD-1 monotherapy and its combination with anti-CTLA-4. After three months of treatment, soluble CD73 activity remains unchanged from baseline, and it is still higher in non-responders than in responders. High CD73 enzymatic activity resulted associated with reduced overall survival and progression-free survival in patients treated with anti-PD-1 monotherapy. Furthermore, CD73 activity emerged as an independent prognostic factor in multivariate cox regression analysis.

The third part of this project was dedicated to the study of the exosomal form of CD73. Firstly, I optimized a protocol, based on Size Exclusion Chromatography and ultrafiltration, to isolate biologically active exosomes from human serum. Analyses were performed in a single-center cohort of 41 patients with melanoma, treated with pembrolizumab or nivolumab. Exosomes express CD73, which maintains its enzymatic activity. In vitro assays revealed that CD73+ exosomes are able to suppress, in presence of AMP, the production of IFN-γ in activated peripheral blood mononuclear cells (PBMCs) isolated from healthy donors. This effect is mediated by the activation of A2A adenosine receptor expressed on PBMCs.

While no differences were observed in terms of exosomal CD73 expression at baseline between patients responding to therapy and those not responding, interestingly, the exosomal CD73 expression significantly increased after 4 weeks of treatment, compared to baseline levels, in non-responders group. These results indicate that exosome-derived adenosine can suppress T cell functions and the expression of CD73 on exosomes may impact the response to anti PD-1 therapy in patients with advanced melanoma.

The last part of the project was focused on studying the expression of CD73 within melanoma lesions, by performing co-detection by indexing multiplexed tissue imaging (CODEX®) using the PhenoCycler™ instrument (Akoya Biosciences). A panel of antibodies enabling the individuation of the different phenotypes populating the tumor lesion has been developed to obtain a complete overview of the CD73 expression in the tumor microenvironment of melanoma, and thus a better understanding of the prognostic value.

Taken together, the results discussed in this thesis indicate that the measurement of CD73 activity and/or expression could be informative to identify a suppressive mechanism that influence the anti-tumor immune response, impairing the therapeutic effectiveness of immune checkpoint inhibitors.

Both the pretreatment activity of serum CD73 and the pretreatment frequency of circulating CD8+CD73+ T cells emerged as prognostic factors, that may guide the therapeutic choices in patients with advanced melanoma. In addition, this thesis strongly supports the notion that targeting CD73 in combination with anti-PD-1 agents could further improve the clinical response in melanoma patients.

Index

1. Introduction

1.1 Biology of melanoma: a brief overview

Melanoma is one of the most aggressive types of cancer and the deadliest among skin cancers. The incidence of melanoma is increasing, while its mortality is decreasing, thanks to improvements in treatments (Siegel et al., 2021;Sung et al., 2021). Historically, it was considered a rare cancer, but in the last 50 years, melanoma incidence increased faster than other tumors (Rigel and Carucci, 2000;Ferlay et al., 2015;Guy et al., 2015;Siegel et al., 2021).

In 2020, approximately 325.000 new melanoma cases were diagnosed worldwide, and over 57.000 deaths were registered (Sung et al., 2021).

Melanoma originates from the uncontrolled proliferation of melanocytes, driven by a series of mutations and epigenetic alterations of tumor suppressor genes and oncogenes (Palmieri et al., 2015). Notably, the mutation rate in melanoma is the highest among any other cancer type (Lawrence et al., 2013). The mitogen-activated protein kinase (MAPK) pathway is dysregulated in 80% melanomas, being crucial for the melanoma genesis (Govindarajan et al., 2003). The most common mutations interest the *BRAF* gene, occurring in about half of melanomas, the *NRAS* gene, observed in almost 30% of melanomas, and *NF1* gene, in about 15% of tumors (Grzywa et al., 2017). The mutation of one of these genes leads to the activation of ERK signaling, which in turn regulates cell proliferation and survival (Gaestel, 2006).

The individuation of these mutations had a great impact on treatment of melanoma, with the introduction of inhibitors of BRAF or mitogen-activated protein kinase kinase (MEK) as therapeutic strategy (Czarnecka et al., 2020). Based on the most common mutations, the Cancer Genome Atlas Network established four melanoma subtypes (mutant *BRAF*, mutant *RAS*, mutant *NF1*, and Triple-wild-type), in order to help clinical decisions on personalized therapies (TheCancerGenomeAtlasNetwork, 2015).

Of relevance, the gene expression analyses of melanoma revealed the existence of two transcriptional programs, classically defined as proliferative phenotype and invasive phenotype.

The proliferative phenotype is characterized by enhanced proliferation of melanoma cells, linked to high expression of microphthalmia-associated transcription factor (MITF), that is involved in the regulation of cell cycle and pigmentation (Arozarena and Wellbrock, 2019). The expression of Melan-A and SOX10, involved in the pigmentation mechanism, is associated with the high expression of MITF. The invasive phenotype is characterized by the absence of MITF expression and an enhanced expression of tyrosine-protein kinase receptor (AXL) and mesenchymal markers (Arozarena and Wellbrock, 2019). While the proliferative phenotype drives the first steps of the tumor growth, the switch to an invasive phenotype allows cells to invade and disseminate (Figure 1) (Hoek et al., 2008). A switch back to the proliferative phenotype may occur again in the metastatic site (Figure 1).

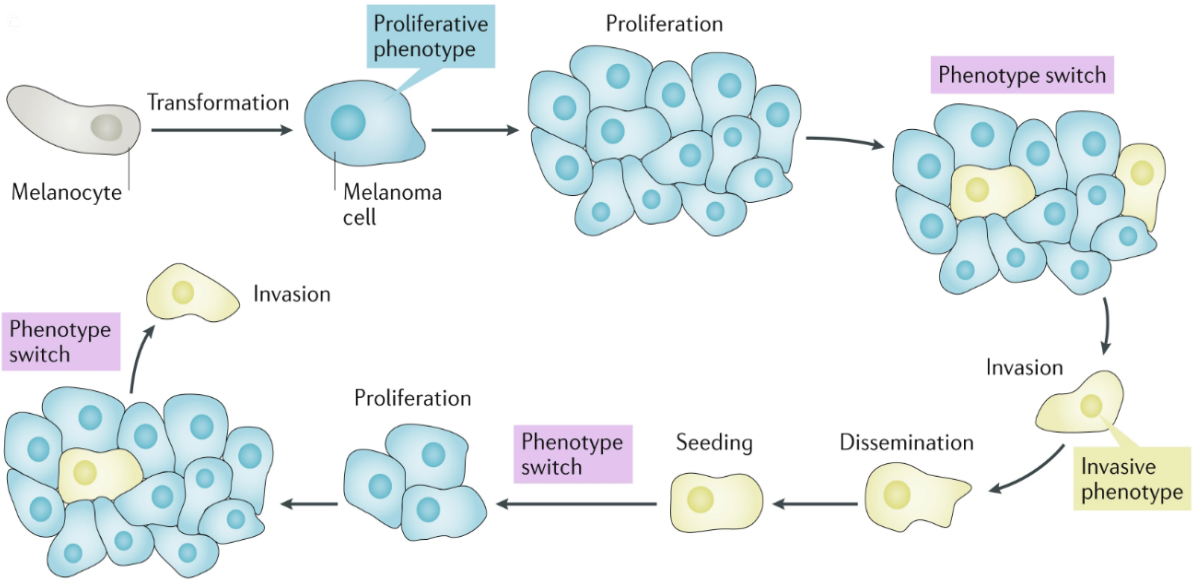

Figure 1 – *Phenotype switch during melanoma progression, modified from Arozarena & Wellbrock, 2019.*

1.2 Advanced melanoma and the revolution of immune checkpoint blockade

Early-stage melanoma is successfully treated with surgical resection, however unresectable stage III or stage IV melanoma have always represented a challenging tumor to treat, being resistant to classical anticancer therapies. Before the approval of targeted therapy and immune checkpoint inhibitors, there were a few treatment options for advanced melanoma, mostly based on dacarbazine chemotherapy, and the chance of tumor regression was less than 10% (Czarnecka et al., 2020;Carlino et al., 2021).

As anticipated, the identification and characterization of common mutations in melanoma led to the development of specific drugs that importantly changed the management of this tumor type. Vemurafenib was the first BRAF inhibitor to be approved by the FDA, in 2011. After that date, other BRAF inhibitors, encorafenib and dabrafenib, were approved, as well inhibitors of MEK, cobimetinib, trametinib and binimetinib (Czarnecka et al., 2020).

The introduction of targeted therapy improved overall survival of patients with advanced melanoma, from a median of 6.2 months (Korn et al., 2008) obtained with classical therapy, to a median of 16.9–33.6 months (Ascierto et al., 2020). To date, three BRAF and MEK inhibitors combination are approved for the treatment of *BRAF* mutated metastatic melanoma: dabrafenib plus trametinib, vemurafenib plus cobimetinib, and encorafinib plus binimetinib.

Unluckily, clinical response to targeted therapy is not durable and acquired resistance appear in half of patients receiving these agents. Importantly, the analyses of melanoma biopsies from patients who progressed on BRAF-targeted therapy revealed a tumor microenvironment characterized by features commonly associated to resistance to immune checkpoint inhibitors (Hugo et al., 2015). Furthermore, preliminary results indicate that long-term overall survival is higher in patients treated with immune checkpoint inhibitors compared to those treated with combined BRAF and MEK inhibitors (Larkin et al., 2019;Robert et al., 2019a;Carlino et al.,

2021). Thus, the use of immune checkpoint inhibitors as first line is currently preferred by clinicians in most cases.

Immune checkpoint inhibitors are a class of drugs blocking signals that otherwise would shut down the activity of immune cells. Physiologically, immune checkpoints have an important role in the control of the immune activation and in the maintenance of self-tolerance, avoiding autoimmunity. The axis PD-1/PD-L1 (programmed cell death protein 1/ programmed cell death ligand 1) has emerged to have an important role in the immune evasion mechanisms that lead to tumor growth (Figure 2). PD-1 is up-regulated in activated T cells and the binding to its ligand PD-L1 inhibits the effector T-cell functions, by terminating the TCR transduction signaling (Figure 2) (Sheppard et al., 2004;Sharpe et al., 2007). Normally, PD-L1 is found on other cells of the immune system or vascular endothelial cells, and it prevents the over activation of T cells (Sharpe et al., 2007). PD-L1 may be expressed by cancer cells, that take advantage from the PD-1/PD-L1 interaction, protecting themselves from T cells.

Another checkpoint of great relevance is CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), also known as CD152, which is commonly expressed by T cells immediately upon activation. As CD28, CTLA-4 can bind two ligands: CD80 (B7-1) and CD86 (B7-2), found on professional antigen-presenting cells, such as dendritic cells, monocytes and activated B cells (Figure 2) (Sansom, 2000). Although they are analogous receptors, CD28 and CTLA-4 mediate opposite effects. The stimulation of CD28, simultaneously with TCR stimulation, leads to T cell activation; whilst CTLA-4 mediates an inhibitory signal that inactivates T cells (Figure 2) (Sansom, 2000).

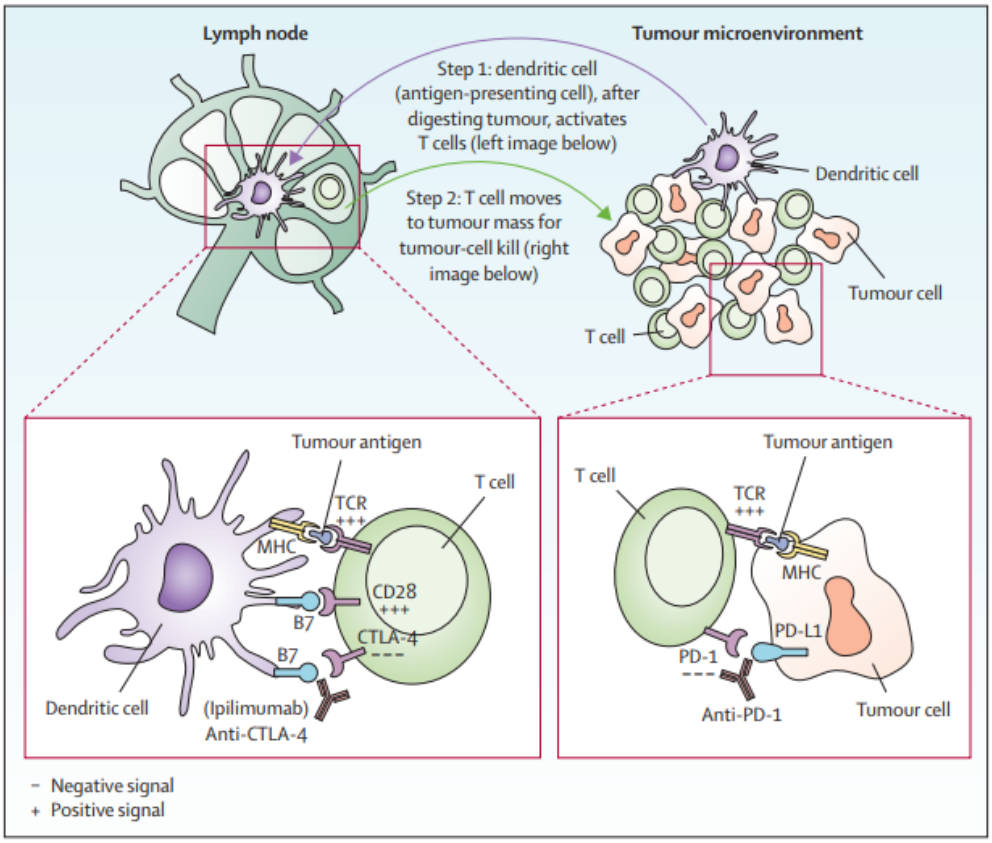

Figure 2 – *Mechanism of action of anti-PD-1 and anti CTLA-4 agents. (Carlino et al., 2021)*

Restoring the anti-tumor immune response, by inhibiting the immune checkpoints signaling, has widely been proven as effective against many cancers, including melanoma.

Among the immune checkpoint inhibitors, the first approved drug for melanoma treatment was an anti-CTLA4 agent: ipilimumab. In 2010, encouraging results were published from the pivotal MDX010-020 trial, comparing the overall survival of patients who received ipilimumab plus the gp100 vaccine (3 mg/kg and 1mg/kg every three weeks for four doses; n=403), ipilimumab alone (3 mg/kg every three weeks for four doses; n=137) and the control therapy of gp100 alone (n=136) (Hodi et al., 2010). The results showed that ipilimumab improved survival of patients and constituted the rationale for the drug to be reviewed with priority by FDA. Ipilimumab was then approved in 2011 both by FDA (25 March 2011) and by EMA (25 July 2011). This was the first step for a decade full of discoveries for science and hope for

patients. Three years later (4 September 2014), the first anti-PD-1 agent, pembrolizumab, was approved by FDA for the treatment of advanced melanoma. In December of the same year, nivolumab, another anti-PD-1 agent, was approved by FDA. In 2015, pembrolizumab and nivolumab received the approval from EMA, as well.

Curran and colleagues, in a study involving melanoma animal models, showed that the simultaneous blockade of CTLA4 and PD-1 could lead to a better tumor regression and it increased the amount of CD8+T cells and CD4+ T cells infiltrating the tumor (Curran et al., 2010). In 2013, another research group showed that concurrent treatment of MC38 tumors with the murine homologous of anti-PD-1 (4H2-mIgG1) and anti-CTLA-4 (9D9-mIgG2b) resulted in a synergistic antitumor activity (Selby et al., 2013). These results obtained in preclinical models represented the rationale to investigate the safety and the efficacy of the combination in a clinical trial, which results were published in 2013, in the *New England Journal of Medicine* (Wolchok et al., 2013). The combination resulted to be more effective than either single agent alone. Importantly, higher rates of immune-related toxicities were observed, compared to the either antibody alone (Wolchok et al., 2013).

In 2015, the FDA approved nivolumab in combination with ipilimumab for the treatment of BRAF V600 wild type melanoma. One year after, the approval was extended to unresectable or metastatic melanoma, across the status of BRAF.

Despite the revolution that these agents represented, the percentage of patients with melanoma not responding to the treatment with immune checkpoint inhibitors is still high. Two settings of resistance have been described: an innate or primary resistance, in which there is no response to therapy, or an acquired resistance, in which tumor progresses after an initial positive response.

The response rate is 19% for ipilimumab, 46% for pembrolizumab, 45% for nivolumab and 58% for nivolumab plus ipilimumab (Carlino et al., 2021). Regarding survival, the 5-year

landmark overall survival is 26% for ipilimumab, 43% for pembrolizumab, 44% for nivolumab and 52% for nivolumab plus ipilimumab (Larkin et al., 2019;Robert et al., 2019b).

The toxicity induced by immune checkpoint inhibitors is another important issue to consider, since it affects between 10-60% of melanoma patients receiving these agents and the development of adverse events of high severity could lead to therapy suspension or, in the most severe cases, to death of patients (Kumar et al., 2017;Postow et al., 2018;Larkin et al., 2019). By activating the immune system, immune checkpoint inhibitors can induce toxicities that mimic autoimmune diseases, that appears most frequently early during treatment and affects mainly skin, gastrointestinal tract, liver and endocrine system. Less frequently, adverse events may affect also the nervous system, heart and hematological system (Carlino et al., 2021). Importantly, anti-PD-1 monotherapy showed less toxicity compared with ipilimumab and with the combination anti-PD-1 plus anti-CTLA-4 (Khoja et al., 2017).

Given the great percentage of patients who face innate or acquired resistance, and given the toxicity that immune checkpoint inhibitors imply, the identification of biomarker of response to therapy and the setting of new therapeutic strategies are a priority, to better guide the clinical choices.

Many factors have been addressed as possible biomarkers of response to immune checkpoint inhibitors in melanoma: tumor mutational burden, PD-L1 expression, exosomal PD-L1, tumor T-cell infiltration, interferon gamma signature, the host's gastrointestinal microbiome. To date, no single factor is sufficiently sensitive or specific and the evaluation of clinical characteristics of patients still represents the best way to select treatment for patients. Among the clinical characteristics, the *BRAF* mutation status gained great interest: patients with BRAF mutation had a better overall survival at 5 years, compared to patients without mutation (60% and 48%, respectively, in the nivolumab-plus-ipilimumab group; 46% and 43% in the nivolumab group; and 30% and 25% in the ipilimumab group) (Larkin et al., 2019). Furthermore, patients with

BRAF^{V600K} mutation showed a higher response rate to anti-PD-1 monotherapy than patients with *BRAF*^{V600E} mutation (Pires da Silva et al., 2019). The mechanisms behind these results still need to be clarified.

Interestingly, immune checkpoint inhibitors are more effective in elderly patients (Kugel et al., 2018;Li et al., 2018;Ridolfi et al., 2020). Furthermore, the site of both the primary lesion and/or the metastases has great importance: higher response rate are observed in patients with a desmoplastic primary melanoma, compared to patients with mucosal or acral primary sites (D'Angelo et al., 2017;Nakamura et al., 2020). The presence of non-lung visceral site, especially liver and brain metastases, are associated to a poorer response to immune checkpoint inhibitors.

High level of serum lactate dehydrogenase represents one more factor associated to poorer response to immune checkpoint inhibitors (Van Wilpe et al., 2020).

Understanding the mechanisms causing the lack of response to treatment with immune checkpoint inhibitors is crucial to extend the effectiveness of treatment to a larger percentage of patients. Recently, the adenosinergic pathway has gained particular interest as mechanism behind the failure of anti-cancer treatments, including immunotherapy and immune checkpoint inhibitors. This topic is discussed in the following paragraph.

1.3 Adenosine pathway: an overview

1.3.1 Enzymes and receptors involved in the adenosine pathway

Adenosine is a purine nucleoside, which production and metabolism are regulated by the activity of many enzymes and transporters (Yegutkin, 2008). Extracellular adenosine can be generated via two different pathways, differing for the starting substrate and the enzymes involved (Ferretti et al., 2019). The canonical pathway starts with the hydrolysis of adenosine-triphosphate (ATP), firstly into adenosine-diphosphate (ADP), then in adenosine monophosphate (AMP). Both the reactions are catalyzed by the ecto-nucleoside triphosphate diphosphohydrolase CD39 (Yegutkin, 2008). AMP in then hydrolyzed into adenosine and inorganic phosphate by the ecto-5′-nucleotidase CD73 (Figure 3) (Yegutkin, 2008).

The non-canonical pathway starts with the hydrolysis of nicotinamide adenine dinucleotide (NAD^+), into ADP-ribose, mediated by the NAD^+-glycohydrolase CD38. The molecule of ADP-ribose is then converted into AMP thanks to the activity of CD203a (Figure 3) (Horenstein et al., 2013). Importantly, also in the non-canonical pathway, AMP is hydrolyzed by CD73, which is accordingly the key enzyme in the extracellular adenosine production, participating both in the canonical and in non-canonical pathway (Figure 3).

Enzymes belonging to the family of alkaline phosphatases can contribute to extracellular adenosine production by dephosphorylating AMP. Four alkaline phosphatase isozymes have been described: three are tissue specific (intestinal, placental and germ cell alkaline phosphatases), while the fourth is tissue-non specific and it is mostly found in bone, liver and kidney (Yegutkin, 2008). Tissue-non specific alkaline phosphatases (TNAP) exist not only anchored to the cell membrane, but also in a soluble form, found in human plasma. This form participate in regulating the levels of inorganic pyrophosphate, while its contribution in ATP metabolism is negligible (Yegutkin, 2008).

Once produced, extracellular adenosine has a very short half-life, being quickly catabolized into inosine by adenosine deaminase (ADA) or transported into cells via nucleoside transporters or captured by its receptors (Figure 3) (Yegutkin, 2008). Four adenosine receptors have been described, all coupled to a G-protein: A1 and A3, $G_{i/o}$-coupled receptors, A2A and A2B, G_s-coupled receptors (Yegutkin, 2008). The affinity of A1, A2A and A3 (K_d of 100, 310, 290 nM respectively) receptors for adenosine is in the nanomolar range, while the affinity of A2B receptor is in the micromolar range (K_d 15 μM). Considered that the physiological concentration of extracellular adenosine in most tissues is in the nanomolar range, A2B receptor is activated only under pathological conditions, such as inflammation or cancer. Physiologically, the adenosine signaling attenuates inflammatory responses and controls the immune response, to protect tissues from damages. In context of cancer, the impairment of the immune cells activity promotes the formation of a pro tumorigenic environment, as briefly summarized in the following paragraph.

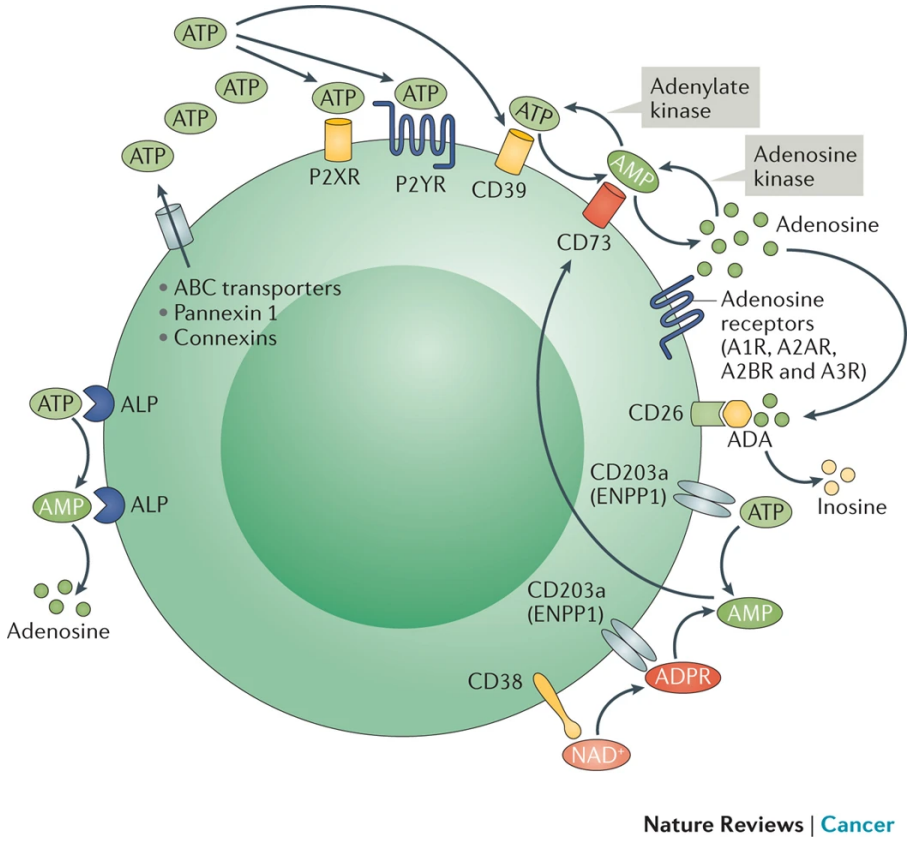

Figure 3 – *Enzymes and receptors involved in extracellular adenosine pathway (Vijayan et al., 2017)*

1.3.2 Adenosine pathway in cancer

In the tumor microenvironment many stimuli promote the accumulation of extracellular ATP, including cell death, hypoxia, tissue injury and chemotherapeutic agents (Di Virgilio et al., 2018). Once released, ATP activates cell surface type 2 (P2) purinergic receptors, including seven ligand-gated ion channel receptors (P2XRs) and eight G-protein-coupled receptors (P2YRs), which stimulation on immune cell types could promote inflammatory responses. However, in context of cancer, the effects mediated by extracellular ATP are influenced by the expression of ectonucleotidases and the properties of the inflammatory cells infiltrating the lesion (Di Virgilio et al., 2018). It is well known that in the tumor microenvironment many factors, including hypoxia or the presence of transforming growth factor or IL-6, can enhance the expression of both CD39 and CD73 (Antonioli et al., 2016;Moesta et al., 2020;Antonioli et

al., 2021), which activity promotes the metabolism of ATP and the accumulation of extracellular adenosine.

In contrast to ATP, which acts as a potent immunogenic signal, extracellular adenosine strongly suppresses the anti-tumor immune response by modulating the activity of different cell types populating the tumor microenvironment, mostly via A2A and A2B-mediated signaling (Allard et al., 2020).

In T cells, the activation of A2A receptor suppresses the effector functions, inhibiting the cell motility, the proliferation and the production of cytokines, promoting at the same time the expression of inhibitory checkpoints (such as PD-1, CTLA-4 and LAG3) (Csoka et al., 2012). On CD4+ T cells, this receptor stimulates the differentiation of regulatory T cells (T regs), which exert suppressive functions and contribute to increase the adenosine production (Allard et al., 2020).

Similarly, the activation of A2A receptor on B cells impairs the survival and the effector functions of cells, by inhibiting the downstream signaling of the B cell receptor (BCR) and the Toll-like receptor 4 (TLR-4) (Allard et al., 2020).

The activation of both A2A and A2B receptors on dendritic cells (DCs) inhibits the production of the proinflammatory mediator interleukin (IL)-12 and the expression of the costimulatory molecule CD80 and CD86, that are crucial for the antigen presentation and the sub-sequential activation of T cells. At the same time, the expression of IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β or cyclooxygenase 2 is enhanced in DCs upon A2A/A2B stimulation (Allard et al., 2020).

Adenosine can also modulate the activity of tumor-infiltrating macrophages, promoting the differentiation of M2-like macrophages, characterized by an increased production of IL-10, VEGF and arginase 1 (Csoka et al., 2012). In contrast to M1-macrophages that express low

levels of CD73, M2-like macrophages highly expressed this enzyme, contributing to adenosine production and in turn to the maintenance of M2-like phenotype (Zanin et al., 2012).

Natural kill (NK) cells express both A2A and A2B receptors, which activation leads to impairment of cell maturation and inhibition of effector functions. Specifically, the production of interferon (IFN)-γ is reduced, whilst the secretion of TGF-β and IL-10 is enhanced (Sorrentino and Morello, 2017;Allard et al., 2020).

Besides modulating the immune response, extracellular adenosine can also impair the functions of stromal cells, promoting the tumor angiogenesis, endothelial vasculogenesis and the formation of new lymphatic vessels and tumor-draining lymph nodes (Wang et al., 2013;Allard et al., 2014b;Allard et al., 2019).

By stimulating its receptors on endothelial cells, adenosine promotes the expression of CD73 (Narravula et al., 2000), VEGF (Khoa et al., 2003;Feoktistov et al., 2004;Acurio et al., 2017), IL-8 (Feoktistov et al., 2004) and fibroblast growth factor (Grant et al., 1999;Feoktistov et al., 2004), while it inhibits the expression of antiangiogenic factor thrombospondin-1 (Katamura et al., 1995;Canale et al., 2018). Importantly, another effect mediated by adenosine on endothelial cells is the downregulation of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin on endothelial cells, impairing leukocyte extravasation (Bouma et al., 1996;Walker et al., 1999;Henttinen et al., 2003;Grünewald and Ridley, 2010;Wang et al., 2011).

Many studies described the role of CD73-derived adenosine in promoting angiogenesis. Wang and collaborators demonstrated that tumor angiogenesis and the size of tumors were reduced in CD73 deficient mice, compared with wild type mice (Wang et al., 2013). In the same study, the authors isolated and cultured pulmonary microvascular endothelial cells (PMECs) from $CD73^{+/+}$ and $CD73^{-/-}$ mice and they observed that CD73 induces the formation of tube-like

structures. Importantly, this effect resulted enhanced upon challenge by cancer cell culture supernatant (Wang et al., 2013).

In a breast cancer mouse model, the treatment with a monoclonal antibody targeting CD73 led to reduced levels of VEGF in tumor and to inhibition of angiogenesis (Allard et al., 2014a). In line with these findings, tumor bearing mice lacking A2B or treated with a selective A2B receptors antagonist showed decreased levels of VEGF in tumor and a reduction of tumor growth (Ryzhov et al., 2008;Sorrentino et al., 2015).

Within tumor microenvironment, cancer associated fibroblasts (CAFs) express CD39 and CD73 and therefore are able to produce adenosine, which not only influences the other cell populations, but modulates the functions of CAFs themselves (Turcotte et al., 2015;Sorrentino et al., 2016;Yu et al., 2020).

Previous data obtained in the laboratory where this PhD project was carried out demonstrated that the blockade of A2B receptor in melanoma bearing mice led to a reduction of fibroblast activation protein (FAP)-expressing cells in tumors, and to a simultaneous reduction of the expression of fibroblast growth factor (FGF)-2 (Sorrentino et al., 2016). The stimulation of A2B receptor stimulated the expression of FGF2, which directly enhanced the proliferation of melanoma cells (Sorrentino et al., 2016). Furthermore, the activation of A2B receptor led to an increased expression of CXCL12 in CAFs, which corresponded to increased tumor growth and angiogenesis (Sorrentino et al., 2016).

Recently, Yu and colleagues demonstrated that CAF-derived adenosine exerts an autocrine effect by activating the A2B receptor, which in turn enhances the expression of CD73 on CAFs, keeping high the production of adenosine mediated by these cells (Yu et al., 2020).

Tumor cells can express A2A receptor, which stimulation promotes their proliferation, invasiveness and motility, as observed in preclinical animal models (Stagg et al., 2010;Beavis et al., 2013).

Importantly, CD73 can influence tumor cells biology not only by producing adenosine, but also independently of its enzymatic activity. In melanoma cell lines, CD73 was observed to modulate cell migration and adhesion by binding the extracellular matrix protein tenascin C (Sadej and Skladanowski, 2012). This latter protein is highly produced in melanoma and its production resulted linked to tumor progression and metastasis dissemination, while its absence defines a benign disease (Ilmonen et al., 2004;Kääriäinen et al., 2006). Thus, in melanoma lesion, CD73 can promote the formation of a protumor environment by suppressing the immune response, but also by directly promoting tumor cell proliferation, motility and invasion.

Being CD73 the main subject of this PhD project, details about this enzyme are discussed in following paragraph.

1.4 CD73: the key enzyme in adenosine production

1.4.1 Biology and function of CD73

Human CD73 is physiologically expressed in several tissues, and it is particularly abundant in lymph nodes, spleen, bone marrow, kidney, lung and brain (Antonioli et al., 2013). CD73 is encoded by the *NT5E* gene, a hypoxia inducible factor (HIF) target gene (Synnestvedt et al., 2002), which expression is up-regulated under hypoxic conditions, typical of the tumor microenvironment. Other factors can promote the expression of this ectonucleotidase, including inflammatory mediators as transforming growth factor β, interferons, interleukin 1β, prostaglandin E2, tumor necrosis factor (Antonioli et al., 2016). The expression of *NT5E* can be regulated by other transcriptional factors, aside from HIF, as c-Jun/AP1, SMAD or SP1(Fausther et al., 2012;Reinhardt et al., 2017).

CD73 is constituted by two identical subunits of 70 kDa, each linked to the cell membrane by a glycosylphosphatidyl inositol (GPI) anchor at C-terminal (Knapp et al., 2012). Each subunit consists of two structural domains: the N-terminal domain (residues 27–317), allows the binding of Zn^{2+} and Co^{2+}, required for the enzymatic activity, and the C-terminal domain (residues 337–549) (Figure 4) (Sträter, 2006;Knapp et al., 2012). The binding site for the substrate AMP is located at the interface between the N- and C-terminal domains and is formed from residues of both domains (Figure 4) (Knapp et al., 2012).

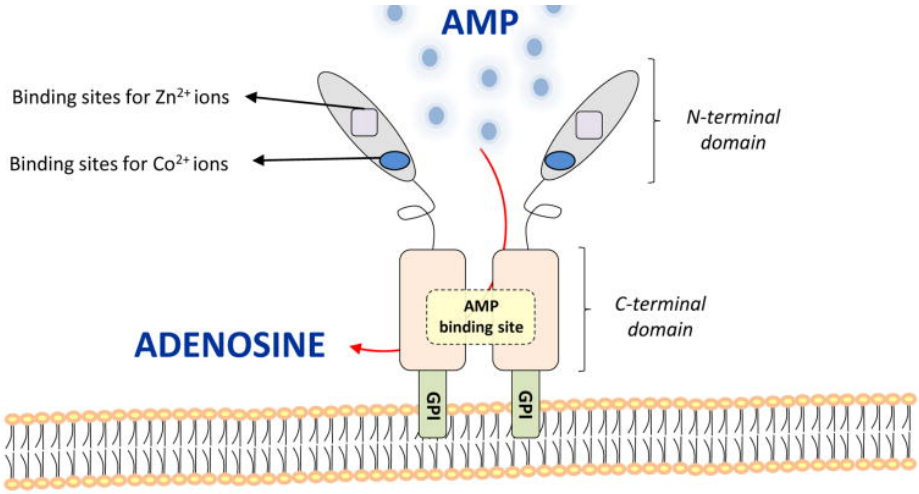

Figure 4 – *Structure of membrane-bound CD73 (Antonioli et al., 2016)*

The activity of CD73 is crucial to protect tissue integrity, maintaining endothelial and epithelial barrier functions, especially after injuries following inflammation, hypoxia, ischemia in heart, lung, kidney, gastrointestinal tract and brain (Colgan et al., 2006). Other functions, independent from the enzymatic activity, have been proposed for CD73, in the regulation of immune-endothelial cells interactions (Colgan et al., 2006).

The relevance of cell-bound CD73 in cancer patients will be discussed in detail in section 1.4.3.

1.4.2 Circulating forms of CD73

The GPI anchor can be cleaved by phosphatidylinositol-specific phospholipase or proteases and once released from the membrane, CD73 can circulate in biological fluids (Figure 5) (Yegutkin, 2008).

Importantly, soluble CD73 maintains its enzymatic activity (Yegutkin, 2008;Heuts et al., 2012), resulting increased in inflammation or cancer (Yegutkin, 2008;Maksimow et al., 2014;Huang et al., 2015;Morello et al., 2017;Persson et al., 2018;Gardani et al., 2019).

Importantly, the non-cell bound fraction of CD73 includes also an exosome-associated form (Figure 5) which, together with the soluble one, contributes to the purine metabolism in biological fluids, including blood (Schneider et al., 2019).

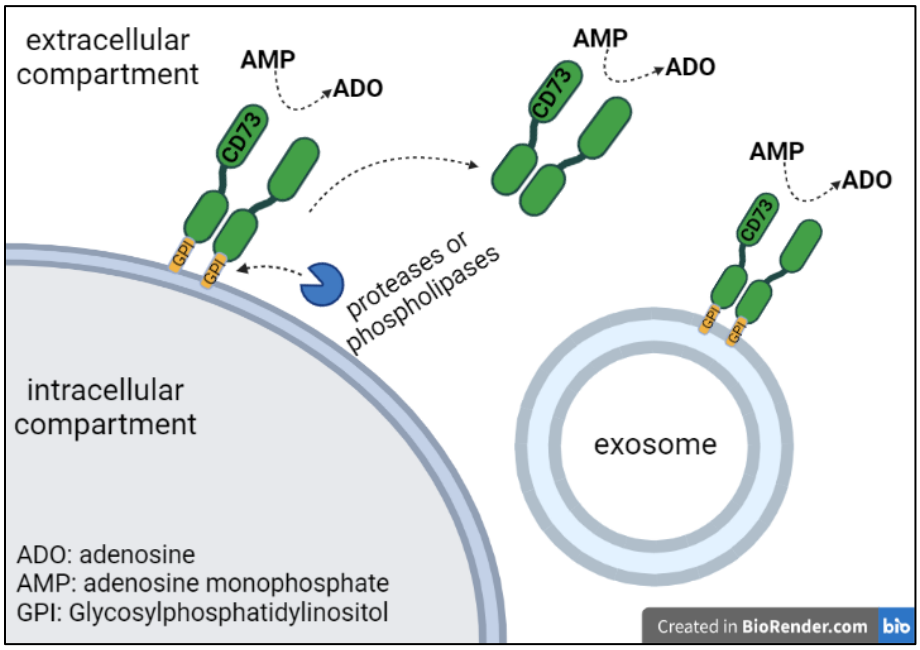

Figure 5 – *CD73 exists anchored to the membrane of cells or exosomes, or in a soluble form.*

Since CD73 is also found on exosomes, a brief introduction on these vesicles is required to the purpose of this thesis.

Exosomes are extracellular vesicles with a diameter of 30-150 nm, which are produced in the endosomal compartment of all cell types, including tumor cells, and are found in many biological fluids (Raposo and Stoorvogel, 2013). These vesicles are composed of an external lipid bilayer, on which many receptors and enzymes are expressed, while the internal compartment contains proteins, RNA and DNA (Kalluri, 2016). Interestingly, the proteins carried by exosomes reflect the profile of the cells that generated them (Kalluri, 2016).

During the last years, exosomes gained a great scientific interest as mechanism involved in tumor progression, being able to influence the activity of cells populating the tumor microenvironment, including immune cells (Kalluri, 2016).

Focusing on melanoma, exosomes have been addressed as potential biomarker of tumor progression and outcomes of patients (Logozzi et al., 2009;Peinado et al., 2012;Pfeffer et al., 2015;Chen et al., 2018;Sharma et al., 2020). Recently, the Whiteside's group showed that

tumor-derived exosomes isolated from plasma of melanoma patients are enriched in immunosuppressive proteins (including CD73) and are able to induce apoptosis and to inhibit the proliferation in CD8+ T cells (Sharma et al., 2020). In another study, the expression of exosomal PD-L1 emerged as potential biomarker of response in melanoma patients receiving anti-PD-1 therapy (Chen et al., 2018).

As anticipated, exosomes can express CD73 on their membrane. Specifically, CD73+ exosomes have been isolated from different human fluids, as plasma and serum (Muller, Hong et al. 2014, Schuler, Saze et al. 2014, Theodoraki, Hoffmann et al. 2018) and pleural fluid (Clayton, Al-Taei et al. 2011). Data published by many groups confirmed that exosomal CD73 is enzymatically active and can impair the anti-tumor immune response (Clayton, Al-Taei et al. 2011, Schuler, Saze et al. 2014, Ludwig, Floros et al. 2017, Zhang, Li et al. 2019).

Recently, Ludwig and colleagues observed that exosomes isolated from UMSCC47 cell lines can produce adenosine, via CD39 and CD73, but can also carry adenosine and inosine in their inner compartment, suggesting that exosomes can promote the formation of pro-tumorigenic niches far from the site they are produced, by transporting and protecting these molecules from metabolism or uptake processes (Ludwig et al., 2020).

The relevance of the two non-cell bound forms of CD73 in cancer patients will be discussed in detail in the following section.

1.4.3 The relevance of CD73 as potential biomarker and therapeutic target in cancer patients

High CD73 expression in tumor tissue is associated with short overall survival and poor prognosis in melanoma (Monteiro et al., 2018), as well as in many other cancer types, such as lymphoma (Wang et al., 2019), breast cancer (Loi et al., 2013;Turcotte et al., 2017;Buisseret et al., 2018;Jiang et al., 2018), head and neck cancer (Ren et al., 2016;Mandapathil et al., 2018), gastric cancer (Lu et al., 2013), non-small-cell lung cancer (Inoue et al., 2017), thyroid

carcinoma (Bertoni et al., 2019), ovarian cancer (Turcotte et al., 2015;Jiang et al., 2018), colorectal cancer (Wu et al., 2012), pancreatic cancer (Chen et al., 2020).

Focusing on melanoma, important results were published by Reinhardt and colleagues, who proposed a dynamic regulation of CD73 expression as an acquired mechanism of resistance to immunotherapy (Reinhardt et al., 2017). In this study, the authors observed that that patients with melanoma who had progression of disease during anti-PD-1 therapy, had also increased CD73 expression in tumor tissue. Notably, patients who were previously treated with inhibitors of MAPK and BRAF were negative for CD73 expression, while patients who did not receive MAPK inhibitor therapy showed CD73 up-regulation at progression (Reinhardt et al., 2017). These results indicate that the expression of CD73 in the tumor tissue is influenced by pharmacological treatments.

As anticipated, CD73 can be cleaved from the cell membrane and the expression and the activity of the resulting soluble form are increased in patients with cancer, as prostate cancer (Gardani et al., 2019), cervical cancer (de Lourdes Mora-García et al., 2019), head and neck cancer (Lal et al., 1989), melanoma (Morello et al., 2017).

In 2017 important results about soluble CD73 in melanoma patients were obtained in the laboratory where this PhD project has been carried out. Morello's group found that CD73 activity in serum correlates with overall survival, progression-free survival, and clinical response to nivolumab treatment, in a single cohort of 37 patients with metastatic melanoma (Morello et al., 2017).

These data constituted the starting point of this PhD thesis, as it will be discussed in the next sections.

New data regarding the soluble form of CD73 were recently published by Stagg's group: patients with colorectal cancer liver metastases with high levels of soluble CD73 showed

shorter survival. Furthermore, the expression of tumor CD73 emerged as a stronger biomarker than the soluble form (Messaoudi et al., 2020).

As described before, CD73 is also expressed on the exosomal membrane. Exosomal CD73 resulted higher in patients with stage III/IV HNSCC than patients classified as stage I/II (Theodoraki et al., 2018). Great interest is growing around this form, that has been addressed as responsible to promote a tumorigenic environment far from the primary tumor site. Further investigations are required to better understand the potential of using exosomal CD73 as predictive factor in cancer patients and this point has been addressed in this PhD project.

All the evidence discussed above constituted the rationale for the development of agents targeting CD73 and for the setting of clinical trials to evaluate these agents. Five anti-CD73 antibodies (BMS-986179, CPI-006, MEDI9447, NZV930, TJ004309) and two small molecules acting as inhibitors (AB122 and LY3475070) are currently in clinical investigation (Boison and Yegutkin, 2019;Allard et al., 2020).

1.5 The blockade of the adenosine pathway to potentiate immune checkpoint inhibitors efficacy

During the last years, there has been a growing interest about the targeting of the adenosine pathway as anti-cancer strategy.

The Sitkovsky group was the first to describe the relevance of the adenosine receptors in regulating immune responses and inflammation (Ohta and Sitkovsky, 2001). The same group was the first to propose the blockade of A2A receptor as new anti-tumor strategy, showing that the genetic deletion of A2AR in melanoma-bearing mice resulted in rejection of tumor; while the blockade of the A2A receptor (by using antagonists or siRNA treatment) led to inhibition of tumor growth, reduction of metastases and prevention of neovascularization (Ohta et al., 2006). The A2B receptor resulted implied in tumor progression as well (Ryzhov et al., 2008): its stimulation with the selective agonist Bay 60-6583 resulted in increased tumor growth, while its blockade with the antagonist PSB1115 led to a significant tumor reduction, in a melanoma mouse model (Iannone et al., 2013).

Given all the evidence supporting the role of the adenosinergic pathway in tumor progression, researchers started studying the potential of targeting the main enzyme responsible for extracellular adenosine production: CD73. The use of a mAb targeting CD73 resulted effective in reducing tumor growth and inhibiting the dissemination of metastases, in a preclinical animal model (Stagg et al., 2010). Another study, from the same group demonstrated that CD73-deficient mice were significantly protected against the development of subcutaneous tumors and experimental lung metastases (Stagg et al., 2011). In line with these results, the blockade of CD73, by administrating α,β-methyleneadenosine 5'-diphosphate (APCP), reduced tumor growth in a melanoma mouse model and increased the frequency of CD8+ T cells and B cells in melanoma tissue (Forte et al., 2012).

Many preclinical studies demonstrated a synergic antitumor activity of combining the blockade of the adenosinergic pathway with immunotherapy, and specifically with immune checkpoint inhibitors (Allard et al., 2020). Beavis et al. demonstrated, in a breast cancer mouse model, that the blockade of A2A receptor and PD-1 inhibited tumor growth by increasing the expression of Granzyme B and IFN- γ in tumor infiltrating CD8+ T cells (Beavis et al., 2015). In line with this, another group showed that the use of an antagonist of A2A receptor (CPI-444), in a colon cancer mouse model, modestly inhibited tumor growth, while the combination with an anti-PD-1 antibody markedly enhanced the anti-tumor activity, compared to anti-PD-1 alone (Leone et al., 2018). Interestingly, in the same study the authors showed that the combination promotes an effector phenotype of tumor-infiltrating CD8+ T cells, while the expression of multiple checkpoints, including PD-1 and LAG-3, is reduced on both CD8+ effector T cells and FoxP3+ CD4+ regulatory T cells (Leone and Emens, 2018).

The enhancement of the efficacy of anti-PD-1 and anti CTLA-4 upon simultaneous blockade of CD73 was demonstrated, in a mouse model, by Allard and colleagues in 2013 (Allard et al., 2013). In another preclinical study, the blockade of CD73 with the selective inhibitor APCP resulted in an enhanced activity of the anti-CTLA-4 antibody, in a melanoma model (Iannone et al., 2014). In the same study, the blockade of A2A was also evaluated and the combination of an antagonist for the A2A receptor (ZM241365) and an anti-CTLA-4 antibody led to a marked inhibition of tumor growth and an increased expression of Granzyme B and and IFN-γ (Iannone et al., 2014). Additionally, the blockade of PD-1 and A2A receptor resulted in a reduced metastatic burden, compared to the use of anti-PD-1 alone (Mittal et al., 2014). This effect was mostly mediated by the activity of NK cells and it was dependent on the expression of CD73 on tumor cells (Mittal et al., 2014).

Given the promising results observed in preclinical models, several clinical trials evaluating the safety and the efficacy of agents targeting the components of the adenosine pathway have been

started (Figure 6), some including the combination with immune checkpoint inhibitors (Allard et al., 2020). Most of the trials are still in early phase (phase I or combined phase I/phase II), as reviewed by Thompson and colleagues (Figure 6) (Thompson and Powell, 2020).

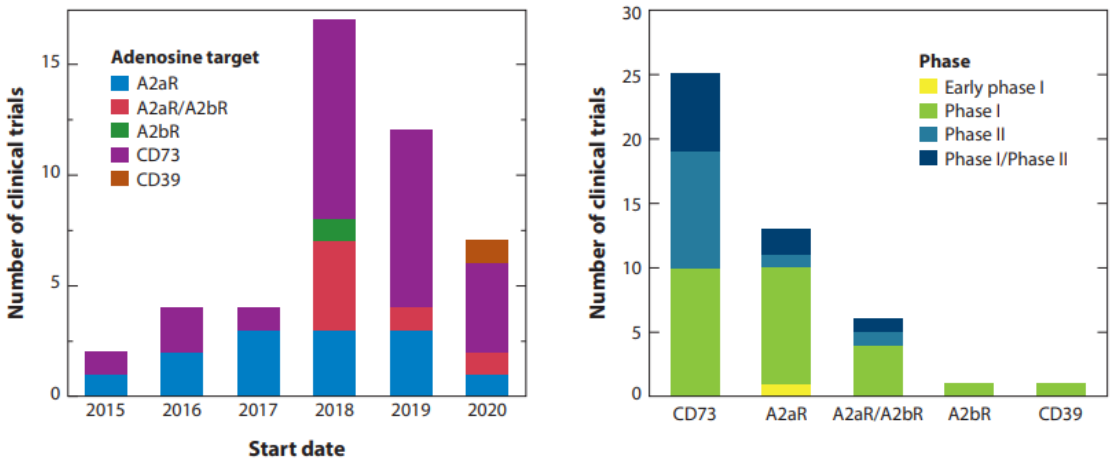

Figure 6 – *Clinical trials evaluating the blockade of the adenosine pathway (Thompson and Powell, 2020)*

In 2016 a trial targeting the A2A receptor, in solid tumors including melanoma, and in combination with a new anti-PD-1 agent was started and it is currently in phase II (NCT03207867). Another trial, evaluating an antagonist for both A2A and A2B, in combination with an anti-PD-1 agent, just concluded the phase I and it involved patients with solid malignancies, including melanoma (NCT03629756).

In 2020, two clinical trials have been started, evaluating the targeting of CD39 in solid tumors and are currently in phase I (NCT04261075;NCT04306900).

Twenty five clinical trials investigating the efficacy and safety of CD73 blockade, alone or in combination with other agents, including ICIs, are ongoing, most of which are in phase I (Thompson and Powell, 2020).

Promising data from a phase I trial started in 2015 (NCT02503774), that have been recently presented, showed that the combination oleclumab (a human IgG1λ mAb targeting CD73) plus

durvalumab (anti-PD-L1) had a tolerable safety profile and showed a good antitumor activity in patients with epidermal growth factor receptor mutation-positive (EGFRm) non-small cell lung cancer (NSCLC) (Bendell et al., 2021).

Other interesting results were obtained about a new monoclonal antibody targeting CD73 (CPI-006), which mechanism of action is based on the inhibition of adenosine production and on the activation of B cells, in an adenosine-independent manner (Luke et al., 2019). This agent is currently in phase I trial, being evaluated alone or in combination with pembrolizumab, with ciforadenant (A2A receptor antagonist) or with pembrolizumab and ciforadenant (NCT03454451). First results showed that the treatment has been well-tolerated, and there is early evidence of anti-tumor activity of CPI-006 monotherapy (Luke et al., 2019).

In 2020, new anti-CD73 agent, a novel antibody and a nucleotide analog, have been proposed and would warrant clinical investigation, considered the promising preclinical results (Jin et al., 2020;Schäkel et al., 2020).

1.6 Aim of the project

The general aim of this PhD project was to study the potential of all the forms of CD73 as predictive factors of response in patients with advanced melanoma receiving anti-PD-1 therapy.

As discussed in the previous sections, anti-PD-1 agents revolutionized the treatment of advanced melanoma, increasing the survival of patients. Further investigations are required to understand the mechanisms behind resistance to these drugs, that may occur in about half of the patients receiving the treatment. The adenosine pathway has emerged as potential mechanism of resistance to anti-PD-1 treatment and CD73 has been addressed as both potential therapeutic target, to be used in combination with immune checkpoint inhibitors, and biomarker of response.

Furthermore, given the increasing interest in CD73 as therapeutic target in cancer, the individuation of patients that would likely benefit from treatment is needed and, at this regard, the measurement of CD73 expression and activity could be informative.

Based on a large body of evidence, previously discussed and in part obtained in the laboratory where this project was carried out, I studied all the forms of CD73 that circulate in blood: including the cell-bound form, the soluble form and the exosomal form. Then I characterized the expression of CD73 in the tumor lesion.

The following four sub-aims were settled for this project.

1. **To evaluate the potential of measuring circulating CD73+ lymphocyte subpopulations to predict clinical outcomes of patients.**

   1.1 Analyze the frequency of CD73+ lymphocytes in association with clinicopathological characteristics of patients and their clinical outcome and survival.

2. **To investigate the predictive potential of CD73 in serum of patients.**

   2.1 Analyze the activity and the expression of serum CD73 in patients with melanoma and healthy donors.

   2.2 Characterize the AMPase activity in serum, evaluating the activity of the enzymes involved in purine metabolism.

   2.3 Analyze serum CD73 levels in association with clinicopathological characteristics of patients and their clinical outcome and survival.

3. **To investigate the role of exosomal CD73 in serum of melanoma patients.**

   3.1 Set up a protocol to isolate intact exosomes.

   3.2 Characterize the activity and the expression of exosomal CD73.

   3.3 Investigate the role of CD73+ exosomes in impairing T cell functions.

   3.4 Analyze exosomal CD73 levels in association with clinicopathological characteristics of patients and their clinical outcomes and survival.

4. **To measure the expression of CD73 in human melanoma lesions.**

   4.1 Characterize the expression of CD73 in the cell types populating the melanoma lesions.

Each part of this project was carried out using human samples belonging to different cohorts. The details of the specimens used, and the characteristics of the patients involved will be provided in each section of the thesis.

Experiments regarding the first part were performed at Nazionale Tumori IRCCS Fondazione "G. Pascale" (Naples), thanks to the collaboration with Dr Paolo Antonio Ascierto, while the experiments regarding the second and the third part of this thesis were performed at the Department of Pharmacy of the University of Salerno, where I was enrolled as PhD student, under the supervision of Professor Silvana Morello. The fourth part was carried out at Institute

of Experimental Oncology of University Clinic of Bonn, thanks to the collaboration with Professor Michael Hölzel and his team.

2. Circulating CD8+CD73+ T cells associate with survival and outcomes of melanoma patients treated with nivolumab

The presence of CD8+PD-1+ T cells in the tumor lesion has been associated with therapeutic efficacy of anti-PD-1 agents (Tumeh et al., 2014;Ngiow et al., 2015;Daud et al., 2016). Tumor lesions of patients responding to therapy with immune checkpoint inhibitors showed abundance of memory-like CD8+T cells, while lesions of non-responders patients showed increased exhausted-like CD8+ T cells (Sade-Feldman et al., 2019).

Given the relevance of T cell populations in influencing the clinical response to immune checkpoint inhibitors, the first part of this PhD project was aimed to investigate the potential of measuring circulating CD73+ lymphocyte subpopulations in patients with advanced melanoma treated with nivolumab, to predict clinical outcomes.

The results here discussed have been published in:

Capone M, Fratangelo F, Giannarelli D, Sorrentino C, **Turiello R**, Zanotta S, Galati D, Madonna G, Tuffanelli M, Scarpato L, Grimaldi AM, Esposito A, Azzaro R, Pinto A, Cavalcanti E, Pinto A, Morello S, Ascierto PA. Frequency of circulating CD8+CD73+T cells is associated with survival in nivolumab-treated melanoma patients. *J Transl Med*. **2020** Mar 11;18(1):121. doi: 10.1186/s12967-020-02285-0. PMID: 32160899; PMCID: PMC7065327.

2.1 Materials and methods

2.1.1 Patients and blood samples

Peripheral blood from melanoma patients were collected at the Unit of Melanoma, Cancer Immunotherapy and Innovative Therapies, of Istituto Nazionale Tumori IRCCS Fondazione "G. Pascale" (Naples). Specifically, blood from 100 melanoma patients (stage III or IV) were collected prior to the start of treatment with nivolumab (baseline), administered at the dosage of 3 mg/kg every two weeks, until disease progression or unacceptable toxicity appeared. Tumor assessment was performed according to response evaluation criteria in solid tumors (RECIST) at baseline and every 12 weeks thereafter (Eisenhauer et al., 2009).

All the characteristics of patients are summarized in table 1. For all patients, clinical data, including serum lactate dehydrogenase (LDH), complete blood count, BRAF status, brain metastasis, lines of prior treatment were collected before starting nivolumab treatment and until last follow-up.

Blood from 20 healthy donors aged > 18 years were collected at Transfusion and Stem Cell Transplantation Unit, of Istituto Nazionale Tumori IRCCS Fondazione "G. Pascale" (Naples).

PBMCs were isolated from blood by Ficoll density gradient and were stored at -80°C until use.

The study was approved by the Ethic Committee of Istituto Nazionale Tumori IRCCS Fondazione "G. Pascale" (Naples); and all individuals provided a signed informed consent for blood donation.

2.1.2 Flow cytometry

Frozen PBMCs were thawed immediately before use, briefly rested and then incubated with antibodies. The following antibodies were used: CD3-V500, CD8-APC Clone BW135/80, PD-1-PE Clone PD1.3.1.3 (all from MiltenyiBiotecS.r.l.) and CD73 PE-Cy7 Clone AD2 (BioLegend UK Ltd). Samples Data were acquired using a FACSAria II (Becton–Dickinson,

USA). Data were acquired using a FACSAria II (Becton–Dickinson, USA). Lymphocytes were firstly gated on forward/side light scatter, according to morphological characteristics, and then gated for the expression of both CD3 and CD8. Then the expression of PD-1 and/or CD73 were assessed on CD3+CD8+ T-cells. Cell viability was measured by staining with 7-AAD (7-Aminoactinomycin D), dead cells were excluded by the gate strategy described above. Results were analyzed using Kaluza 1.2 software (Beckman Coulter).

2.1.3 Statistical analysis

Differences in cells subsets frequencies between groups were evaluated using the Mann–Whitney U test. The Kaplan–Meier method was used to analyze survival of patients and differences were tested using the log-rank tests, at cut-of values identified with the median of frequency of cell populations. OS time was calculated from the date of the first dose of nivolumab to the date of death (due to melanoma) or censored at the date of the last follow-up. Cox regression analysis for survival was performed and reported as hazard ratio (HR). Analyses were performed with GraphPad Prism 7.0 or IBM SPSS version 21.0.

2.2 Results

2.2.1 Clinicopathological characteristics of patients

For the first part of this thesis, 100 patients with melanoma (median age=62 years) were included. The clinicopathological characteristics of patients are summarized in table 1.

Fifty-three patients were male, while 47 were female.

Regarding the stage of disease, 8 patients were assigned to category M1a, 12 to M1b, 78 to M1c, 2 to M0. Twenty-eight patients had brain metastases. BRAF mutation was present in 43% of patients, while 54% of patients had wild-type tumors. The levels of serum lactate dehydrogenase were upper the limit of normal in 34 patients, while 60 patients had normal values.

Patients received nivolumab as first (n=27) or second/third line (n=73) of treatment. To the purpose of the analyses here described, patients were divided into two groups: patients who showed progression of disease (PD) and stable disease (SD) for lesser than 6 months were considered as non-responders (n=61), while patients who showed complete response (CR), partial response (PR) or SD greater than 6 months were considered responders (n=39). The median follow-up was 11 months.

Table 1 – *Clinicopathological characteristics of patients*

	Nivolumab therapy (no, %)
Total	100
Gender Female Male	 47(47) 53(53)
Median age, years (range, 28-90)	62
Serum lactate dehydrogenase	
Normal	60 (60)
Elevated	34 (34)
Unknown	6 (6)
BRAF status	
Mutated	43 (43)
Wild-Type	54 (54)
Unknown	3 (3)
Brain metastasis	28 (28)
M category (AJCC)	
M0	2 (2)
M1a	8 (8)
M1b	12 (12)
M1c	78 (78)
Line of treatment	
First line	27 (27)
Second line or later	73 (73)

2.2.2 Frequency of CD8+ cells in patients with melanoma and healthy donors

The frequency of CD8+ cells was analyzed by flow cytometry and the gating strategy to define the subpopulations of lymphocytes is shown in figure 7.

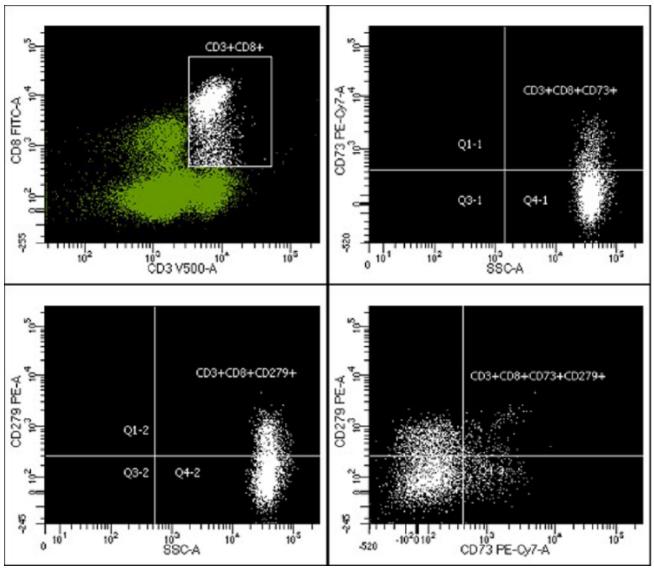

Figure 7 – *Gating strategy used for flow cytometry analyses*

The analysis of CD8+ cells, performed by flow cytometry, revealed that the frequency of total CD8+ cells is significantly decreased in patients with melanoma (25.1%), compared to healthy donors (33.28%) (p=0.008) (figure 8A). No differences were found between the two groups, when considering the frequency of CD8+PD-1+, CD8+CD73+ or CD8+CD73+PD-1+ lymphocytes (figure 8B, C and D, respectively). Specifically, the median frequency for CD8+CD73+ population was 5.8% in patients with melanoma and 7.7% in healthy donors, while the median frequency of CD8+PD-1+ was 9.8% in melanoma patients and 11% in healthy donors. The median frequency of lymphocytes expressing both CD73 and PD-1 was 2.3% in melanoma patients and 1.97% in healthy donors.

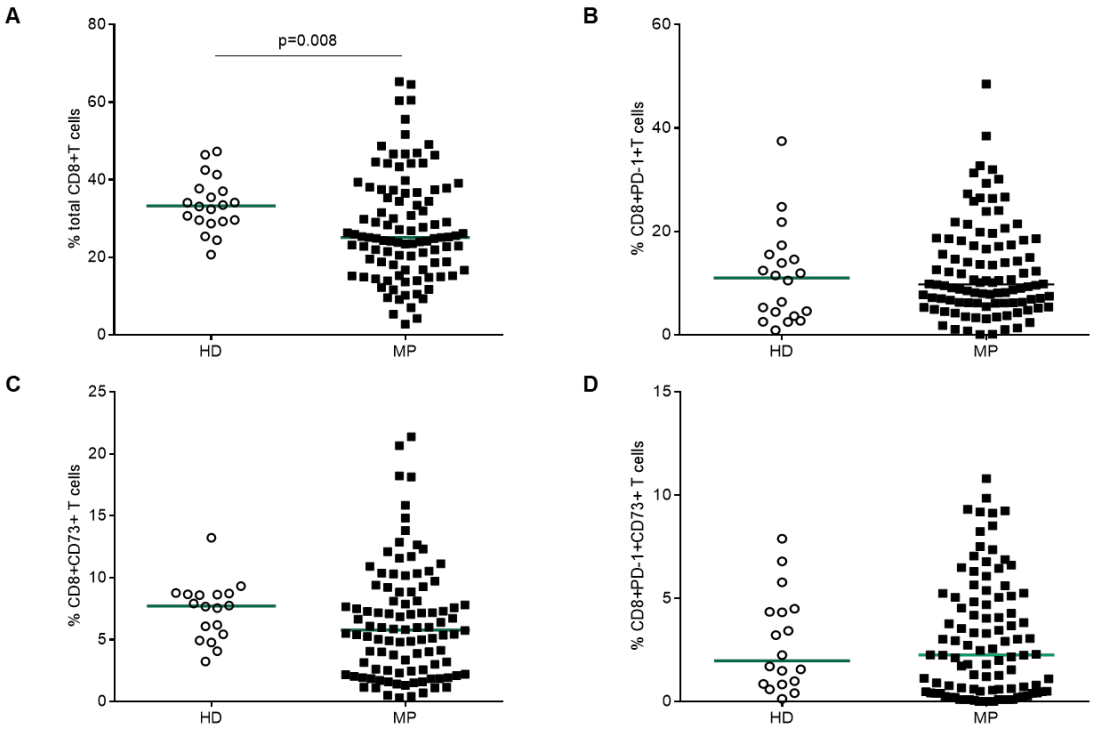

Figure 8 - *Frequency of lymphocytes subsets in melanoma patients (MP, n=100) and healthy donors (HD, n=20). Frequency of CD8+ T cells (A), CD8+PD-1+ T cells (B), CD8+CD73+ T cells (C) and CD8+PD-1+CD73+ T cells. The green line indicates the median value. P value is from Mann-Whitney test.*

2.2.3 Pretreatment frequency of CD8+PD-1+CD73+ T cells associates with overall survival of patients with melanoma

In order to identify association with overall survival, the frequency of CD8+ cells and clinical characteristics of patients were analyzed by Cox regression.

For each T cell subpopulation analyzed, the cut off value was set at its median frequency. Interestingly, CD8+PD1+ T cell frequency < 9.8% (p=0.009) and CD8+PD-1+CD73+ T cell frequency < 2.3% (p<0.001) resulted significantly associated with longer OS, whilst the frequency of lymphocytes positive only to CD73 was not associated with OS (table 2). Survival of patients, according to the frequency of the populations analyzed is summarized in table 3. Patients with low percentage of CD8+PD-1+CD73 lymphocytes showed a longer median

overall survival (22.4 months) than patients with high percentage of these cells (6.9 months). A significant negative correlation was found for high LDH levels (ratio LDH value/LDH normal >2) (p<0.0001) and brain metastases (p<0.008) (table 2).

Table 2 -*Cox regression analysis*

	Overall survival HR (95%CI)
Sex	
Male vs female	0.92 (0.58 – 1.46) P=073
Age	
>62 vs < 62	0.70 (0.44-1.12) P=0.13
BRAF	
mutated vs wild-type	1.06 (0.66-1.70) P=0.81
Serum lactate dehydrogenase	
High vs normal	1.62 (0.86-3.04) P=0.13
Very high vs normal	4.64 (2.52-8.54) P<0.0001
Brain metastasis	
yes vs no	1.92 (1.18-3.11) P=0.008
Previous lines of treatment	
2 vs 1	1.12 (0.62-2.02) P=0.69
>= 3 vs 1	1.38 (0.75-2.54) P=0.30
Metastatic site	
lung vs soft tissue	0.76 (0.30-1.87) P=0.54
visceral vs soft tissue	1.85 (0.93-3.65) P=0.08
Tot CD8+ cells	
(>=25.1 vs <25.1)	1.37 (0.86-2.18) p=0.18
CD8+CD73+ cells	
(>=5.8 vs <5.8)	1.55 (0.97-2.48) P=0.07
CD8+PD-1+ cells	
(>=9.8 vs <9.8)	1.86 (1.17-2.98) P=0.009
CD8+ PD-1+CD73+cells	
(>=2.3 vs <2.3)	2.25 (1.39-3.63) P=0.001

Table 3 – *Overall survival according to the frequency of the reported cell population*

Cell population (Cut-off median frequency)	Median survival (months) (95% CI)	P value
CD8+ lymphocytes		
<25.1%	15.4 (5.8-25.0)	0.18
>25.1%	6.9 (0.1-13.7)	
CD8+CD73+ lymphocytes		
<5.8%	17.9 (4.9-30.9)	0.06
>5.8%	8.0 (0.8-15.2)	
CD8+PD-1+ lymphocytes		
<9.8%	19.2 (10.1-28.3)	0.008
>9.8%	6.0 (0-12.8)	
CD8+PD-1+CD73+ lymphocytes		
<2.3%	22.4 (13.6-31.2)	0.001
>2.3%	6.9 (0.8-13.0)	

The T cell population positive to PD-1 and CD73 was also significantly associated to PFS ($p<0.0001$), as reported in table 4.

Table 4 - *Progression-free survival of patients according to frequency of lymphocyte populations*

Cell populations Cut-off (median frequency)	Median progression-free survival (months) (95%CI)	P value
CD8+ lymphocytes		
<25.1%	3.9 (0.8-7.0)	0.24
>25.1%	3.2 (1.1-5.3)	
CD8+CD73+ lymphocytes		
<5.8%	3.9 (0-10.1)	0.22
>5.8%	3.1 (0.9-5.3)	
CD8+PD-1+ lymphocytes		
<9.8%	5.9 (0.4-11.4)	0.02
>9.8%	2.6 (1.7-3.5)	
CD8+PD-1+CD73+ lymphocytes		
<2.3%	9.0 (0-19.9)	<0.0001
>2.3%	2.7 (2.1-3.3)	

Furthermore, the multivariate model revealed that the frequency of CD8+PD-1+CD73+ lymphocytes was independently associated with longer OS (HR 2.17, CI 1.34 to 3.51, p=0.002).

2.2.4 Pretreatment frequency of CD8+PD-1+CD73+ T cells associates with clinical response to nivolumab

The frequency of circulating T cells was then analyzed according to the clinical outcomes of patients. Interestingly, the frequency of CD8+PD-1+ T cells does not differ between responders (CR/PR, n=39) and non-responders (PD/PR, n=61) (p=0.07), while the percentage of CD8+T cells positive to both CD73 and PD-1 is significantly higher in non-responders (median=3.02%) than in responders (median=0.85%) (p=0.02, Mann-Whitney test) (figure 9). This result was confirmed in patients receiving nivolumab as first line (CR/PR=0.65% vs PD/PR=5.24%; p=0.049, Mann-Whitney test), while in the group treated in second or third line, a tendency to a higher frequency of this population is observed in non-responders but significance is not reached (figure 9D). Overall, the median frequency of CD8+CD73+PD-1+ T cell was lower in patients receiving nivolumab as first line (1.8%), than in patients treated as second line (2.26%), but the difference is not significant.

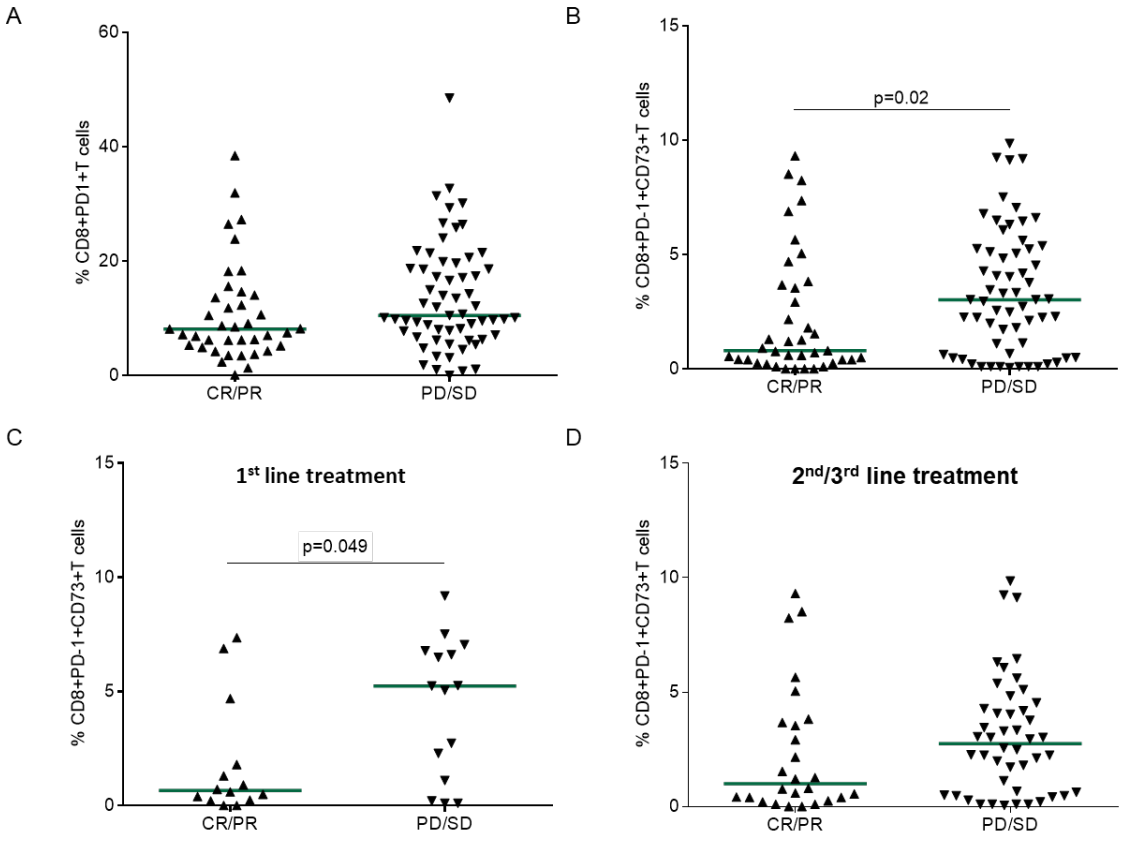

Figure 9 – *Pretreatment frequencies of T cells subpopulations in patients with melanoma treated with nivolumab, according to clinical outcomes. Frequency of CD8+PD+1 T cells (A) and CD8+PD-1+CD73 T cells (B) considering all the patients. Frequency of CD8+PD-1+CD73+ T cells for patients treated as first line (C) and second or third line (D). The green line indicates the median value. P values are from Mann-Whitney test. CR=complete response; PR= partial response; PD=progressive disease; SD=stable disease; Pi= inorganic phosphate*

3. Serum CD73 is associated with clinical outcomes of melanoma patients receiving anti-PD-1 treatment and is an independent prognostic factor

After investigating the role of circulating CD73+ lymphocytes, the second part of this thesis was focused on the study of non-cell bound CD73 in serum samples.

The soluble form of CD73 has been described to be increased in patients with different types of cancer and many studies indicated that soluble CD73 may serve as potential biomarker of response of cancer patients, as discussed in the introduction. Data previously published by Morello's group showed that CD73 activity in serum correlates with overall survival, progression-free survival, and clinical response to nivolumab treatment, in a single cohort of 37 patients with metastatic melanoma (Morello et al., 2017).

The validation of these finding in a greater number of patients, including different multicenter cohorts, was the starting point of the second part of this thesis.

The results here discussed have been published in:

Turiello R, Capone M, Giannarelli D, Morretta E, Monti MC, Madonna G, Mallardo D, Festino L, Azzaro R, Levesque MP, Imhof L, Weide B, Amaral T, Chevrier M, Sucker A, Rutkowski P, Schadendorf D, Lebbe C, Luke JJ, Wistuba-Hamprecht K, Dummer R, Pinto A, Morello S, Ascierto PA. Serum CD73 is a prognostic factor in patients with metastatic melanoma and is associated with response to anti-PD-1 therapy. *J Immunother Cancer*. **2020** Dec;8(2):e001689. doi: 10.1136/jitc-2020-001689. PMID: 33361405; PMCID: PMC7759961.

3.1 Materials and methods

3.1.1 Patients and serum samples

Serum samples from a total of 546 melanoma patients (stage III or IV) were collected at six different centers, prior to the start of treatment with pembrolizumab, nivolumab, or nivolumab in combination with ipilimumab. Patients received treatment as first, second or third line.

One hundred patients were from the Istituto Nazionale Tumori—IRCCS—Fondazione "G. Pascale", Naples (Italy), 53 patients from the University Hospital Essen (Germany), 111 patients from the University Hospital Tübingen (Germany), 88 patients from the Hospital Saint Louis of Paris (France), 144 patients from the University of Zurich (Switzerland) and 50 patients from the University of Chicago (USA).

Serum samples from patients undergoing anti-PD-1 therapy (3 months) were also available: 41 patients from the Istituto Nazionale Tumori—IRCCS—Fondazione "G. Pascale" (Naples) and 19 patients from the University of Chicago.

Most of the patients (496/546, 91%) were diagnosed with stage IV melanoma, according to VII edition of American Joint Committee on Cancer (Balch et al., 2009). Response to therapy was evaluated according to RECIST V.1.1 criteria (Eisenhauer et al., 2009).Tumor response was assessed every 12 weeks. Characteristics of patients are summarized in table 5.

Sera from 96 healthy donors (69 males and 27 females), aged >18 years, were collected at Istituto Nazionale Tumori—IRCCS—Fondazione "G. Pascale", Naples, (Italy) and used as controls.

All sera were stored at -80°C until use.

The study was conducted according to the provisions of the Declaration of Helsinki, and it was approved by the Ethic Committee of Istituto Nazionale Tumori IRCCS Fondazione "G. Pascale" (Naples); and all individuals provided a signed informed consent for blood donation.

3.1.2 Colorimetric assay with Malachite Green

AMPase activity in sera, from both melanoma patients and healthy donors, was measured by a colorimetric assay based on the quantization of inorganic phosphate (Pi) (Morello et al., 2019).

Sera were thawed and protein concentration was measured by Bradford assay. For measuring the AMPase activity, 100 µg of each sample were used. Specifically, 100 µg of proteins were incubated at 37°C, for 10 minutes in 100 µL of a Pi-free buffer, whose composition was the following: 10 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, 50 mM Tris–HCl, pH 7.4. Then, AMP was added as substrate, at a final concentration of 2 mM and samples were incubated again at 37°C for 40 minutes. For each sample, the experiment was performed also in absence of AMP, in order to exclude the baseline amount of Pi. The reaction was quenched using trichloroacetic acid (TCA, final concentration 5%) and samples were centrifuged at 3000 g. Supernatants were then used to measure the Pi, by means of Malachite Green Phosphate Assay Kit (8118, ScienCell Research Laboratories). The net value of Pi was calculated by subtracting the baseline Pi from the Pi produced after the hydrolysis of AMP.

To confirm that the Pi measured was derived from the reaction catalyzed by CD73, 71 randomly chosen samples were also tested in presence of 100 µM adenosine 5'-α,β-methylene diphosphate (APCP, Sigma Aldrich), a potent CD73 inhibitor. For this set of experiments, samples were incubated with APCP for 30 minutes, at 37 °C, prior to the adding of the substrate.

Results were expressed as pmol of Pi produced in 1 minute, by 1 mg of proteins (pmol/min/mg proteins).

3.1.3 Multiple reaction monitoring mass spectrometry

To confirm the results obtained with the colorimetric assay, the AMPase activity in sera was also measured by Multiple Reaction Monitoring Mass Spectrometry (MRM-MS), using randomly chosen samples from melanoma patients and healthy donors. As described above, 100 µg of proteins were equilibrated for 10 minutes, at 37°C, in the reaction buffer. Samples

were treated with APCP inhibitor (100 µM) or with the anti-human CD73 mAb (Clone 7G2) (5 µg/mL) for 30 minutes at 37 °C. Then, ^{15}N-AMP (25 µM; Sigma-Aldrich) was used as substrate and samples were incubated again at 37°C for 120 minutes. Finally, 25 µL of each sample were collected and added to 25 µL of ice-cold TCA (final concentration 5%), to stop the reaction. Samples were centrifuged 3000 g, 10 min, 4°C and the supernatants were dried in Vacuum Concentrator Savant (ThermoFisher). The pellets were dissolved in 50 µL of 10 mM Ammonium Acetate (AmAC, Sigma-Aldrich) containing 0.1% acetic acid (AA; Sigma-Aldrich). The obtained samples were submitted to ultra-performance liquid chromatographyelectrospray ionization (UPLC-ESI)-MRM-MS analysis to quantify the ^{15}N-AMP. UPLC-ESI-MRM-MS analyses were performed on a 6500 Q-TRAP from AB Sciex equipped with Shimadzu LC-20A and Autosampler systems, injecting 2 µL of each sample. UPLC separation was performed on a Luna Omega Polar 1.6 µm C18 100 Å column (50×2.10 mm, Phenomenex, Torrance, California, USA) at a flow rate of 400 µL/min; 10 mM AmAc/0.1% AA in H2 O (phase A) and 0.1% AA in MeOH (phase B) were used as mobile phases and the following gradient was exploited: 0% B from 0 to 0.5 min, 0% to 5% B over 2.5 min, 5% to 95% B over 2 min, then held at 95% B for 2 min and re-equilibrated to 0% B over 5 min. Q-TRAP 6500 was operated in positive MRM scanning mode, with declustering potential set at 80 V, entrance potential at 10 V, collision energy at 30 V and cell exit potential at 12 V. 15N-AMP was monitored through the 353.19/140.00 transition: the area of its related peak in each sample was measured using the Analyst Software from AB Sciex.

3.1.4 Tissue non-specific alkaline phosphatases activity

The activity of tissue non-specific alkaline phosphateses (TNAP) was evaluated incubating 100 µg of proteins from serum of 5 melanoma patients, with p-nytrophenolphosphate (pNPP), used at four different concentrations: 0.5 mM, 1 mM, 2.5 mM, 5 mM. The reaction buffer was the same one used for measuring the activity of CD73, reported above. The experiments were

performed both at pH=7.4 and pH=9.8. Samples were incubated at 37°C, for 40 minutes. The hydrolysis of pNPP leads to the formation of p-nitrophenolate, a colored product which can be measured by spectrophotometer at 405 nm.

3.1.5 Enzyme-linked immunosorbent assay for CD73 expression

CD73 was quantified in randomly selected sera of 83 patients with melanoma and 38 healthy subjects, using a commercially available ELISA kit (ab213761, Abcam), following the manufacturer's instructions.

3.1.6 CD39 activity assay

To evaluate the ATPase activity in serum samples, 100 µg of proteins were incubated for 10 minutes at 37°C, in the reaction buffer ($CaCl_2$ 5 mM, NaCl 120 mM, KCl 5 mM, Glucose 60 mM, Tris HCl 50 mM, pH 7.4). Then, the CD39 inhibitor ARL67156 (100 µm) or the vehicle (PBS) were added to the samples. After 30 minutes of incubation at 37°C, ATP (2 mM) was added and samples were incubated again at 37°C, for 40 minutes. The reaction was stopped by adding TCA (final concentration: 5%) and samples were then centrifuged at 3000 g. The supernatants were used to measure the levels of ATP, by the ATP assay kit (ab83355, Abcam). Experiments were performed using 26 sera from melanoma patients.

3.1.7 Enzyme-linked immunosorbent assay for CD39 expression

CD39 expression was measured in serum of 31 patients with melanoma and 10 healthy donors, using a commercially available ELISA kit (Human CD39 ELISA kit, LS-F25268, LifeSpan BioSciences, Inc).

3.1.8 Statistical analyses

Results are presented using absolute frequencies and percentages when referring to categorical variables, and median and range when considering quantitative variables. Survival time was analyzed with the Kaplan Meier method, and log-rank test was used to test for differences. Proportional hazard models were used to estimate HRs and their 95% CIs. Multivariate analysis

was performed using a forward stepwise method based on Wald statistics with p=0.05 and p=0.10 as enter and remove limit. IBM SPSS V.21.0 statistical software was used for these analyses. All other analyses were performed using GraphPad Prism V.7.0. In figures, mean ± SD is shown. The Mann-Whitney test or analysis of variance test were used for two groups comparison or three or more groups comparison, respectively.

3.2 Results

3.2.1 Clinicopathological characteristics of patients

For this part of the PhD project, a total of 546 patients (median age: 65 years) with melanoma were included. The characteristics of patients are summarized in table 5.

Three hundred twenty-four patients were male (59.3%), while 222 (40.7%) were female. Mutation of *BRAF* was present in 180 patients (33%), while 315 patients (57%) had wild-type tumors and the *BRAF* status was unknown for 51 patients (9.3%). Brain metastases were present in 87 patients, while bone metastases were present in 93 patients.

Most of the patients (83%) received PD-1 monotherapy, nivolumab (n=182) or pembrolizumab (n=271), and 17% received nivolumab plus ipilimumab (n=93). Regarding the line of treatment, 218 patients received first-line PD-1 monotherapy, 134 patients received second-line PD-1 monotherapy and 101 patients received PD-1 monotherapy in third line or later. Thirty-eight patients received first-line nivolumab plus ipilimumab, 27 patients second line and 28 patients third line or later. The baseline characteristics of the patients' groups were comparable (table 5). The median progression-free survival (PFS) was 7.1 months (95% CI: 5.3 to 8.8) and the median overall survival (OS) was 27.0 months (95% CI: 22.7 to 31.3).

Excluding patients who were lost to follow-up, in the group of patients receiving anti-PD-1 agents in monotherapy (n=447), a total of 154 patients (35%) had a complete response (CR, n=87) or partial response (PR, n=67), while the others 293 (65%) showed progressive disease

(PD, n=205) or stable disease (SD, n=88). In the subgroup of patients receiving nivolumab plus ipilimumab (n=90), 28 patients (32%) presented CR (n=3) or PR (n=25), while 62 patients (68%) had SD (n=16) or PD (n=46). The median follow-up for patients receiving nivolumab or pembrolizumab in monotherapy was 29 months; for patients receiving nivolumab in combination with ipilimumab, the median follow-up was 22 months.

Table 5 - *Clinicopathological characteristics of melanoma patients*

	Nivolumab or pembrolizumab therapy (no, %)		**Nivolumab + ipilimumab therapy (no, %)**		**TOTAL (no, %)**	
Total	453	100	93	100	546	100
Median age, years (range, 19-94)	65		61		64.5	
Gender						
Male	271	59.8	53	57.0	324	59.3
Female	182	40.2	40	43.0	222	40.7
Stage						
III	48	10.6	4	4.3	52	9.5
IV	402	88.7	89	95.7	491	89.9
unknown	3	0.66	0		3	0,5
Serum lactate dehydrogenase						
< ULN	308	74.0	51	54.8	359	65.8
≥ ULN	116	19.6	41	44.1	157	28.7
unknown	29	6.4	1	1.1	30	5.5
BRAF status						
wt	270	59.6	45	48.4	315	57.7
mut	147	32.5	33	35.5	180	33.0
unknown	36	7.9	15	16.1	51	9.3
Brain metastasis						
yes	78	17.2	9	9.7	87	15.9
no	307	67.8	41	44.1	348	63.7
unknown	68	15.0	43	46.2	111	20.3
Bone metastasis						
yes	80	17.6	13	14.0	93	17.0
no	302	66.7	37	39.8	339	62.1
unknown	71	15.7	43	46.2	114	20.9
Line of treatment						
1	218	48.1	38	40.9	256	46.9
2	134	29.6	27	29.0	161	29.4
≥ 3	101	22.3	28	30.1	129	23.6

3.2.2 Baseline AMPase activity in serum is higher in melanoma patients than in healthy donors and it is CD73-dependent

The AMPase activity was measured in sera of melanoma patients collected prior to the start of treatment with anti-PD-1 agents and in sera of healthy donors. The colorimetric assay with Malachite Green revealed that the AMP hydrolysis is higher in melanoma patients (n=546, mean: 37.85 ± 44.31 pmol/min/mg proteins) than in healthy donors (n=96, mean: 7.88 ± 15.5 pmol/min/mg protein) ($p<0.0001$, two-sided Mann-Whitney test) (figure 10A).

Additional experiments using sera from melanoma patients (n=71) were performed in presence of APCP (100 µM), a chemical inhibitor of CD73. In this condition, the AMPase activity resulted significantly reduced (mean: 49.98±29.59 pmol/min/mg protein) compared to the control (mean: 26.55±27.74 pmol/min/mg protein) ($p<0.0001$, two-sided Mann-Whitney test) (figure 10B), suggesting the relevant role of CD73 in hydrolyzing AMP.

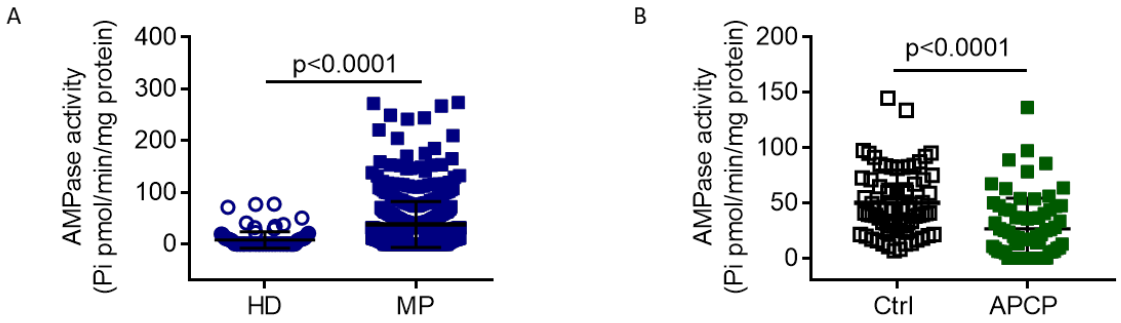

Figure 10 – *A) The AMPase activity in serum, measured by colorimetric assay with Malachite green, in melanoma patients (MP, n=546) and healthy donors (HD, n=96). B) The inhibitor of CD73, APCP (100 µM), significantly reduced the hydrolysis of AMP (n=71). Mean±SD is shown. P values are from two-sided Mann-Whitney test (A, B).*

The presence of soluble enzymes able to metabolize purines in bloodstream has been documented; among these enzymes, tissue non-specific alkaline phosphatases (TNAP) deserve particular attention, being these phosphatases able to hydrolyze AMP (Yegutkin, 2008).

Thus, to verify whether TNAP could influence the AMPase activity in human serum, samples from melanoma patients (n=7) were incubated with para-nitrophenolphosphate, which can be hydrolyzed by these enzymes. The product of the reaction is a colored molecule, para-nitrophenolate, which can be measured by spectrophotometry (405 nm). The formation of the colored product was observed only at pH=9.8 and not at pH=7.5 (figure 11). Importantly, the experiments performed to measure the AMPase activity were carried out at physiological pH, which is the optimal condition for the activity of CD73, a condition in which the TNAP activity can be considered as negligible. This result confirmed that TNAP are not active in the experimental condition used to measure the AMPase activity, excluding any relevant role for these enzymes in producing adenosine in serum samples.

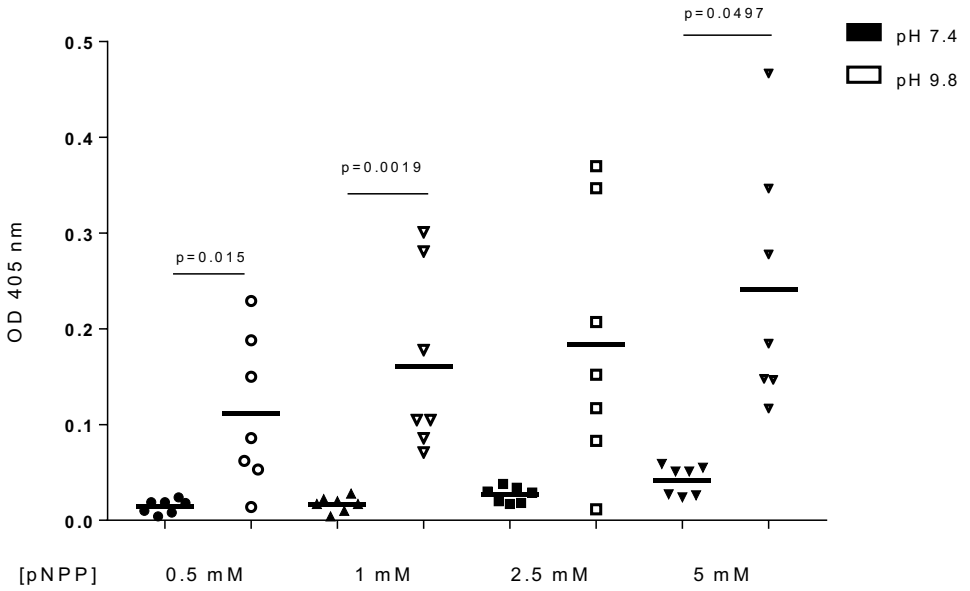

Figure 11 – *TNAP activity in serum of melanoma patients (n=5), in presence of p-nytrophenolphosphate, pH=9.8 or pH=7.4. Black lines represent mean. P values are from Kruskal-Wallis test.*

Then, to validate the results obtained with the colorimetric assay and to further confirm that the AMPase activity is dependent on CD73, serum samples from melanoma patients (n=13) were incubated with ^{15}N-AMP, in presence or absence of APCP or anti-human CD73 monoclonal

antibody (clone 7G2). The reduction of the substrate was measured by MRM-MS and it resulted significantly reduced in presence of APCP, as well as in presence of the antibody, compared with the control (figure 12).

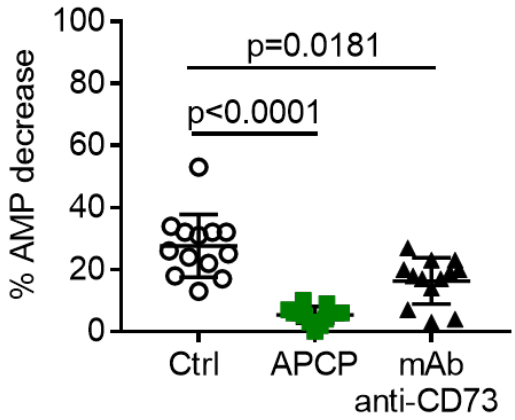

Figure 12 – *Percentage of 15N-AMP decrease in sera of melanoma patients (n=13), measured with multiple reaction monitoring mass spectrometry, in presence of APCP (100 µM) or the mAb anti-CD73 (clone 7G2, 5 µg/ml), or PBS (ctrl). Mean±SD is shown. P values are from one-way analysis of variance test.*

In samples from healthy donors, the reduction of ^{15}N-AMP was nearly absent (not shown).

Altogether, these results confirm that the AMPase activity in serum is higher in melanoma patients and it is mostly dependent on CD73.

3.2.3 The expression of CD73 in serum is higher in melanoma patients than in healthy donors

The expression of CD73 was measured by ELISA, in sera of 83 melanoma patients and 38 healthy donors, resulting significantly higher in patients (mean: 6.30 ng/mL) than in healthy subjects (3.02 ng/mL) (p=0.0369, two-sided Mann-Whitney test) (figure 13).

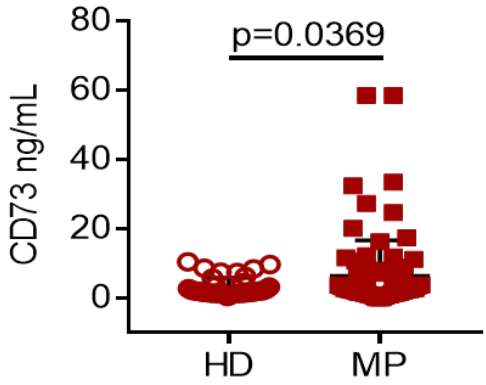

Figure 13 – *Expression of CD73 in sera of 83 melanoma patients (MP) and 38 healthy donors (HD), measured by ELISA. Mean ± SD is shown. P value is from two-sided Mann-Whitney test.*

3.2.4 The enzymatic activity of CD73 in serum better stratifies melanoma patients from healthy donors than its expression

The values of activity and expression of CD73 in healthy donors and melanoma patients were plotted in Receiver Operating Characteristic (ROC) curves, showed in figure 14. The analysis of the curves revealed that the enzymatic activity (AUC=0.7751) better stratifies patients from healthy donors than the expression (AUC=0.6169). Thus, all the subsequential analyses were focused on the activity of CD73, rather than its expression.

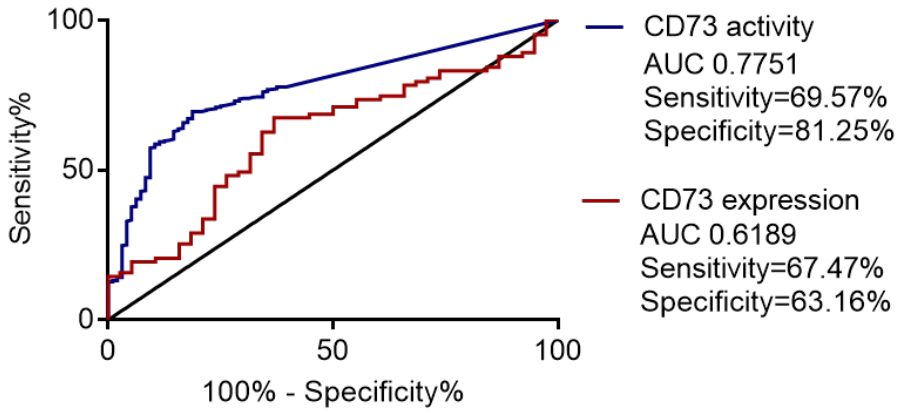

Figure 14 - *Receiver operating characteristic curve for CD73 expression (red) and CD73 activity (blue) in patients with melanoma versus healthy donors. The area under the curve (AUC), sensitivity and specificity values are calculated using GraphPad Prism 7.*

3.2.5 The role of soluble CD39 in the production of adenosine in serum is negligible

The production of extracellular adenosine is mainly mediated by CD73, whose substrate AMP can derive from the hydrolysis of ATP catalyzed by CD39. This latter enzyme can be cleaved from cell membrane, and it is found in bloodstream as well as CD73. Thus, the ATPase activity and CD39 expression in serum was also evaluated.

The expression of CD39 was measured by ELISA and no differences were observed between melanoma patients (n=31) and healthy donors (10) (figure 15A).

The ATPase activity was evaluated in samples from melanoma patients (n=26) by using a commercially available kit (ab83355 Abcam), after incubating ATP with sera. Experiments were also performed in presence of ARL 67156, a CD39 inhibitor. The reduction of substrate resulted negligible (figure 15B).

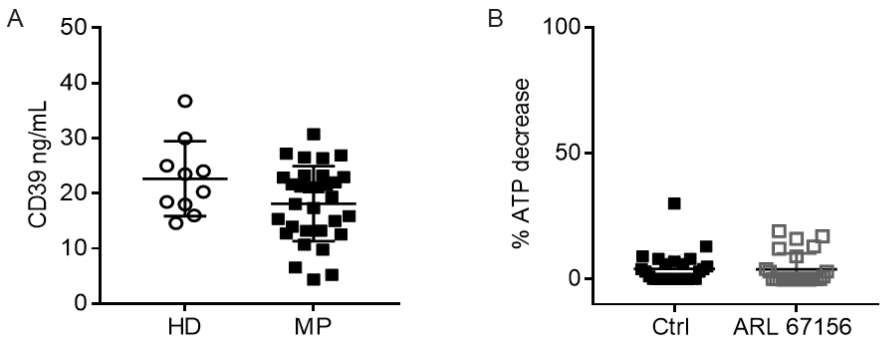

Figure 15 – *Expression and activity of CD39 in serum. A) CD39 protein levels in healthy donors (HD, n=10) and melanoma patients (MP, n=31) measured by ELISA. B) Percentage of ATP decrease in serum of melanoma patients (n=26), in presence of ARL 67156 (100 μM) or PBS (ctrl), measured by colorimetric assay. Mean ± SD is shown.*

These results rule out any relevant CD39 activity in serum of patients with melanoma, thus this enzyme was not considered for further analyses or experiments.

3.2.6 Pretreatment CD73 activity correlates with response to therapy with anti-PD-1 agents

The enzymatic activity of CD73 resulted higher in non-responders (PD or SD) than in responders (CR or PR) (p>0.0001) (figure 16A). The result was confirmed both for the group treated with anti-PD-1 monotherapy (nivolumab or pembrolizumab) (p=0.0013) (figure 16B) and for the group treated with the combination with anti-CTLA-4 (nivolumab plus ipilimumab) (p=0.0052) (figure 16C).

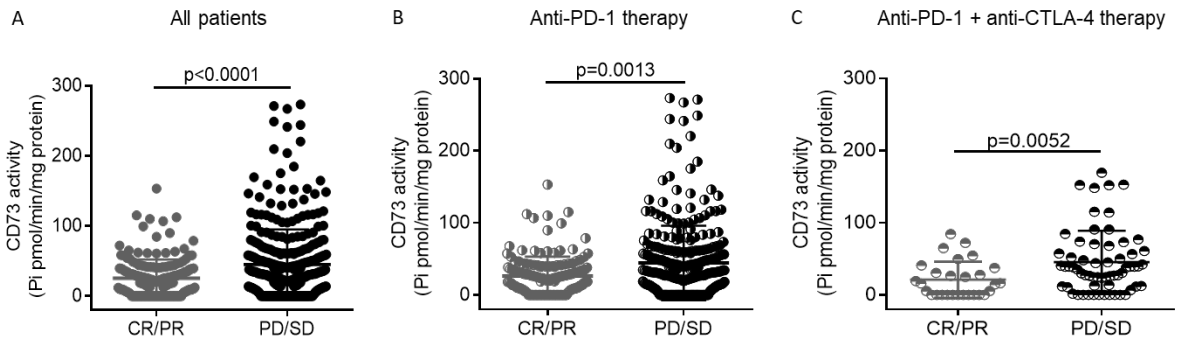

Figure 16 – *Baseline CD73 activity in melanoma patients according to clinical outcomes. A) CD73 activity in all patients receiving anti-PD-1 treatment (n=447); B) CD73 activity in patients receiving the monotherapy; C) CD73 activity in patients treated with nivolumab plus ipilimumab. Mean ± SD is shown. P values are from two-sided Mann-Whitney test. CR=complete response; PR= partial response; PD=progressive disease; SD=stable disease; Pi= inorganic phosphate.*

A further stratification of patients treated with monotherapy was performed according to the line of treatment. Among patients who received anti-PD-1 therapy as first line, the pretreatment levels of CD73 activity were significantly higher in patients who did not respond to therapy than those who responded (p=0.0195) (figure 17A). Considering the group treated as second line, the same tendency was observed (p=0.0545) (figure 17B). This analysis was not performed for the patients treated with the combination with anti-CTLA-4, due to the small number of patients included in this group.

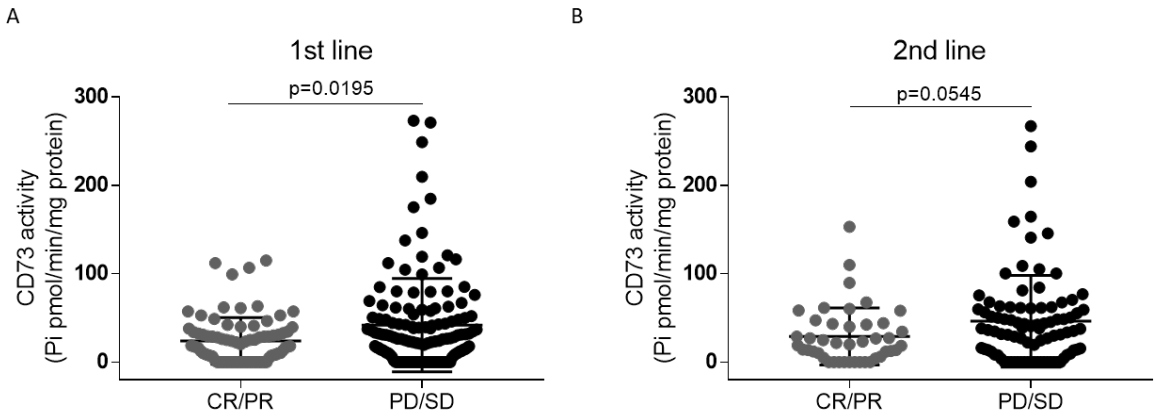

Figure 17 - *Baseline CD73 activity in patients treated with anti-PD-1 monotherapy according to the clinical outcomes and the line of treatment. A) CD73 activity in patients receiving anti-PD-1 as first line; B) CD73 activity in patients receiving anti-PD-1 as second line. Mean ± SD is shown. P values are from two-sided Mann-Whitney test. CR=complete*

response; PR= partial response; PD=progressive disease; SD=stable disease; Pi= inorganic phosphate.

3.2.7 CD73 activity is higher in non-responders than in responders after 3 months of treatment

The activity of CD73 was also evaluated in samples collected after 3 months from the start of therapy with anti-PD-1 monotherapy. Considering all patients together, no differences were observed between the two time points (figure 18A), but interestingly, when dividing patients according to the response to therapy, the activity of CD73 resulted higher in non-responders than in responders, not only at baseline but also after 3 months of treatment (figure 18B).

This analysis was not performed for the group receiving the combination with ipilimumab due to the small number of available samples.

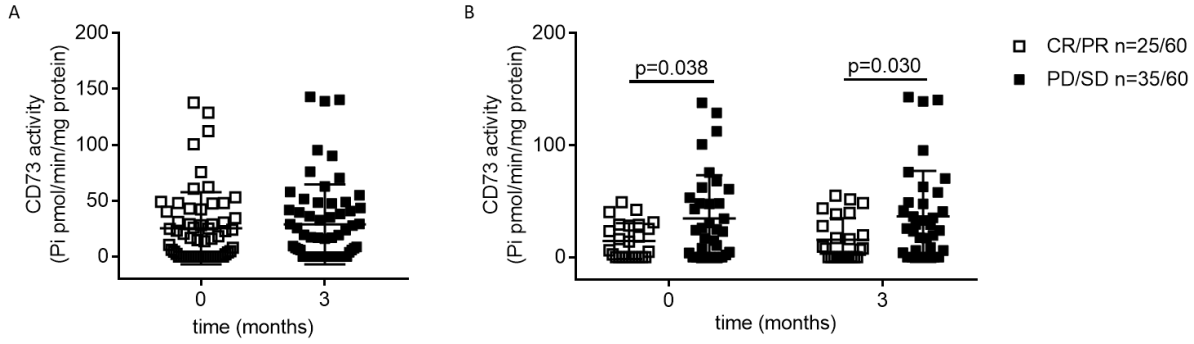

Figure 18 – *A) CD73 activity at baseline and after 3 months of treatment, in melanoma patients receiving anti-PD-1 monotherapy. B) Pretreatment and on-treatment CD73 activity, according to the clinical outcomes of patients. Mean ± SD is shown. P values are from two-sided Mann-Whitney test. CR=complete response; PR= partial response; PD=progressive disease; SD=stable disease; Pi= inorganic phosphate.*

3.2.8 Associations of pretreatment CD73 activity levels with survival

The next step was to analyze the associations between the enzymatic activity of CD73 and the survival of patients.

The discriminative cut-off value was set at 2 standard deviation (SD) from the mean of CD73 activity measured in healthy donors (7.88 ± 15.5 pmol/min/mg protein): 38.8. The patients were accordingly classified in two groups: patients with high CD73 (activity levels > 38.8 pmol/min/mg protein) and patients with low CD73 (activity levels < 38.8 pmol/min/mg protein).

High levels of CD73 activity resulted to be associated with worse OS (HR=1.36; 95% CI 1.03 to 1.78; p=0.03) (figure 19) and PFS (HR=1.42; 95% CI 1.13 to 1.79; p=0.003) in patients treated with nivolumab or pembrolizumab alone.

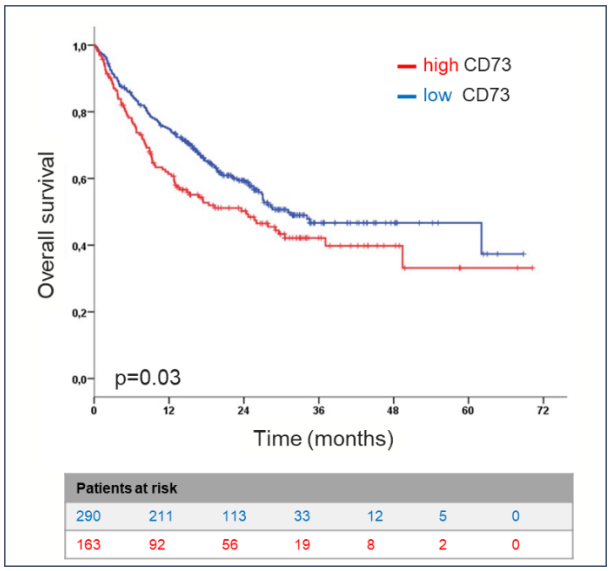

Figure 19 – *Kaplan-Meier curve for the overall survival of melanoma patients treated with anti-PD-1 monotherapy, according to the pretreatment CD73 activity. High CD73 (red line) is > 38.8 Pi pmol/min/mg protein; Low CD73 (blue line) is < 38.8 Pi pmol/min/mg protein. P value is from log rank test.*

Among patients receiving anti-PD-1 monotherapy as first line, the median PFS was significantly shorter in patients with high CD73 (4.2 months, 95% CI 2.4 to 6.0) than patients with low CD73 (12.3 months, 95% CI 6.6 to 17.9) (p<0.001) (figure 20A). In this group, patients with low CD73 showed a tendency to a longer OS (27.9 months, 95% CI 22.5 to 33.2)

than patients with high CD73 but significance was not reached (18.4 months, 95% CI 0.4 to 36.4) (p=0.09) (figure 20B).

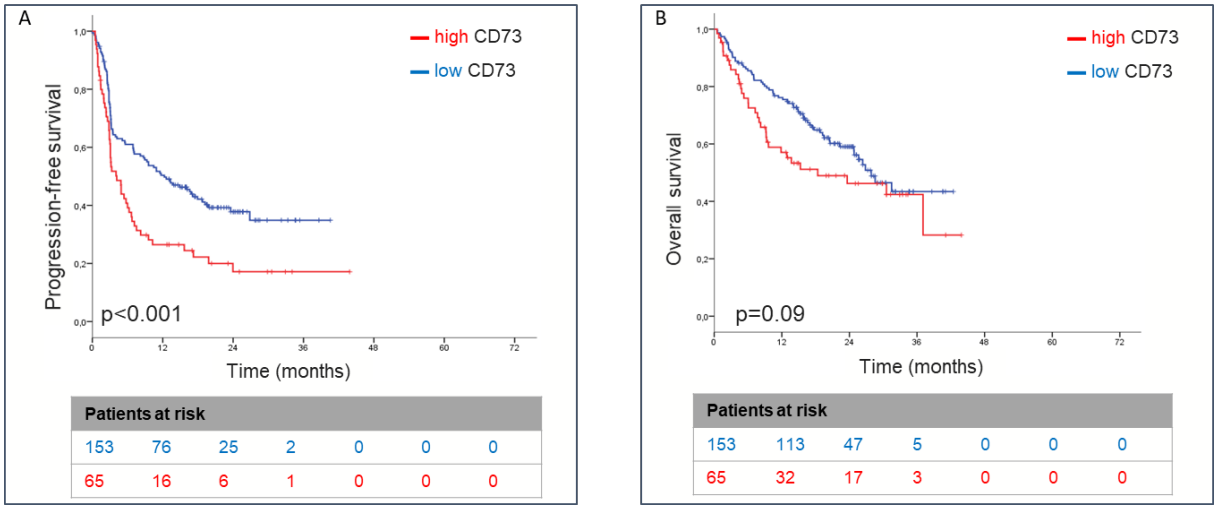

Figure 20 – *Kaplan-Meier estimating progression free survival (A) and overall survival (B) of melanoma patients receiving anti-PD-1 monotherapy as first line. High CD73 (red line) is > 38.8 Pi pmol/min/mg protein; Low CD73 (blue line) is < 38.8 Pi pmol/min/mg protein. P value is from log rank test.*

Regarding the patients treated with anti-PD-1 monotherapy as second line, no significant differences were observed for PFS (p=0.17) or OS (p=0.19) (figure 21).

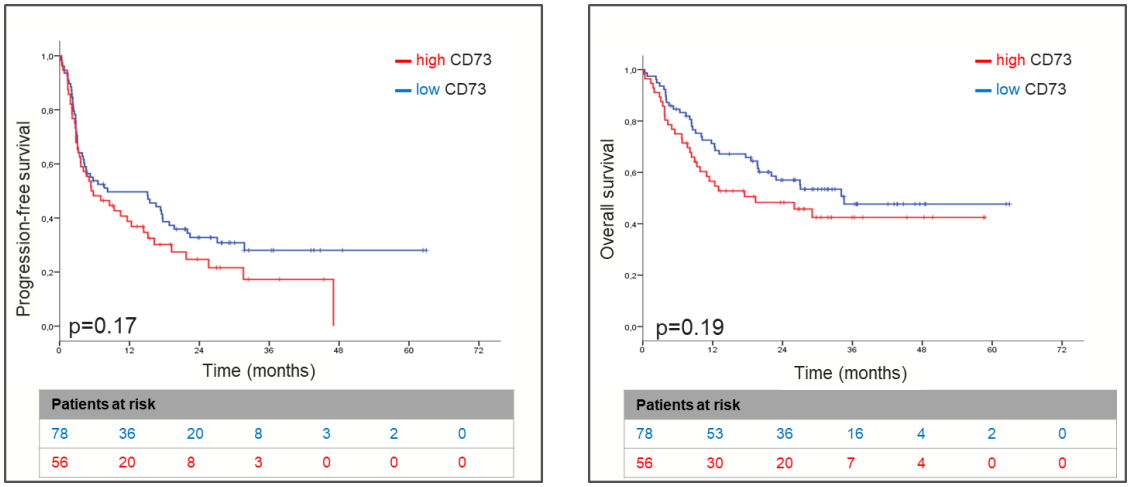

Figure 21 - *Kaplan-Meier estimating progression free survival (A) and overall survival (B) of melanoma patients receiving anti-PD-1 monotherapy as second line. High CD73 (red line) is*

> 38.8 Pi pmol/min/mg protein; Low CD73 (blue line) is < 38.8 Pi pmol/min/mg protein. P value is from log rank test.

Analyzing the patients receiving nivolumab in combination with ipilimumab, no association was found for OS (HR 1.41; 95% CI 0.82 to 2.44; p=0.23) or PFS (HR 1.31; 95% CI 0.82 to 2.08; p=026).

Additional analyses were performed for the group of patients treated with nivolumab or pembrolizumab alone, considering sex, age, *BRAF* mutation, LDH levels, brain or bone metastases.

Among female patients, a negative correlation with OS was found for high CD73 (HR=1.69; 95% CI 1.11 to 2.59; p=0.01) (figure 22A), while no correlation was found in the male patients group.

Patients aged <65 years with high CD73 showed a shorter OS, than patients aged <65 but with low CD73 (HR=1.87; 95% CI 1.26 to 2.78; p=0.002) (figure 22B), while no differences were observed among patients aged >65 years.

A negative correlation between high CD73 and OS was also found for patients with BRAF mutated tumors (HR=1.85; 95% ci 1.11 to 3.08; p=0.02) (figure 22C), but not for patients with BRAF wild-type tumors.

Patients with high LDH levels and high CD73 showed a shorter OS than patients with high LDH levels but low CD73 (HR=2.94; 95% CI 1.86 to 4.64; p<0.0001) (figure 22D), while no correlation was found in the group of patients with normal levels of LDH.

The OS resulted shorter for patients who had both high CD73 and bone metastases (HR=2.41; 95% CI 1.27 to 4.57; p=0.006) (figure 22E), compared with patients with bone metastases but low CD73. A similar tendency was observed comparing patients with high CD73 and brain

metastases versus patients with brain metastases but low CD73 (HR=1.74, 95% CI 0.96 to 30.15; p=0.062) (figure 22F).

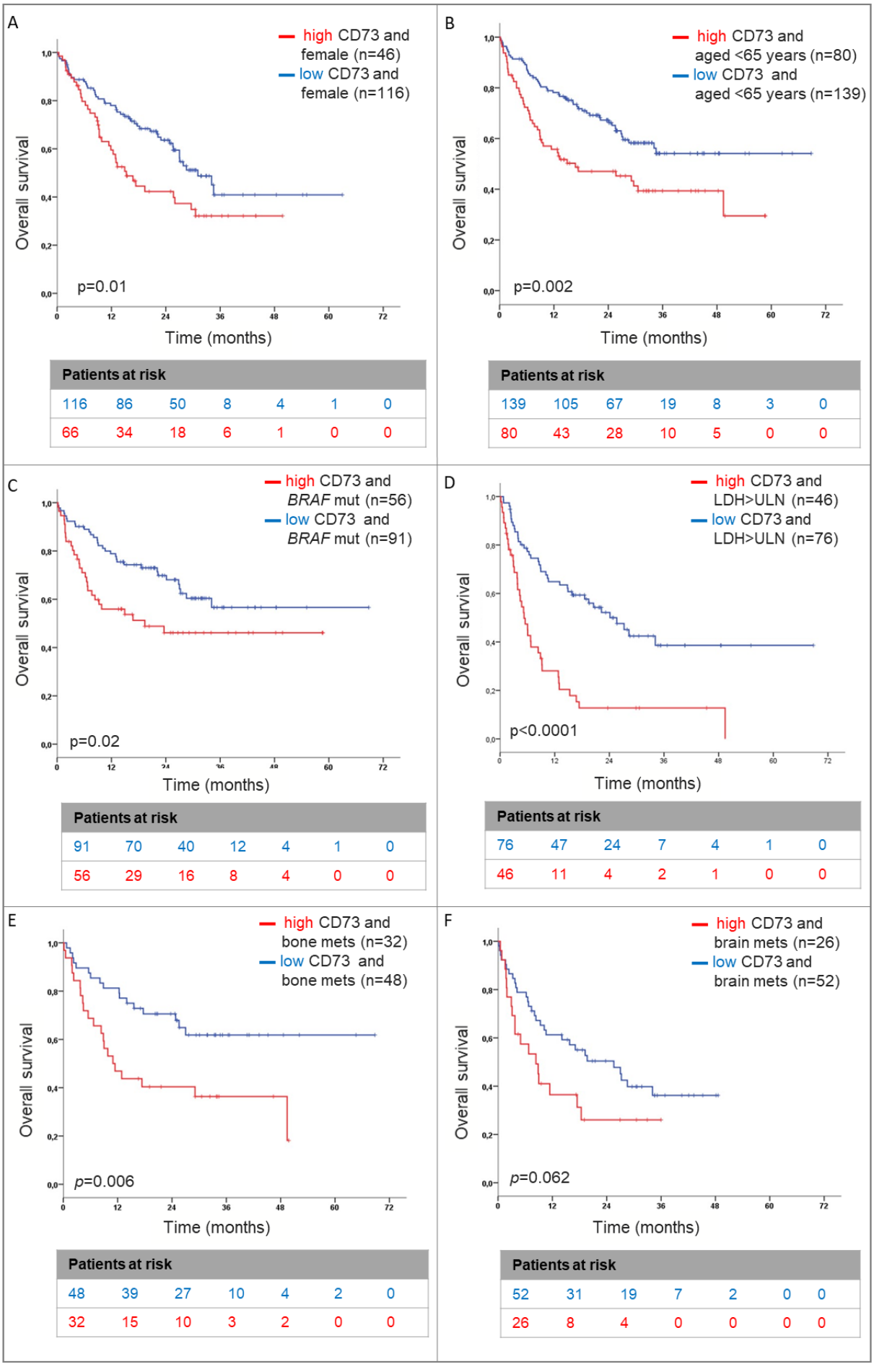
A
high CD73 and female (n=46)
low CD73 and female (n=116)
Overall survival
p=0.01
Time (months)
Patients at risk
116 86 50 8 4 1 0
66 34 18 6 1 0 0
B
high CD73 and aged <65 years (n=80)
low CD73 and aged <65 years (n=139)
Overall survival
p=0.002
Time (months)
Patients at risk
139 105 67 19 8 3 0
80 43 28 10 5 0 0
C
high CD73 and BRAF mut (n=56)
low CD73 and BRAF mut (n=91)
Overall survival
p=0.02
Time (months)
Patients at risk
91 70 40 12 4 1 0
56 29 16 8 4 0 0
D
high CD73 and LDH>ULN (n=46)
low CD73 and LDH>ULN (n=76)
Overall survival
p<0.0001
Time (months)
Patients at risk
76 47 24 7 4 1 0
46 11 4 2 1 0 0
E
high CD73 and bone mets (n=32)
low CD73 and bone mets (n=48)
Overall survival
p=0.006
Time (months)
Patients at risk
48 39 27 10 4 2 0
32 15 10 3 2 0 0
F
high CD73 and brain mets (n=26)
low CD73 and brain mets (n=52)
Overall survival
p=0.062
Time (months)
Patients at risk
52 31 19 7 2 0 0
26 8 4 0 0 0 0

Figure 22 – *Kaplan-Meier showing overall survival in subgroups of patients with melanoma receiving anti-PD-1 monotherapy. A) Female patients; B) patients aged <65 years; C) patients with BRAF mutation; D) patients with high lactate dehydrogenase (LDH) levels; E) patients with bone metastases; F) patients with brain metastases. High CD73 (red line) is > 38.8 Pi pmol/min/mg protein; Low CD73 (blue line) is < 38.8 Pi pmol/min/mg protein. P value is from log rank test. ULN= upper limit of normal.*

3.2.9 High CD73 activity is prognostic in melanoma

Finally, a Cox regression analysis was performed to evaluate potential associations of age, disease stage, sex, BRAF mutation status, serum LDH levels, line of treatment, brain or bone metastases and baseline serum CD73 activity with OS and PFS, among patients receiving nivolumab or pembrolizumab monotherapy (n=453). The univariate analysis showed that high levels of LDH, the presence of brain metastases and high levels of serum CD73 enzymatic activity were significantly associated with a shorter OS and PFS (table 6). In the multivariate model, CD73 emerged as an independent prognostic factor (OS: HR=1.52, 95% CI 1.11 to 2.08; p=0.009; PFS: HR=1.58 95% CI 1.22 to 2.05, p=0.001), besides LDH and brain metastasis (table 6).

Table 6 – *Cox regression analyses*

	Overall survival		Progression-free survival	
	UNIVARIATE HR (95% CI)	**MULTIVARIATE HR (95% CI)**	**UNIVARIATE HR (95% CI)**	**MULTIVARIATE HR (95% CI)**
Age, years (≥ 65 vs <65)	1.18 (0.90-1.54) P=0.23		1.01 (0.81-1.26) P=0.41	
Gender (male vs female)	0.95 (0.72-1.25) P=0.72		1.01 (0.80-1.27) P=0.95	
Serum lactate dehydrogenase (>UNL vs <UNL)	2.22 (1.67-2.95) P<0.0001	2.40 (1.74-3.31) P<0.0001	1.46 (1.14-1.88) P=0.003	1.61 (1.21-2.14) P=0.001
***BRAF* status** (mut vs wt)	0.79 (0.58-1.07) P=0.12		1.18 (0.93-1.52) P=0.16	
Brain metastasis (yes vs no)	1.78 (1.28-2.48) P=0.001	1.79 (1.27-2.53) P=0.001	1.62 (1.21-2.15) P=0.001	1.76 (1.31-2.36) P<0.0001
Bone metastasis (yes vs no)	0.96 (0.67-1.37) P=0.81		0.99 (0.74-1.34) P=0.97	
Line of treatment (1 vs >1)	1.06 (0.81-1.38) P=0.69		0.97 (0.77-1.21) P=0.77	
CD73 activity (>38.9 vs <38.9)	1.36 (1.03-1.78) P=0.03	1.52 (1.11-2.08) P=0.009	1.42 (1.13-1.79) P=0.003	1.58 (1.22-2.05) P=0.001

4. Serum-derived exosomes suppress lymphocyte functions in a CD73-dependent manner and associate with resistance to anti-PD-1 therapy

As anticipated, the circulating portion of non-cell bound CD73 includes not only the soluble form, but also an exosomal form. To date, less is known about the exosomal form of CD73, and further investigations are required to better understand the role of CD73+ exosomes in cancer, evaluating the possibility to use these vesicles as biomarkers of prognosis.

In melanoma, circulating tumor-derived exosomes are enriched in immunosuppressive proteins (including CD73) and impair CD8+ T cell functions (Sharma et al., 2020).

The expression of exosomal PD-L1 emerged as potential biomarker of response in melanoma patients receiving anti-PD-1 therapy, resulting increased in responders patients (Chen et al., 2018).

The third part of this thesis was aimed to investigate the role of CD73+ exosomes in impairing T cell functions, in melanoma patients receiving anti-PD-1 therapy. The expression and the activity of exosomal CD73 were analysed in order to evaluate their potential as predictive factors.

The results here discussed have been published in:

Turiello R, Capone M, Morretta E, et al. Exosomal CD73 from serum of melanoma patients suppresses lymphocyte functions and is associated with therapy resistance to anti-PD1 agents. *Journal for ImmunoTherapy of Cancer* **2022***; 10:e004043* PMID: 35273100 PMCID: PMC8915288 DOI: 10.1136/jitc-2021-004043.

4.1 Materials and methods

4.1.1 Serum samples

For the third part of this project, 41 melanoma patients and 19 healthy donors (aged > 18 years) were included. Sera were collected at Istituto Nazionale Tumori-IRCCS-Fondazione "G. Pascale" Naples (Italy).

In details, patients were treated with nivolumab (n=21) or pembrolizumab (n=20) in first, second or third line, and response to therapy was evaluated according to RECIST V.1.1 criteria and tumor response was assessed every 12 weeks (Eisenhauer et al., 2009).

Eighteen patients were classified as responders (PR=14; CR=4), while 23 patients were classified as non-responders (PD=19; SD=4).

Sera from patients were collected prior to the start of treatment (baseline) and after 4 weeks.

The study was approved by the Ethics Committee of Istituto Nazionale Tumori-IRCCS-Fondazione "G. Pascale", Naples (Italy). Written consent was obtained from each subject for blood donation.

All samples in 1ml aliquots were stored at -80°C and thawed immediately prior exosomes isolation.

4.1.2 Exosomes isolation

Exosomes were isolated by using size exclusion chromatography as described by Hong and collaborators (Hong et al., 2017). The protocol was modified as follows.

4.1.2.1 Sample preparation

The first steps of the protocols were aimed to clear samples from contaminants possibly present, such as cellular debris or big vesicles. Briefly, sera were thawed and filtered by using 70 μm pores filter. Then, the samples were centrifuged for 10 minutes at 2000g, 4°C and then again for 30 minutes at 10000 g, 4°C. Supernatants were ultrafiltered by using Spin-X Centrifuge

Tubes filter (Corning Incorpored), equipped with a 0.22 μm pore membrane, which allows the exosomes pass through the pores, while retains on the top the bigger vesicles and/or other contaminants. Volume of the samples was adjusted, if necessary, to 1 mL, adding PBS.

4.1.2.2 Mini Size exclusion chromatography (mini-SEC)

The samples were then loaded onto a column (Hercules Econo-pac, Bio-Rad) packed with 10 mL of Sepharose (CL-2B300, Sigma). Importantly, the Sepharose we used was provided as alcoholic mixture (80% Sepharose, 20% ethanol). To pack each column, the required amount of Sepharose was moved into a beaker and let it rest for 1h, at room temperature. Then the alcoholic phase was removed and the Sepharose was washed by adding 3 mL of PBS and gently mixed. The Sepharose was then let at room temperature for 30 minutes and the washing was repeated two more times.

The Sepharose was eventually poured into the column, which was already filled with PBS. The lower cap was immediately removed from the column and the Sepharose was let packing up to 10 mL.

PBS was used as eluent buffer, and specifically, 1 mL was used to elute each fraction. As already described (Hong et al., 2017;Sharma et al., 2018;Theodoraki et al., 2018), exosomes are eluted mostly in the fraction # 4, that was used for all the experiments we describe here. Once collected, the fraction #4 was then concentrated using Amicon Ultracel-100k tubes (Millipore #UFC510024).

4.1.3 BCA-protein assay

The protein concentration of the purified exosomal samples was determined by Pierce © BCA Protein Assay Kit (Thermo Scientific). Results were expressed as μg of proteins/ mL of serum.

4.1.4 Exosomes proteins characterization

Protein expression from purified exosomal samples was examined by Western blot. Ten micrograms of proteins were prepared in Laemmli buffer and loaded into a 10% polyacrylamide gel. The primary antibodies used were the following: CD9 (1: 1000; C-4: sc-13118, SantaCruz Biotechnology), CD-81 (1:1000; 1.3.3.22: sc-7637, SantaCruz Biotechnology), Calregulin (1:1000; A-9: sc-166837, SantaCruz Biotechnology), detected with the secondary antibody goat anti-mouse IgG1k HRP-conjugated (1:20000; A90-105P, Bethyl Laboratories) using the chemiluminescence detection system. Bands we visualized using Las4000 Imaging System (GE Healthcare Life Sciences).

4.1.5 Dynamic light scattering (DLS)

The size distribution of vesicles was measured by DLS using a Zetasizer Ver. 7.01, Malvern Instrument (Malvern, UK). Each sample was dispersed in deionized water and the intensity of the scattered light was measured with a detector at 90° angle at room temperature. Mean diameter and size distribution were the mean of three measures.

4.1.6 Scanning electron microscope (SEM)

Visualization of the isolated exosomal samples was performed by SEM. Samples were diluted (1:2) in ultrapure water (Milli-Q UF Plus, Millipore, Molsheim, France) and spotted onto carbon-coated aluminium stabs. Solvent was evaporated under nitrogen flux overnight (O/N). SEM images of the samples were then acquired using a Tescan Solaris microscope equipped with secondary electron and backscattered electron detectors (TESCAN, Brno, Czech Republic). Analyses were conducted both at 1.5 and 5 keV without any coating of the particles, respectively.

4.1.7 Beads-assisted flow cytometry

To analyse by flow cytometry the proteins expressed by exosomes, it was required to conjugate exosomes with magnetic beads, being these vesicles too small to be detected by a flow

cytometer. Specifically, the ExoCap™ Streptavidin Kit (MEX-SA) from MBL was used, adapting the protocol described by Theodoraki et al., (Theodoraki, Hoffmann et al. 2018) as follows. Fifty microliters of exosomes fraction #4 were incubated for 2h with 0.5 µg of anti CD63 biotinylated antibody (BioLegend, Clone: H5C6, 353017), at room temperature on rotary mixer (20 rpm). Then, 10 µL of the streptavidin magnetic beads from the kit were added and samples were incubated for 2h at room temperature, on rotary mixer. During these 2 hours, the streptavidin on the beads can bind the anti CD63 antibody, creating in turn a complex with exosomes. The complexes were then captured using a magnet (EasySep magnet, StemCell Tecnologies) and the supernatant was discarded. Samples were then re-suspended in 250 µL of PBS, and the suspension was divided into 5 aliquots of 50 µL. Each aliquot was used for the staining with one antibody. Specifically, the antibodies used were the following: anti-human CD73 FITC (Invitrogen, AD2, 11-0739-42) (0.250 µg/sample); anti-human PD-L1 APC (Invitrogen, B7-H1, 17-5983-42) (0.250 µg/sample); while the control isotypes were the following: mouse IgG1K APC (Invitrogen, 174714-82) (0.250 µg/sample), mouse IgG1K FITC (Invitrogen, 17-4714-42) (0.250 µg/sample). Samples were incubated for 1h at room temperature, repaired from the light, on rotary mixer. Then, the supernatant was discarded and exosomes were firstly washed using the buffer provided by the kit and eventually suspended in 300 µL of PBS.

For each sample, 10000 events were acquired using a BD FACScalibur (Becton Dickinson). Results were analysed by using BD CellQuest Pro Software. The mean fluorescence intensity (MFI) of positive beads, gated on forward and sideward light scatter, was measured. Results are expressed as relative fluorescence intensity (RFI), calculated as ratio between the MFI of the target of interest and the MFI of the control isotype. Values greater than 1 were considered positive.

4.1.8 CD73 enzymatic activity measurement

The activity of CD73 in purified exosomes was evaluated by MRM-MS, as described in the previous chapter and as previously published (Turiello et al., 2020). A total of 5 µg of exosomes proteins in 50 µL total volume of PBS were incubated at 37°C for 10 minutes, and then treated or not with CD73 inhibitors for 30 minutes at 37°C. Specifically, we used 5' α,β-methylene-ADP (APCP; Sigma-Aldrich) (100 µM) or the anti-human CD73 mAb (Clone 7G2; Thermo Fisher Scientific) (5 µg/mL). Then, ^{15}N-AMP substrate (10µM; Sigma-Aldrich) was added and substrate and samples were incubated for 120 minutes at 37°C. Immediately after adding the substrate and after 120 minutes of reaction, 15 µL of each sample were collected and treated with ice-cold trichloroacetic acid (TCA; 5% final concentration; Sigma-Aldrich) to quench the reaction. Samples were then centrifuged at 3000 g for 10 minutes, 4°C. The supernatants were dried in Concentrator Plus (Eppendorf) and dissolved in 25 µL of 10 mM Ammonium Acetate (AmAC, Sigma-Aldrich) containing 0.1% acetic acid (AA; Sigma-Aldrich). The samples were used to perform ultra-performance liquid chromatography-electrospray ionization (UPLC-ESI)-MRM-MS analysis, in order to quantify the ^{15}N-adenosine produced. Briefly, experiments were performed using a 6500 Q-TRAP from AB Sciex equipped with Shimadzu LC-20A and Autosampler systems, injecting 3 µL of each sample. UPLC separation was performed on a Luna Omega Polar 1.6 µm C18 100 Å column (50×2.10 mm, Phenomenex, Torrance, California, USA) at a flow rate of 400 µL/min and using 10 mM AmAC/0.1% Acetic acid in H_2O (A) and 0.1% Acetic acid in methanol (B) as mobile phases. The gradient was set as follows: 0.5 min at 0% B, 0.5 min to 3 min at 5% B, 3 min to 5 min at 95% B, 5 min to 7 min at 95% B and then back to 0% B for a 5 min re-equilibration step.

^{15}N-AMP was monitored through the 353.19/140.00 transition and ^{15}N-Adenosine through the 273.00/141.00 one; the areas of their related peaks in each sample were measured using the Analyst Software from AB Sciex.

4.1.9 Functional assays

Peripheral blood mononuclear cells (PBMCs) of healthy subjects were isolated by Ficoll density gradient (Histopaque-1077, Sigma–Aldrich). After isolation, cells were incubated in RPMI 1640 (Euroclone S.p.A.) supplemented with 10% (v/v) heat-inactivated FBS (Euroclone S.p.A.), 1% (v/v) penicillin–streptomycin (Euroclone S.p.A.), 1% (v/v) MEM non-essential amino acids (MEM NEAA; Euroclone S.p.A.), and 1% (v/v) sodium pyruvate (Euroclone S.p.A.). After 24 hours, 2 x 10^5/well cells were incubated in supplemented RPMI without serum, and then exosomes from serum of melanoma patients were added at concentration of 100 µg/ml. PBS was added to the cells instead of exosomes as control. AMP (50 µM) was added and cells were incubated for 1 h, at 37C, 5% CO_2. At the end of this incubation, cells were activated using ImmunoCult Human CD3/CD28 T cell activator (25 µg/ml), according to the manufacturer's instructions (StemCell Technologies). For some experiments, cells were pre-treated with the CD73 inhibitor APCP (100µM) or the A2A adenosine receptor antagonist ZM 241385 (1µM) (Sigma Aldrich) before adding the substrate AMP.

Supernatants from cells were collected after 72h and used to quantify the levels of IFN-γ, using DuoSet ELISA, following the manufacturer's protocol (R&D Systems).

Cells collected 24h later treatments described above were lysate to analyse the expression of Granzyme B (1:1000; BD Pharmingen, #550558) by Western blot.

4.1.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism 7.0. To compare two groups the Mann-Whitney test or the paired t-test was used.

The two-tailed unpaired *t*-test was used to analyse results from the *in vitro* experiments with PBMCs. To compare the expression of CD73 or PD-L1 between two time points, the Wilcoxon matched-pairs signed rank test was used. P values <0.05 were considered as significant.

4.2 Results

4.2.1 Clinicopathological characteristics of patients

The third part of this thesis included 41 patients (median age 64 years) with stage III (14.6 %) or stage IV (85.4%) (VII edition AJCC) melanoma, treated with nivolumab (n=21) or pembrolizumab (n=20), as first line (41.5%), second line (41.5%) or third line (17%). Eighteen patients responded to therapy, showing partial response (PR, n=14) or complete response (CR, n=4); while 23 patients did not benefit from treatment, showing progressive disease (PD, n=19) or stable disease (SD, n=4).

Patients showing CR or PR were grouped together as "responders", while patients showing PD or SD were grouped as "non-responders.

Characteristics of patients are summarized in table 7.

Table 7 – *Clinicopathological characteristics of patients*

	Nivolumab or Pembrolizumab N (%)
Total	41 (100%)
Median age Range (23-85)	64
Gender Male Female	 26 (63.4) 15 (36.6)
Disease stage III IV	 6 (14.6) 35 (85.4)
Line of treatment I II >III	 17 (41.5) 17 (41.5) 7 (17)

4.2.2 Characterization of exosomes purified from human serum

Dynamic light scattering showed that vesicles eluted in the fraction #4 of the mini-SEC have a mean diameter of 105.5 ± 21.99 nm (n=4) (figure 23A), which is the diameter expected for exosomes. Vesicles were visualized by Scanning Electron Microscopy experiments, which

confirmed the results from DLS (figure 23B). Furthermore, exosomes appeared intact (figure 23B) and this is of great importance, considering that exosomes were also used to treat cells or to measure the activity of CD73 expressed on their membrane, as discussed in the next sections.

The presence of the exosomal markers CD9 and CD81 and the absence of the negative marker calregulin was confirmed by Western Blot, as shown in figure 23C.

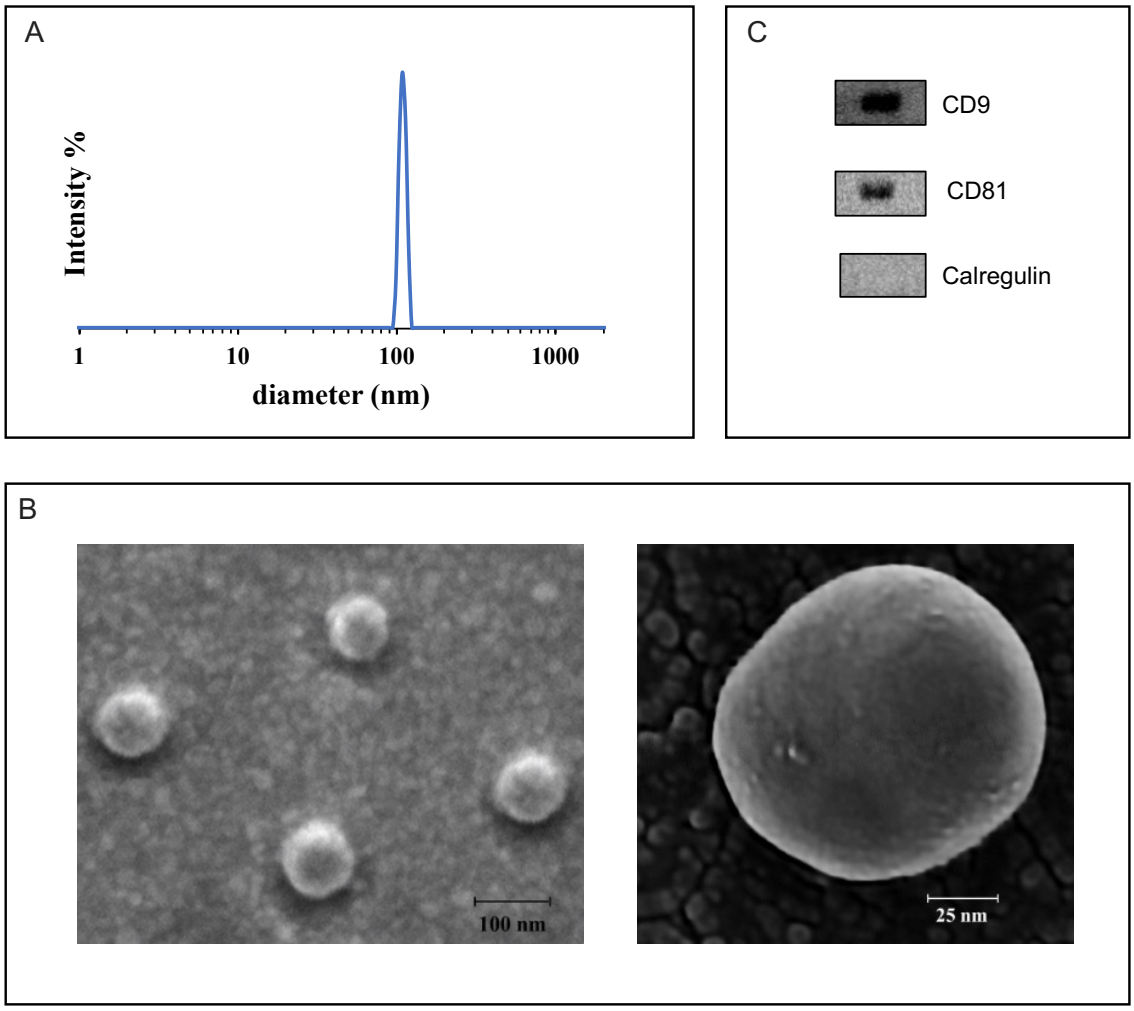

Figure 23 – *Exosomes characterization by A) Dynamic light scattering; B) Scanning Electron Microscopy; C) Western blot.*

4.2.3 Exosomes express CD73 and produce adenosine by hydrolysing AMP

The expression of CD73 on exosomes was firstly confirmed by Western blot (figure 24A). Then, CD73 expression was further analysed by beads-assisted flow cytometry (figure 24B).

CD73 was detected on both exosomes from healthy donors (n=9, mean RFI=1.351) and melanoma patients (n=25, mean RFI=1.338), without any significant differences between the two groups (Figure 24C).

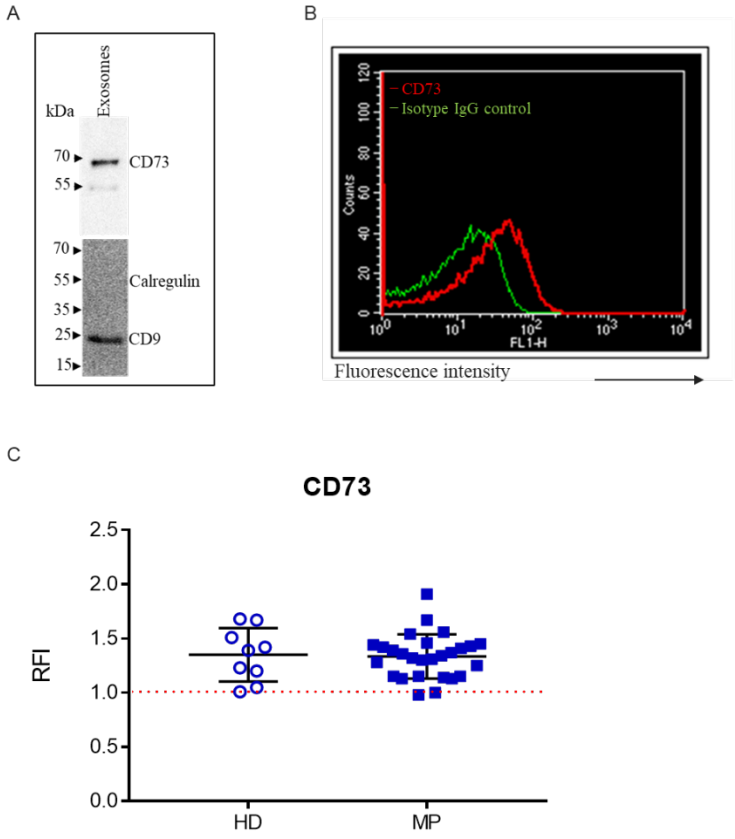

Figure 24 – *CD73 expression in exosomes isolated from melanoma patients (MP) and healthy donors* (HD). *A) A representative Western blot image for CD73 in purified exosomes. Calregulin is a negative marker for exosomes, while CD9 is a positive marker, confirming the presence of exosomes in samples. B) A representative dot blot showing the expression of CD73 (red line) on exosomes, measured by flow cytometry, in comparison with the signal of the isotype IgG control (green line). C) Comparison of exosomal CD73 expression between HD (n=9) and MP at baseline (n=27). Data are expressed as Relative Fluorescence Intensity and mean±SD is shown.*

Then, the activity of exosomal CD73 was evaluated by multiple reaction monitoring mass spectrometry, after incubating exosomes with ^{15}N-AMP. The production of labelled adenosine was detected in all exosome samples, both from healthy donors and melanoma patients.

Exosomes from melanoma patients produce more adenosine than those isolated from healthy donors, after 2 hours of incubation with ^{15}N-AMP (p=0.020, Mann-Whitney test). Specifically, patients derived exosomes produce 4.15 ng (mean value, n=6), while exosomes from healthy donors produce 1.55 ng (figure 25A).

Importantly, when exosomes were pre-treated with APCP or the mAb anti CD73, the production of adenosine resulted significantly reduced compared to the control (figure 25B-C).

This result clearly confirm that exosomes produce adenosine in a CD73-dependent manner.

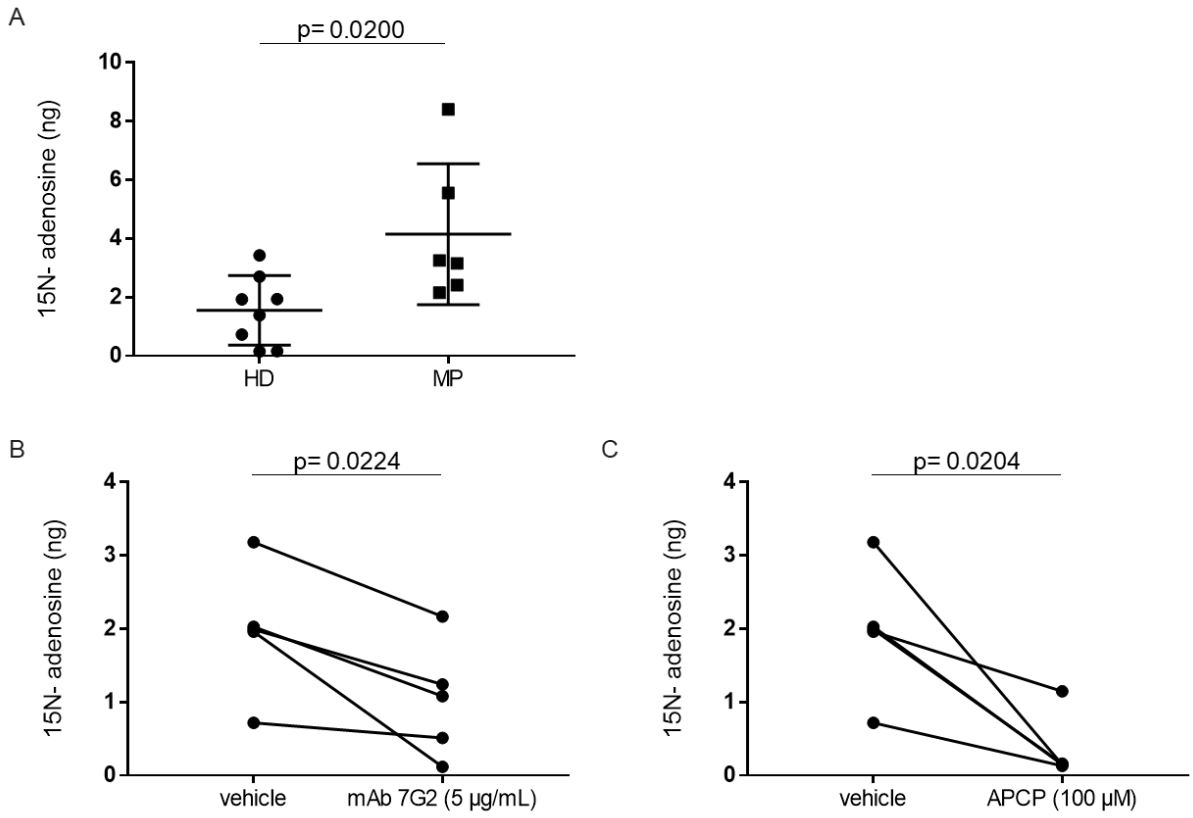

Figure 25 – *CD73 activity in exosome samples (5 µg) measured by MRM mass spectrometry, after 2h incubation with ^{15}N AMP (10 µM). A) ^{15}N-adenosine production in healthy donors*

(HD, n=8) and melanoma patients (n=6); B) ^{15}N-adenosine production in MP (n=5), in presence or absence of APCP (100 µM); C) ^{15}N-adenosine production in MP in presence or absence of the mAb anti CD73 7G2 (5 µg/mL). Mean ± SD is shown in A. P values are obtained from a Mann-Whitney test (A) or a paired t test (B-C).

Furthermore, the adenosine production in exosomes samples from melanoma patients, at baseline, was paired to the enzymatic activity measured in total serum (as described in methods section 3.2.3) and no correlation was observed (Pearson correlation r=-0.04131; P=0.9226, n=8) (figure 26).

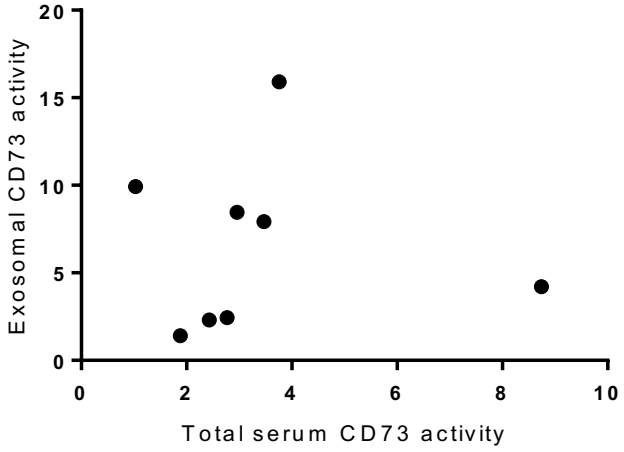

Figure 26 - *Pearson correlation between the fold change in 15N-adenosine production in exosomes and serum from patients with melanoma (n=8).*

4.2.4 Exosomes suppress T-cell functions in a CD73-dependent manner

After confirming the presence of active CD73 on exosomes, the next step was to evaluate whether exosomal CD73 could impair the function of T-cells. For this purpose, PBMCs isolated from healthy donors were activated with anti CD3/CD28 T cell activator, in presence of not of melanoma patients derived exosomes and AMP. As expected, control cells, that were not treated with CD3/CD28, did not produce IFN-γ (figure 27). The production of IFN-γ resulted significantly reduced in presence of AMP, in cells pre-treated with exosomes (figure 27),

compared with control or cells treated with exosomes alone, while the treatment of activated cells with exosomes alone did not cause any difference in comparison with control. Thus, any direct effect of exosomes on T cells is excluded.

Importantly, levels of IFN-γ restored in presence of APCP (figure 27), confirming that the suppressive effect on T cells is mediated by the adenosine produced in a CD73-dependent manner. To verify whether the CD73 expressed by T cells could have a role in the impairment of IFN-γ production, activated cells were incubated with AMP, in presence or absence of APCP, without exosomes. In these conditions, the levels of IFN-γ resulted unchanged compared to the activated control cells (figure 27). This result confirmed that the reduction of IFN-γ is mainly mediated by the activity of exosomal CD73, while CD73 expressed by T-cells has no relevant role.

To further clarify the role of CD73-derived adenosine, cells were also treated with the selective A2A antagonist ZM 241385. In this condition, the inhibitory effect of AMP in presence of exosomes resulted abrogated (figure 27).

Altogether these results indicate that adenosine generated by exosomal CD73 is able to inhibit the production of IFN-γ by stimulating A2A receptor on T-cells.

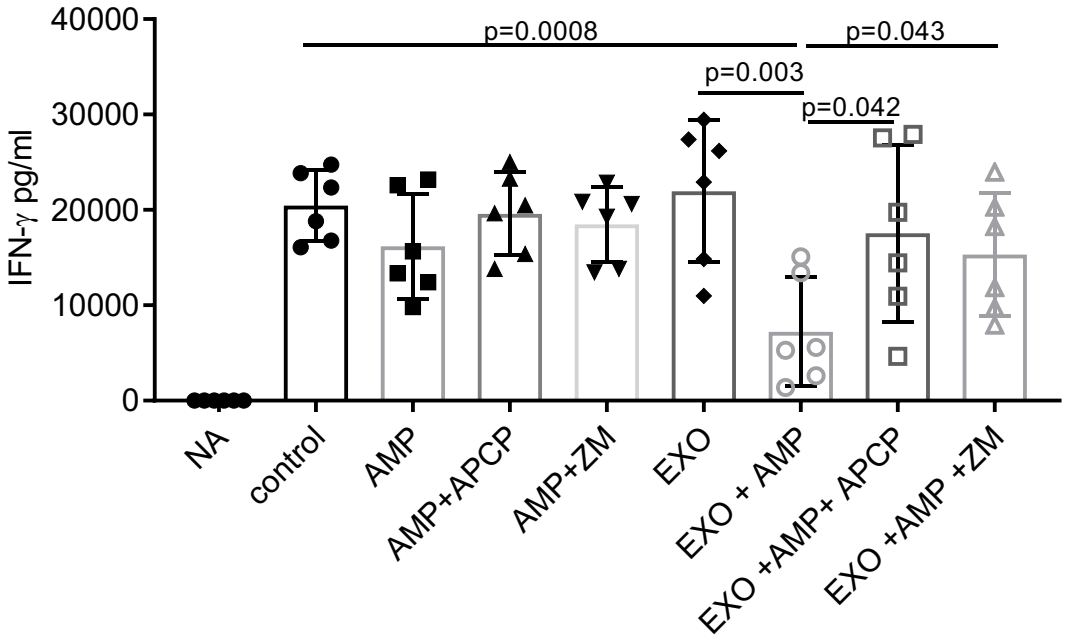

Figure 27 – *IFN-γ levels measured by ELISA in the supernatant of CD3/CD28 activated PBMCs from healthy donors with or without (100 µg/mL) patients derived exosomes (EXO) in presence or in absence of AMP (50 µM). Experiments were also performed in presence of the CD73 inhibitor APCP (100 µM) or the A2A receptor antagonist ZM241386 (ZM, 1 µM). Mean ± SD is shown. P values are obtained from a two-tailed unpaired t-test (n=6 independent biological experiments).*

To further explore the effects of exosomes-derived adenosine on T-cells, the expression of Granzyme B in CD3/CD28 activated cells was also evaluated (figure 28), and it resulted significantly reduced in cells treated with exosomes in presence of AMP, compared to the condition with exosomes alone (P=0.02, two-tailed unpaired t-test) (figure 28). This result further confirms that exosomes can suppress the effector functions of T-cells, in presence of AMP.

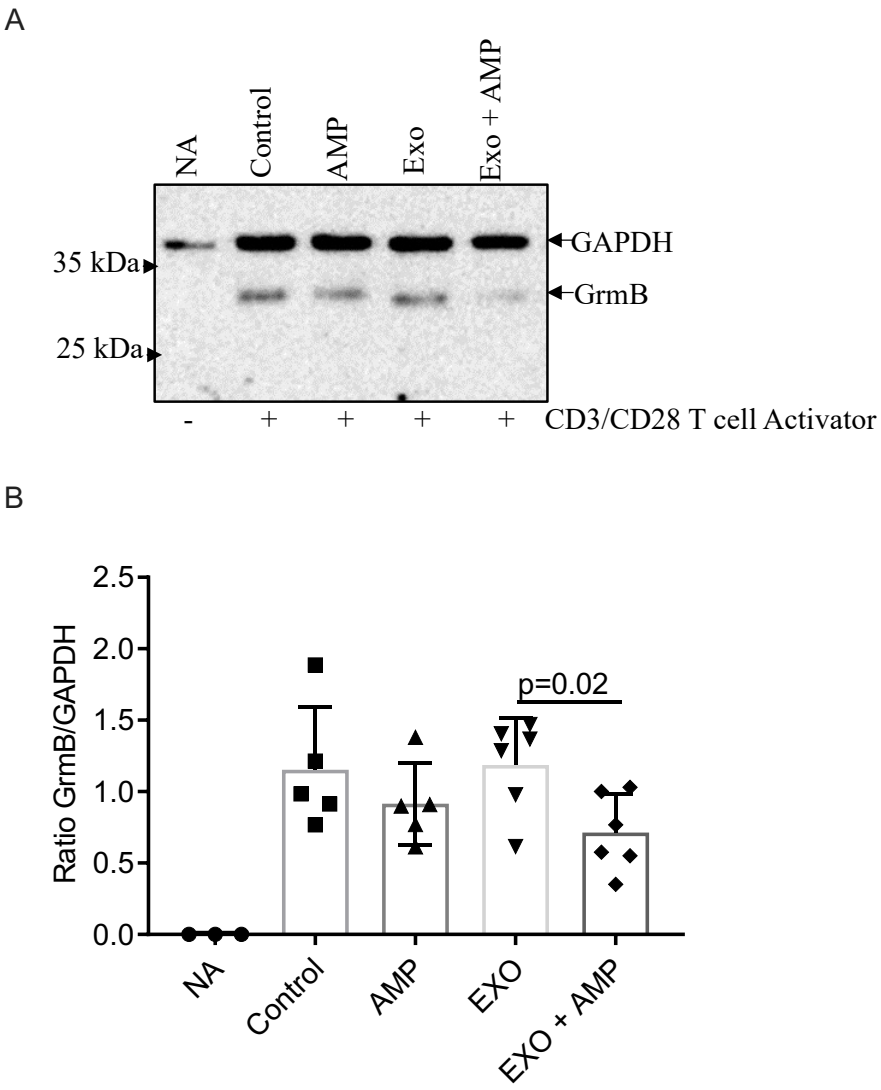

Figure 28 – *Expression of granzyme B in CD3/CD28 activated PBMCs from healthy donors with or without (100 µg/mL) patients derived exosomes (EXO) in presence or in absence of AMP (50 µM). A) Representative Western blot image for granzyme B (Grm B). GAPDH was used as protein loading control. B) Ratio Grm B/GAPDH. Mean ± SD is shown. P values are obtained from a two-tailed unpaired t-test (n=6 independent biological experiments).*

4.2.5 Associations between exosomal CD73/PD-L1 and clinical outcomes of patients

To investigate the clinical relevance of exosomal CD73 in melanoma patients treated with anti-PD-1 monotherapy, its expression at baseline and on-treatment (week 4) was analysed in association with outcomes of patients.

For these analyses, the expression of exosomal CD73 was measured in a total of 25 patients with melanoma, both at baseline and after 4 weeks from the start of treatment. Fourteen patients did not respond to treatment (PD=12, SD=2, grouped as non-responders), while 11 patients had benefit from therapy (PR=8, CR=3, grouped as responders).

CD73 expression resulted no different between responders (mean RFI=1.41) and non-responders (mean RFI= 1.30) at baseline (figure 29).

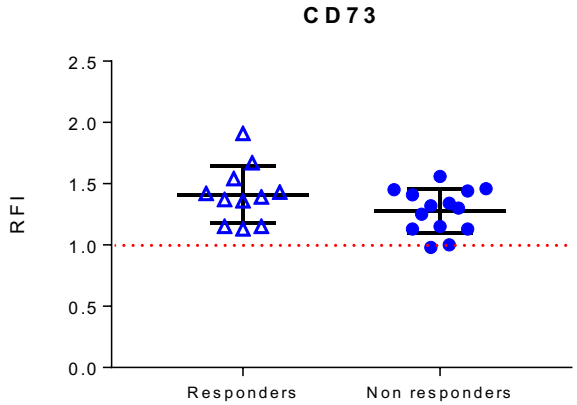

Figure 29 – *Expression of exosomal CD73 in melanoma patients, according to the clinical outcomes (responders, n=11 vs non responders, n=14). Data are expressed as Relative Fluorescence Intensity (RFI). Mean ± SD is shown.*

No differences in CD73 expression were observed also when dividing patients according to the line of treatment (figure 30), indicating that previous treatments do not influence the expression of CD73 on exosomes at baseline or at week 4.

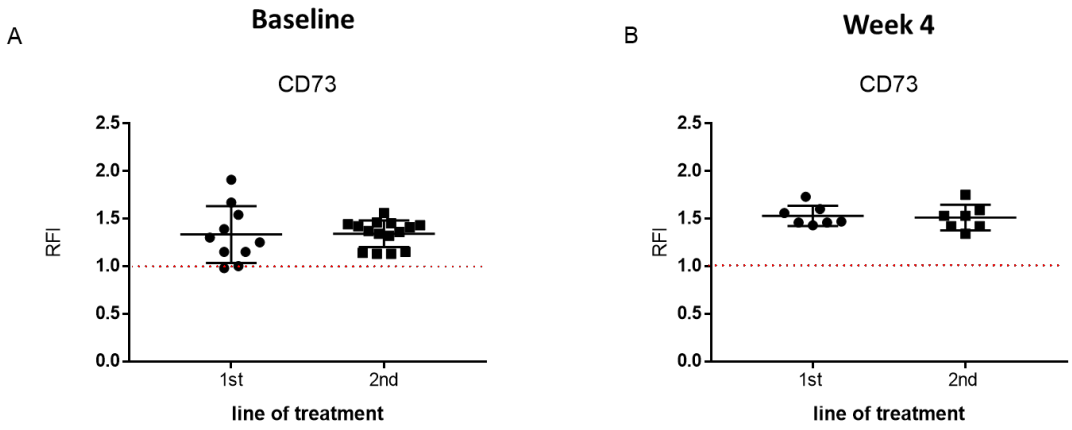

Figure 30 – *Expression of exosomal CD73 in melanoma patients receiving anti-PD-1 monotherapy according to the line of treatment, at baseline (1st line, n=10; 2nd line, n=15) and at week 4 (1st line, n=7; 2nd line, n=7). Data are expressed as Relative Fluorescence Intensity (RFI). Mean ± SD is shown.*

Comparing the expression of CD73 at baseline with week 4, in all patients (n=15) a significant increment was measured on treatment (p=0.0015, Wilcoxon matched pairs signed rank test) (figure 31A).

Recent data published by others (Chen et al., 2018), revealed that exosomal PD-L1 correlates with response to therapy in melanoma patients treated with anti-PD-1 monotherapy. Thus, exosomal PD-L1 was included in the analyses. As observed for CD73, exosomal PD-L1 resulted increased at week 4, compared to baseline, considering all the patients (figure 31B). Notably, the total amount of proteins does not differ from exosomes isolated at baseline (78.69 ± 16.8 µg/mL, n=13) and samples obtained at week 4 (68.15 ± 20.35 µg/mL, n=10) (figure 31C).

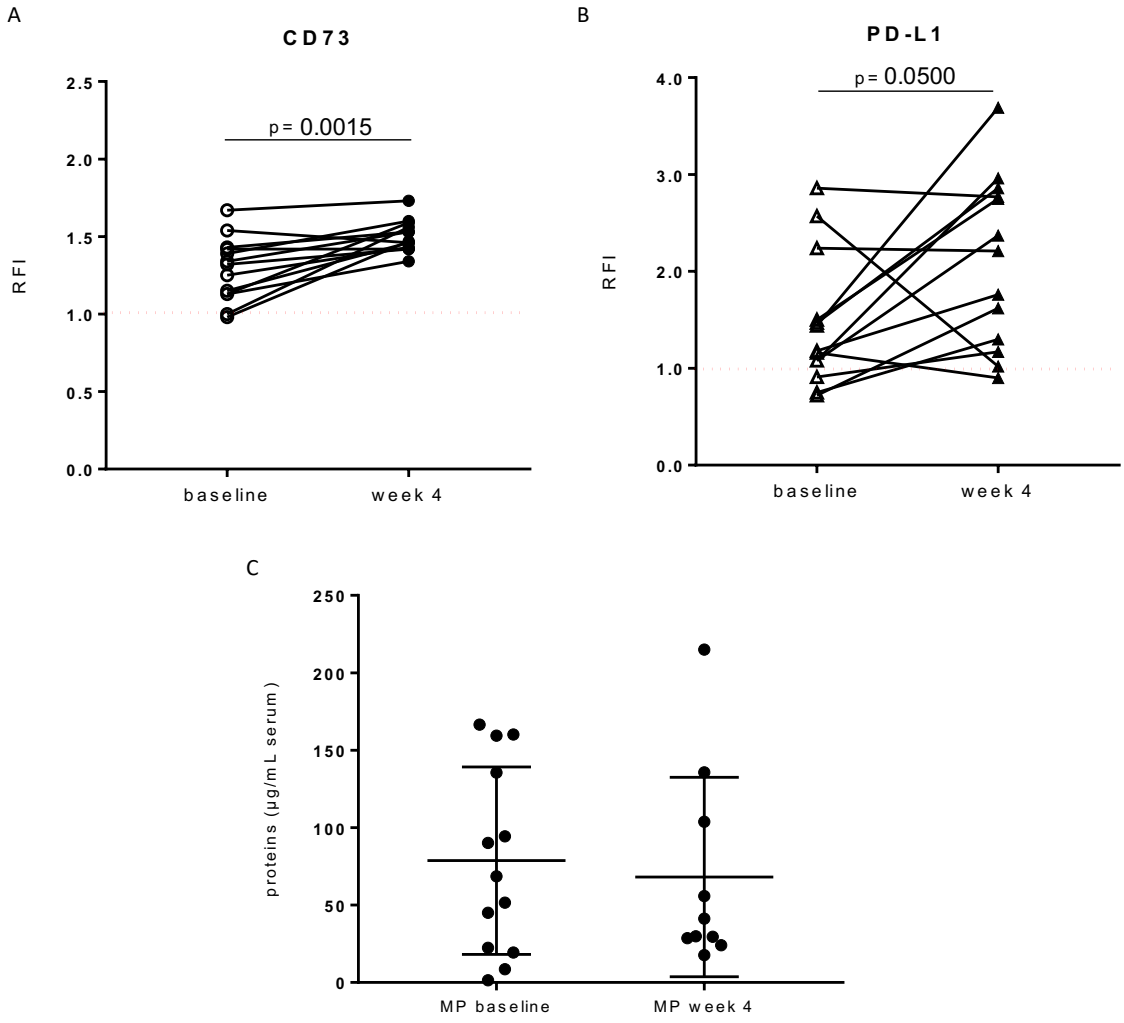

Figure 31 – *Expression of exosomal CD73 and PD-L1 in melanoma patients undergoing anti-PD-1 monotherapy treatment. CD73 expression (A) and PD-L1 expression (B) at baseline and at week 4 in matched samples (n=15). Data are expressed as Relative Fluorescence Intensity (RFI). C) Total protein amount measured by BCA assay in purified exosomes isolated from sera at baseline and week 4. P values are obtained from Wilcoxon matched-pairs signed rank test (A, B). Data are Mean ± SD (C).*

Interesting results were observed when dividing the patients according to their clinical outcomes. The expression of CD73 after four weeks is unchanged in patients responding to therapy, compared to baseline, while it is significantly increased in non-responders (p=0.041, Wilcoxon matched-pairs signed rank test) (figure 32).

In line with previously published data (Chen et al., 2018), the expression of exosomal PD-L1 resulted significantly increased in responders patients (figure 32A) but not in non-responders (figure 32B).

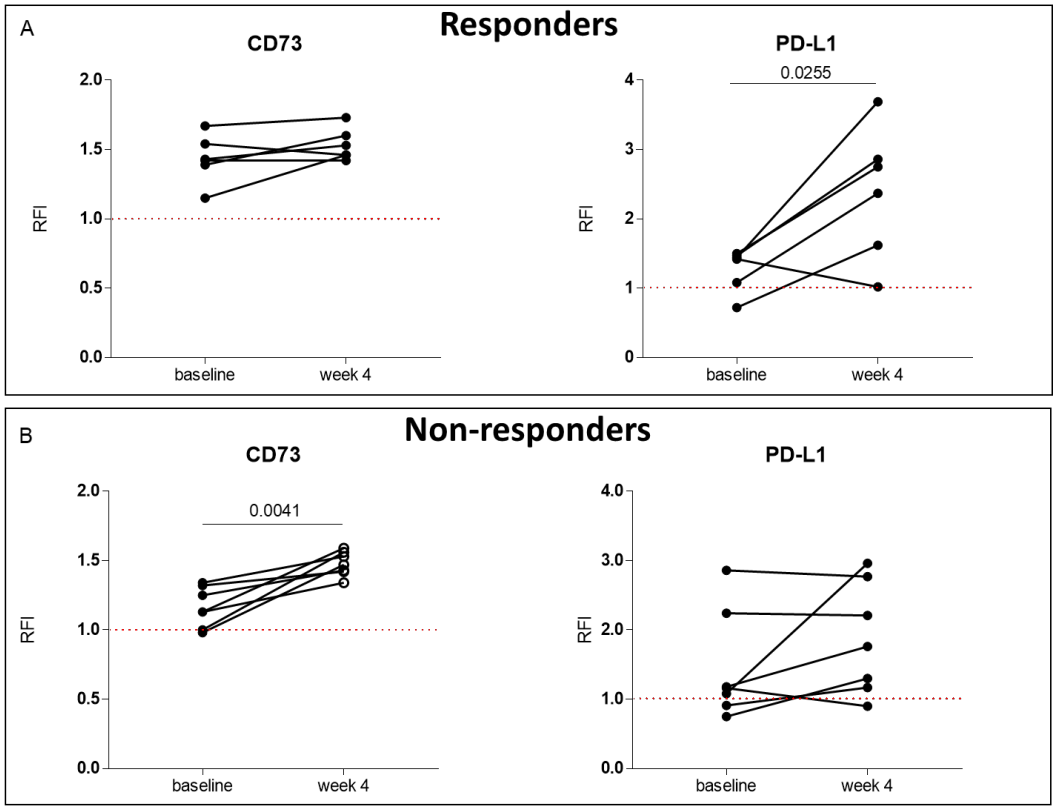

Figure 32 – *Changes in expression of exosomal CD73 and PD-L1 in melanoma patients, after 4 weeks of treatment with anti-PD-1 monotherapy in responsers (n=6) (A) and non-responders (n=7) (B). Data are expressed as Relative Fluorescence Intensity (RFI). P values are obtained from Wilcoxon matched-pairs signed rank test.*

5. Analysis of CD73 expression in human melanoma lesions by multiplexed tissue imaging

The analysis of CD73 expression in melanoma lesions was performed by co-detection by indexing (CODEX®) using the PhenoCycler™ instrument (Akoya Biosciences): CODEX® is a new multiplexed tissue imaging technology, recently developed by Garry P. Nolan's research group and licensed by Stanford University to Akoya Biosciences (https:\\www.akoyabio.com/). This technology, described for the first time in 2018, is based on the detection of DNA-conjugated antibodies and was initially used to create multiplexed datasets of normal and lupus murine spleen (Goltsev et al., 2018). The protocol used in this first study was based on the use of dNTP analogs and DNA polymerase primer extension. Then, a new version of the technology was adapted, which does not require the use of DNA polymerase and it allows the detection of up to 60 markers in a single staining procedure in human formalin-fixed, paraffin-embedded (FFPE) samples (Black et al., 2021).

Briefly, the procedure is based on the use of DNA-conjugated antibodies which are detected by the cyclic addition and removal of three fluorescent dye-conjugated oligonucleotides at one time. In presence of DMSO, a chaotropic solvent, each fluorescent dye-conjugated oligonucleotide can hybridize, at room temperature, to its unique complementary oligonucleotide-conjugated antibody, together with the nuclear stain DAPI. For each cycle, the signals of three antibodies are acquired, using a fluorescent microscope. Then, the fluorescent dye-conjugated oligonucleotides are removed by higher concentration of the solvent, the tissue is subsequential washed and a new cycle with three differ fluorescent dye-conjugated oligonucleotides can start (Figure 33).

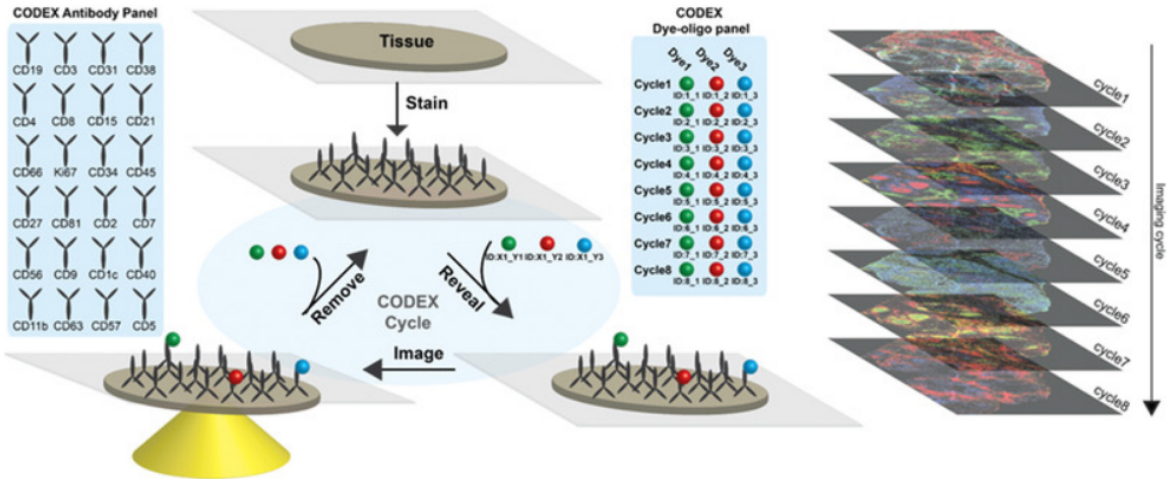

Figure 33 – *Schematic summary of PhenoCycler® procedure (Kennedy-Darling et al., 2021).*

5.1 Materials and methods

5.1.1 Patients and tissue samples

In the fourth part of this thesis, FFPE tissues from 10 melanoma patients were included. Specifically, the samples were provided by Prof. Dr. med. Jennifer Landsberg (Universitätsprofessorin für Dermato-Onkologie Leitung des Hauttumorzentrums Klinik und Poliklinik für Dermatologie und Allergologie, Universitätsklinikum Bonn).

Tissues were collected prior to the start of treatment with anti-PD-1 agents.

5.1.2 CO-detection by indexing tissue imaging

For the experiments here described, the protocol published by Garry P. Nolan group was followed and slightly adapted (Black et al., 2021).

5.1.2.1 Antibody conjugation with malemide-modified DNA oligonucleotides

Antibodies were conjugated with DNA oligonucleotides as described by Black and colleagues (figure 34) (Black et al., 2021).

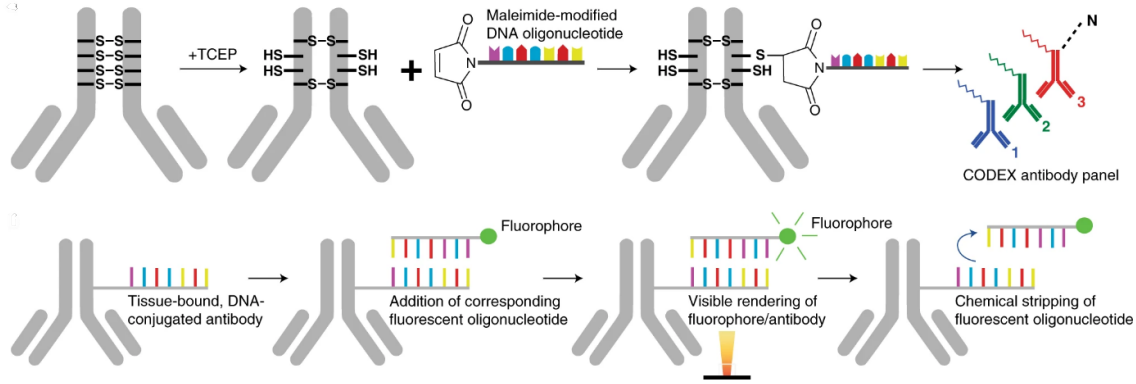

Figure 34 – *Schematic summary of antibody conjugation with DNA-oligonucleotides and detection with fluorescent dye-conjugated oligonucleotides (Black et al., 2021).*

Briefly, 100 µg of each purified antibody were added into a 50 kDa filter column and concentrated by centrifuging at 12000 g, for 8 minutes, at RT, and the flow through was discarded. Then, 360 µL of TCEP + EDTA solution (Table 8) were added into each column and antibodies were incubated for 30 minutes at RT, then centrifuged again at 12000 g, for 8 minutes, at RT. To stop the reduction reaction and to remove any TCEP residues, 400 µL of Buffer C (Table 8) were added to the antibodies, which were then centrifuged at 12000 g, for 8 minutes, at RT. This step was repeated for a total of two times and the flow through was discarded.

Finally, 200 µg of DNA oligonucleotide solution (0.5 µg/µL, in 1X PBS) were added to the respective antibody and the mixtures were incubated 2h at RT. At the end of the incubation, tubes were centrifuged at 12000 g, for 8 min at RT and antibodies were washed three times by adding 450 µL of high-salt PBS to each column, then centrifuged at 12000 g, for 8 min at RT.

After the last centrifugation, antibodies (Table 9) were resuspended in 200 µL of antibody stabilizer buffer (Table 8), collected in new tubes and stored at 4°C.

Table 8 – *Buffers required for the antibody conjugation steps*

Buffer	Composition
TCEP+EDTA solution	2.5 mM TCEP+ 2.5 mM EDTA pH 8, in ddH_2O
Buffer C	1 mM Tris pH 7.0, 1 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8, 0.02% wt/vol NaN_3, in ddH_2O
High salt PBS	Add 45 mL of 5M NaCl and 25 mL of 10x DPBS to 180 mL of ddH_2O, add NaN_3 to 0.02% (wt/vol) final concentration
Stock antibody stabilizer solution	Add 0.02% NaN3 to Candor PBS antibody stabilizer solution (purchased from Thermo Fisher)
Working antibody stabilizer solution	500 mM NaCl, 500 mM EDTA (pH 8.0), in stock antibody stabilizer solution (Candor)

Table 9 – *List of antibodies used to stain tissues*

Antibody	**Clone**	**Company**	**Working dilution**
Foxp3	236A/E7	Invitrogen	1:50
CD4	A17070D	BioLegend	1:50
Axl	Polyclonal Goat IgG	R&D	1:50
GP100	HMB-45	BioLegend	1:50
PD-1	D4W2J	Cell Signaling Technology	1:50
CD8	C8/144B	BioLegend	1:50
PD-L1	E1L3N®	Cell Signaling Technology	1:50
Antibody	**Clone**	**Company**	**Working dilution**
CD11b	EP1345Y	abcam	1:50

CD14	EPR3653	abcam	1:50
CD73	AF5795 (RD)	R&D	1:50
SOX 10	SOX10/1074	abcam	1:50
Tyrosinase	T311	Santa Cruz Biotechnology	1:50
CD39	A1	BioLegend	1:50
Granzyme B	D6E9W	Cell Signaling Technology	1:50
CD11c	EP1347Y (abcam)	abcam	1:50
CD45	92B11 + PS7/26 (novus	NovusBio	1:50
CD3e	EP449E	abcam	1:50
CD68	KP-1	BioLegend	1:100
CD56	E7X9M (CellSignal.)	R&D	1:50
CD163	EDHu-1	NovusBio	1:50
CD206	5C11	Abnova	1:50
NGFR/p75NTR	D4B3	Cell Signaling Technology	1:50
MITF	D5	abnova	1:50
A2A	7GF-G5-A2	Santa Cruz Biotechnology	1:25
A2A	EPR23731-50	abcam	1:25
CD20	L26	Invitrogen/ThermoFisher	1:50
Ki67	B56	BD Biosciences	1:50
vimentin	O91D3	BioLegend	1:50
αSMA	1A4	Invitrogen/ThermoFisher	1:50
NKG7	2G9A10F5	Beckman Coulter	1:50
MUC-1	E29	Santa Cruz Biotechnology	1:100
CD31	EP3095	abcam	1:50

5.1.2.2 Tissue specimen preparation

FFPE tissues were heated at 55°C, for 30 minutes, on a slide warmer heating plate in order to melt the paraffine. Tissues were then let cool down at RT and rehydrated in the following solutions:

- 100% xylene, 5 minutes, two times
- 100% ethanol, 5 minutes, two times
- 90% ethanol, 5 minutes
- 70% ethanol, 5 minutes
- ddH2O, 5 minutes, two times

5.1.2.3 Antigen retrieval

Heat-induced antigen retrieval was performed using a pressure cooker (PC). The PC tank was filled with ddH2O, while tissues were immersed into a beaker containing the antigen retrieval solution (Tris/EDTA buffer, pH=9, purchased from Agilent Dako), placed into the PC tank. Antigen retrieval was carried out at high-pressure, at 97°C, for 20 minutes.

Tissues were then let cool down at RT.

5.1.2.4 Bleaching of tissues and staining with DNA-conjugated antibodies

After antigen retrieval, in order to reduce autofluorescence of tissues, samples were bleached by placing the coverslips into 2 mL of bleaching solution and between two LED lamps, for 90 minutes, changing the solution after 45 minutes. The bleaching solution is composed by 20 mM NaOH, 4.5% (v/v) H_2O_2, in PBS.

After the bleaching process, tissues were washed in 1X TBS IHC buffer with Tween 20 (table 10) for 10 minutes at RT. Then tissues were covered with 100 µL of blocking solution (table 10) and incubated for 1 h, at RT, in a humidity chamber. The blocking solution is composed of mouse IgG, rat IgG, sheared salmon sperm and a mixture of 57 non-modified DNA

oligonucleotides, and it was prepared as described by Black and colleagues (Black et al., 2021). At the end of incubation, the blocking solution was removed and 100 µL of the conjugated antibodies, at the proper dilution (table 9) were added on the tissues and incubated overnight at 4°C, in a humidity chamber.

Tissues were then washed in CODEX® staining buffer 2 (S2) (table 10) and then fixed for 10 minutes in 1.6% paraformaldehyde solution (table 10). Tissues were washed in PBS (2 minutes, three times) and then incubated in ice-cold 100% methanol, for 5 minutes at 4°C. Tissues were washed again three times in PBS, then placed in a humidity chamber. One hundred microliters of final BS3 fixative solution (Table 10) were added on tissues and samples were incubated for 20 minutes at RT. At the end of incubation, tissues were washed again three times in PBS and stored in S4 buffer until the image acquisition.

Table 10 – *Buffers required for the staining of tissues*

Buffer	Composition
1X TBS IHC buffer with Tween 20	Dilute 20x TBS IHC buffer with Tween 20 (purchased from Cell Marque) in ddH_2O
Blocking solution	B1 reagent (1:20), B2 reagent (1:20), B3 (1:20), BC4 (1:30) in S2 buffer
Blocking reagent 1 (B1)	1 mg/mL mouse IgG (purchased from Sigma) in S2 buffer
Blocking reagent 2 (B2)	1 mg/mL rat IgG (purchased from Sigma) in S2 buffer
Blocking reagent 3 (B3)	10 mg/mL sheared salmon sperm in ddH_2O (purchased from ThermoFisher)
BC4 solution	Mixture of the 57 nonmodified CODEX® DNA oligonucleotides, each at the final concentration of 0.5 mM, in 10 mM Tris + 5 mM EDTA (pH 8.0) buffer
CODEX® staining buffer 1 (S1)	5 mM EDTA (pH 8.0), 0.5 % (wt/vol) BSA, 0.02% NaN_3, in ddH_2O
CODEX® staining buffer 2 (S2)	2.5 mM EDTA (pH 8.0), 0.25 % (wt/vol) BSA, 250 mM NaCl, 61 mM Na_2HPO_4, 40 mM NaH_2PO_4, in ddH_2O
CODEX® staining buffer 4 (S4)	500 mM NaCl, in S1
Paraformaldehyde solution	1.6% paraformaldehyde in S4 buffer
BS3 fixative	Dissolve BS3, bis(sulfosuccinimidyl) suberate (purchased from ThermoFisher) in DMSO, at a final concentration of 200 mg/mL.
Final BS3 fixative	Dilute BS3 fixative 1:50 in 1X PBS, immediately prior to use.

5.1.2.5 Preparation of the fluorescent oligonucleotides plate

Each CODEX® cycle is based on the acquisition of the signal of up to three fluorescent oligonucleotides, specifically binding to three individual antibodies, and the nuclear stain DAPI. The fluorescent oligonucleotides were diluted in a total volume of 250 µL of plate buffer (5 mg/mL sheared salmon sperm, DAPI nuclear stain 1:300, in H2 buffer), at a concentration of 400 nM, in a Corning black 96-well plate. Each well corresponds to one cycle and contains up to three spectrally distinct fluorescent oligonucleotides, ATTO550 (Absorption max. 554 nm, Emission max. 579 nm), DY647P1 (Absorption max. 653 nm, Emission max. 672 nm), DY747P1 (Absorption max. 747 nm, Emission max. 769 nm), in addition to DAPI (Absorption max. 358 nm, Emission max. 461 nm) for nuclear detection. The first and the last cycles of the cyclic run do not contain fluorescent oligonucleotides, but only DAPI (1:300). The plate was eventually sealed with aluminum film and stored at 4°C until the image acquisition.

In the following table 11, the pairs of antibody-DNA oligonucleotide and the fluorescent DNA-oligonucleotides chosen for the experiments are summarized. The DNA oligonucleotides were purchased from Biomers.

Table 11 – *List of the antibodies included in the panel and the respective malemide-DNA oligonucleotides and fluorescent DNA oligonucleotides.*

Antibody	DNA Oligonucleotide identification number	DNA Oligonucleotide Sequence (5'-3')	Fluorescent DNA oligonucleotide sequences (5'-3')
Foxp3	45	/mal/GCCAGAATGCCA	/5ATTO550N/TGGCATTCTGGC
CD4	20	/mal/ATGTAGGATGGTCTC	/5ATTO550N/GAGACCATCCTACAT
hAxl	75	/mal/CGCTTGGGTGTTTA	/5ATTO550N/TAAACACCCAAGCG
GP100	77	/mal/ATTTCAACAAATATTGTT	/5ATTO550N/AACAATATTTGTTGAAAT
PD-1	23	/mal/GGTTTCCTCAGACAC	/5ATTO550N/GTGTCTGAGGAAACC
CD8	8	/mal/CGCAGATGAATATTC	/5ATTO550N/GAATATTCATCTGCG
PD-L1	11	/mal/GGGTTATACACTCGT	/5ATTO550N/ACGAGTGTATAACCC
Melan-A	58	/mal/CGTCAGTACTTTCAG	/5ATTO550N/CTGAAAGTACTGACG
CD11b	28	/mal/TGGTCCACTAACGTA	/5ATTO550N/TACGTTAGTGGACCA
CD14	5	/mal/TCTCCCATTAGTCGG	/5ATTO550N/CCGACTAATGGGAGA
CD73	44	/mal/TCACTACTATTAGTACT	/5DY647P1/AGTACTAATAGTAGTGA
SOX 10	72	/mal/AACGCGACGGAT	/5DY647P1/ATCCGTCGCGTT
Tyrosinase	76	/mal/CTGTCGGTCGTCA	/5DY647P1/TGACGACCGACAG
CD39	24	/mal/ATAAGGGCTCATTGT	/5DY647P1/ACAATGAGCCCTTAT
Granzyme B	81	/mal/CAAGGAACTACCGA	/5DY647P1/TCGGTAGTTCCTTG
CD11c	49	/mal/ATAACGCCTCGTATC	/5DY647P1/GATACGAGGCGTTAT
CD45	56	/mal/GGTCACATGGTCGTT	/5DY647P1/AACGACCATGTGACC
CD3e	69	/mal/CCCGGCAGTT	/5DY647P1/AACTGCCGGG
CD68	70	/mal/AACCAAACTGACCG	/5DY647P1/CGGTCAGTTTGGTT
CD56	29	/mal/ATAGGGCATTTGAAG	/5DY647P1/CTTCAAATGCCCTAT
CD163	43	/mal/GACATTATCCGTGAT	/5DY647P1/ATCACGGATAATGTC
CD206	42	/mal/TCTAAGTCAGAGAGC	/5DY647P1/GCTCTCTGACTTAGA
NGFR/p75NTR	15	/mal/CTGTAATAGGCACTA	/5DY-747P1/TAGTGCCTATTACAG
MITF	38	/mal/GCGTCTACTTATAAG	/5DY-747P1/CTTATAAGTAGACGC
A2A	62	/mal/TAGGGGAACAGGTTG	/5DY-747P1/CAACCTGTTCCCCTA
CD20	48	/mal/GCACGGCAAAGTG	/5DY-747P1/CACTTTGCCGTGC
Ki67	6	/mal/TGGATGTGTTACGAT	/5DY-747P1/ATCGTAACACATCCA
vimentin	7	/mal/CGCTAAGATATTCTAAG	/5DY-747P1/CTTAGAATATCTTAGCG
αSMA	33	/mal/TTATCATGAGGAGCG	/5DY-747P1/CGCTCCTCATGATAA

Antibody	DNA Oligonucleotide identification number	DNA Oligonucleotide Sequence (5'-3')	Fluorescent DNA oligonucleotide sequences (5'-3')
NKG7	66	/mal/TACGTGCTTTGGTT	/5DY-747P1/AACCAAAGCACGTA
MUC-1	41	/mal/TGTATGAGTAGTAATCT	/5DY-747P1/AGATTACTACTCATACA
CD31	68	/mal/CTTCTTGTGGAACC	/5DY-747P1/GGTTCCACAAGAAG

5.2.2.6 Image acquisition and data analysis

Images were acquired using a Zeiss Axio Observer 7 inverted microscope, with Colibri 7 as the LED Light source and the Plan-Apochromat 20X/0,8 M27 (a=0,55 mm) as objective. The microscope used is equipped with the Prime BSI PCIe camera.

Once acquired, the images were converted to TIF files by using Codex Manager Instrument® (Akoya Biosciences).

Then, images were processed by using CODEX® Processor 1.8, and the following methods were applied:

- Image stitching
- Deconvolution
- Background subtraction
- Shading correction
- Tile processing (cycle alignment, background subtraction, deconvolution, extended depth of field)
- Region processing (shading correction, tile registration, overlap cropping).

Images were analyzed using Halo® v3.3.2541.256 (Indica Labs). Cell segmentation was performed using the Nuclei Segmentation Classifier, while the algorithm HighPlex FL v4.1.3 was used to analyze cell phenotypes.

Specifically, the following phenotypes were considered:

- T cells: CD45+CD3+
- CD8+ T cells: CD45+CD3+CD8
- CD4+ T cells: CD45+CD3+CD8-
- NK cells: CD45+CD56+CD3-
- CD31+ endothelial cells: CD31+CD45-
- Tumor cells (proliferative phenotype): MITF+ or Melan-A+, or SOX10+
- Tumor cells (invasive phenotype): Axl+ or NGFR+

For each phenotype, the expression of CD73 was also analyzed.

5.2 Results

5.2.1 Clinicopathological characteristics of patients

For this part of the thesis, tissues from 10 patients were analyzed. Seven patients were male and 3 patients were female, with a median age of 74 years. All the patients were diagnosed as stage IV melanoma, according to AJCC, VIII edition (Keung and Gershenwald, 2018). The mutation of *BRAF* was present in 2 patients, while 7 patients had wild-type tumors and for one patient the information was unknown. All patients received anti-PD-1 monotherapy (pembrolizumab, n=10; nivolumab, n=1) as first line for most of them (n=10), while only one patient had previously received ipilimumab.

Six patients showed clinical benefits from treatment with anti-PD-1 agents (CR=4, PR=2), while 4 patients had progression of disease.

LDH levels were upper the limit of normal in 3 patients, while all the other patients had normal values (n=7).

Importantly, all the tissues analyzed were metastases from skin (n=5) or lymph nodes (n=5).

Characteristics of patients are summarized in the following table 12.

Table 12 - *Clinicopathological characteristics of patients*

	Nivolumab or Pembrolizumab N (%)
Total	10 (100%)
Median age Range (49-88)	74
Gender Male Female	 7 (70%) 3 (30%)
Disease stage IV	 10 (100%)
Line of treatment I II	 9 (90%) 1 (10 %)
BRAF status wild-type mutated unkown	 7 (70%) 2 (20%) 1 (10%)
Serum lactate dehydrogenase >ULN < ULN	 3 (30%) 7 (70%)

5.2.2 Validation of antibodies

All the antibodies listed in table 9 were tested using different melanoma tissues (n=3) in two conditions of antigen retrieval, in presence of citrate pH=6 or EDTA pH=9. The antigen retrieval at acidic pH involved a loss of signal for some of the antibodies, compared to basic pH, thus this latter condition was chosen to perform all the experiments here described. All the antibodies were also tested using a multi-tumor tissue microarray and tonsils, to verify the specificity of each signal.

For A2A receptor, two antibodies were tested, two clones from two different companies (table 9) and none produced a reliable staining, in the tested experimental conditions. Thus, even if the target would be of great interest to the purpose of this thesis, it has not been included in the analyses.

The antibody targeting CD39 did not work the experimental conditions tested and this target was also excluded from the analyses. For these two targets, more clones will be tested in the near future.

Regarding the setting of the acquisition parameters, after testing different times of exposure and intensities of LEDs, the following set up was selected as the best one and then used for the next experiments.

Table 13 – *Settings for LED intensity and exposure time*

Channel	LED intensity	Exposure time (ms)
DAPI	40%	20
Cy3	65%	400
Cy5	50%	500
Cy7	40%	350 for most of the antibodies 500 for NGFR and MITF

Representative images obtained in melanoma tissues are shown in the next pages (figure 35 to 40).

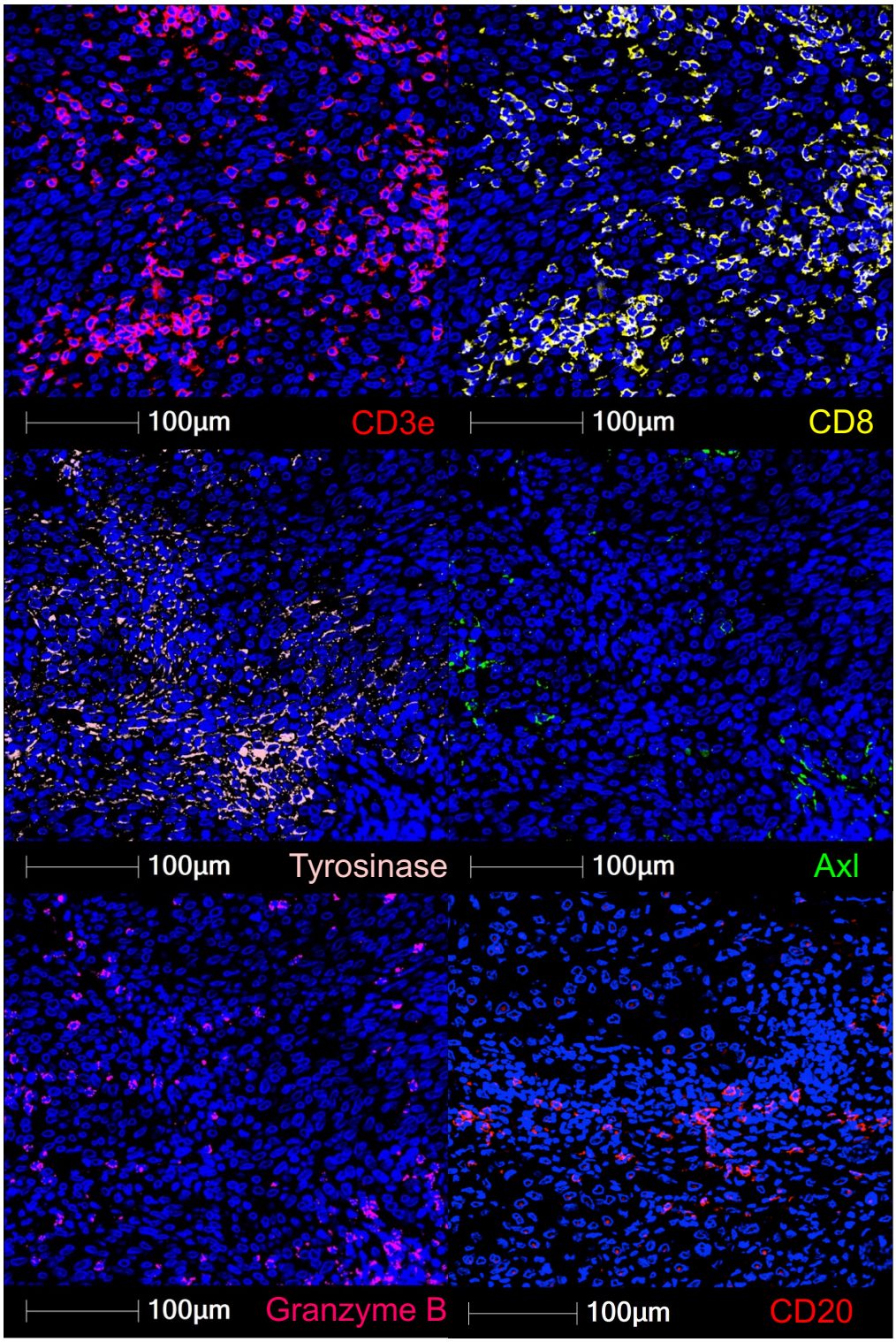

Figure 35 – *Representative images for six antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

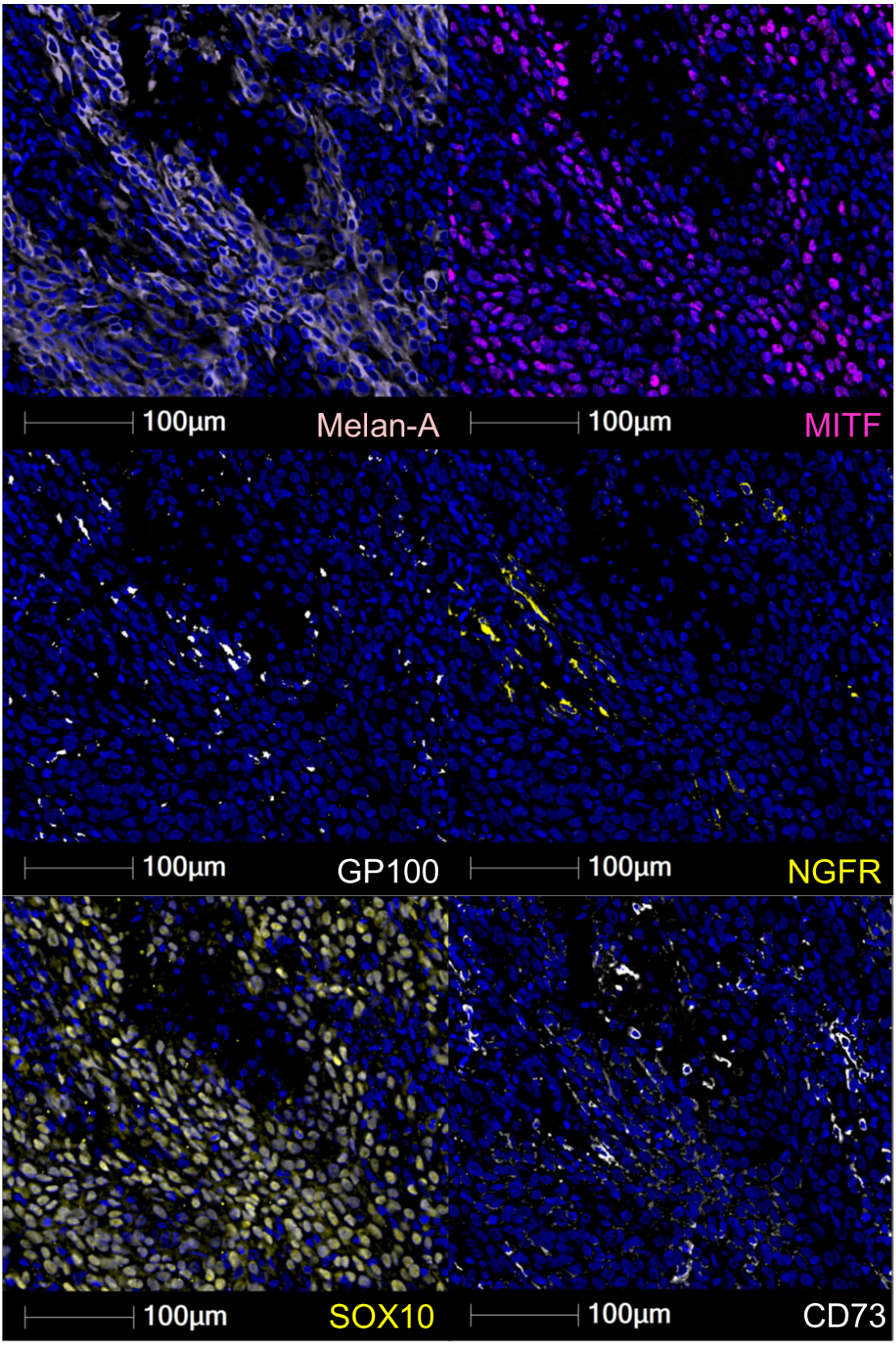

Figure 36 – *Representative images for 6 antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

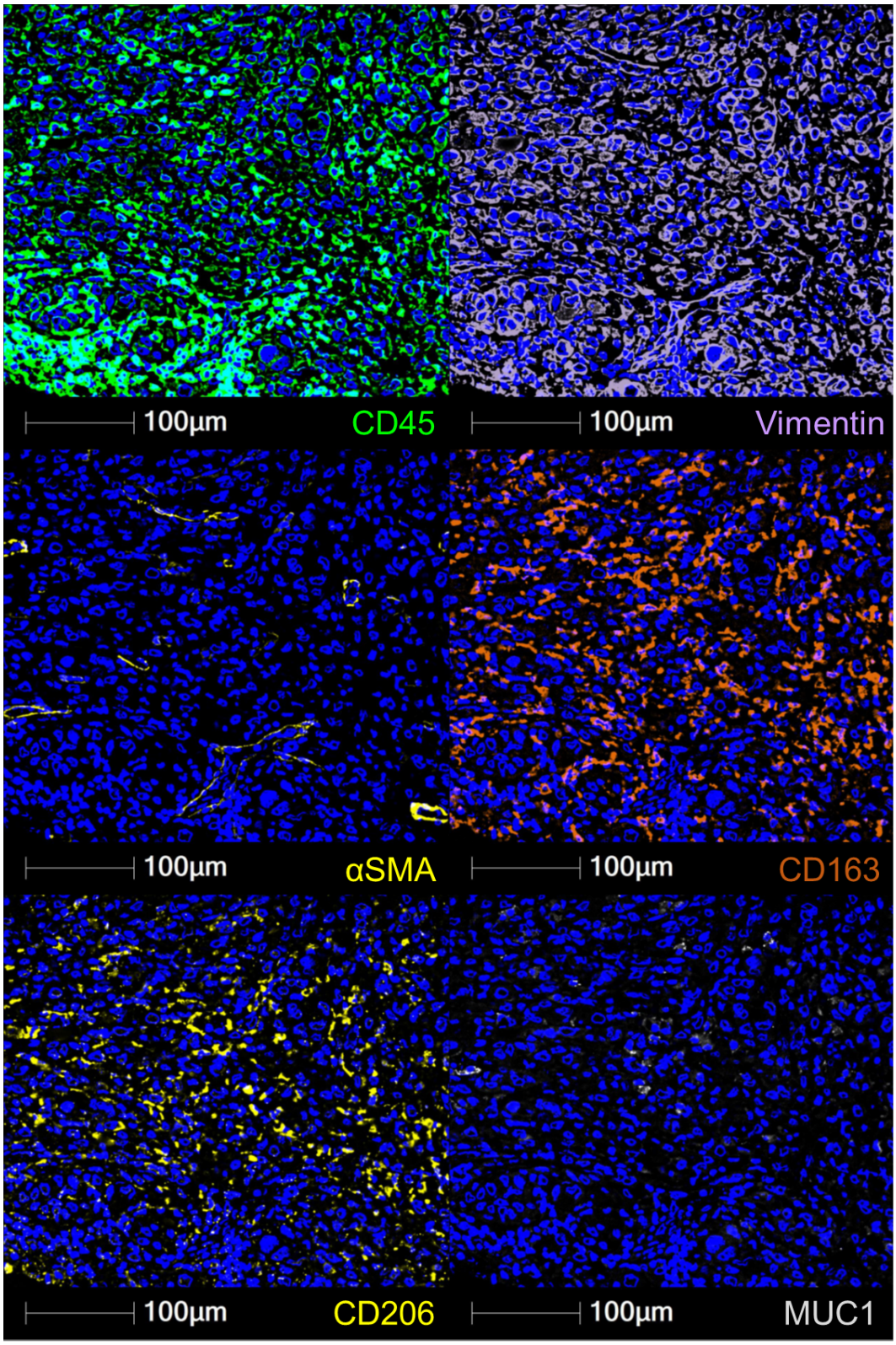

Figure 37 – *Representative images for 6 antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

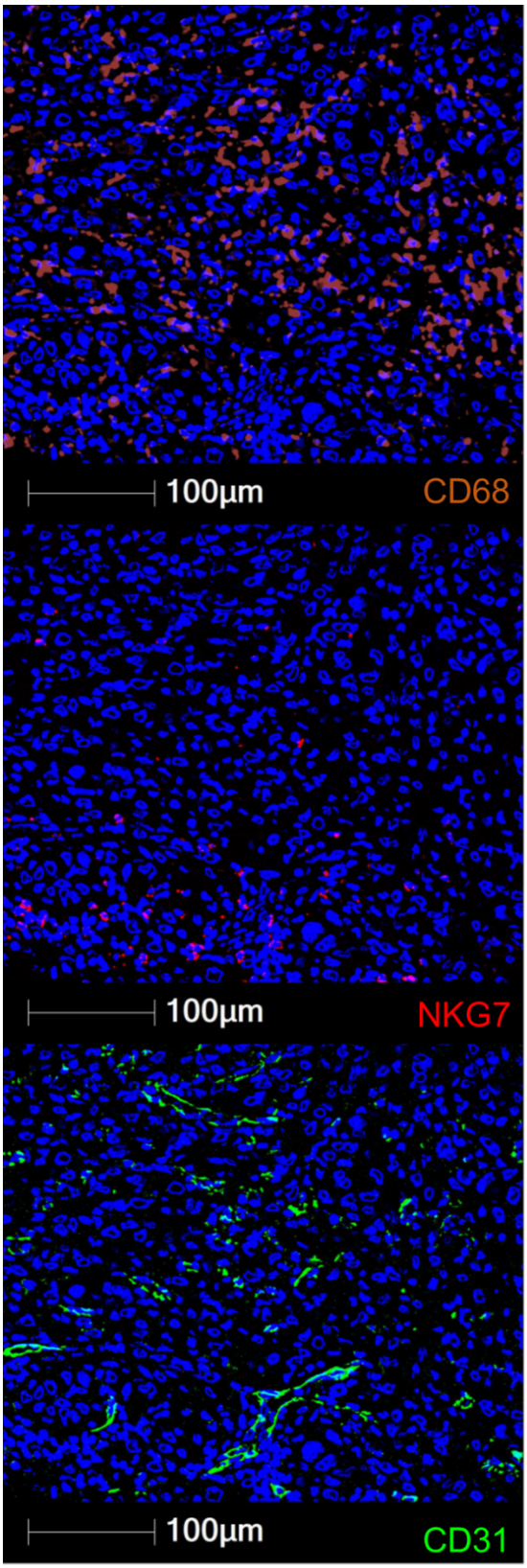

Figure 38 – *Representative images for 3 antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

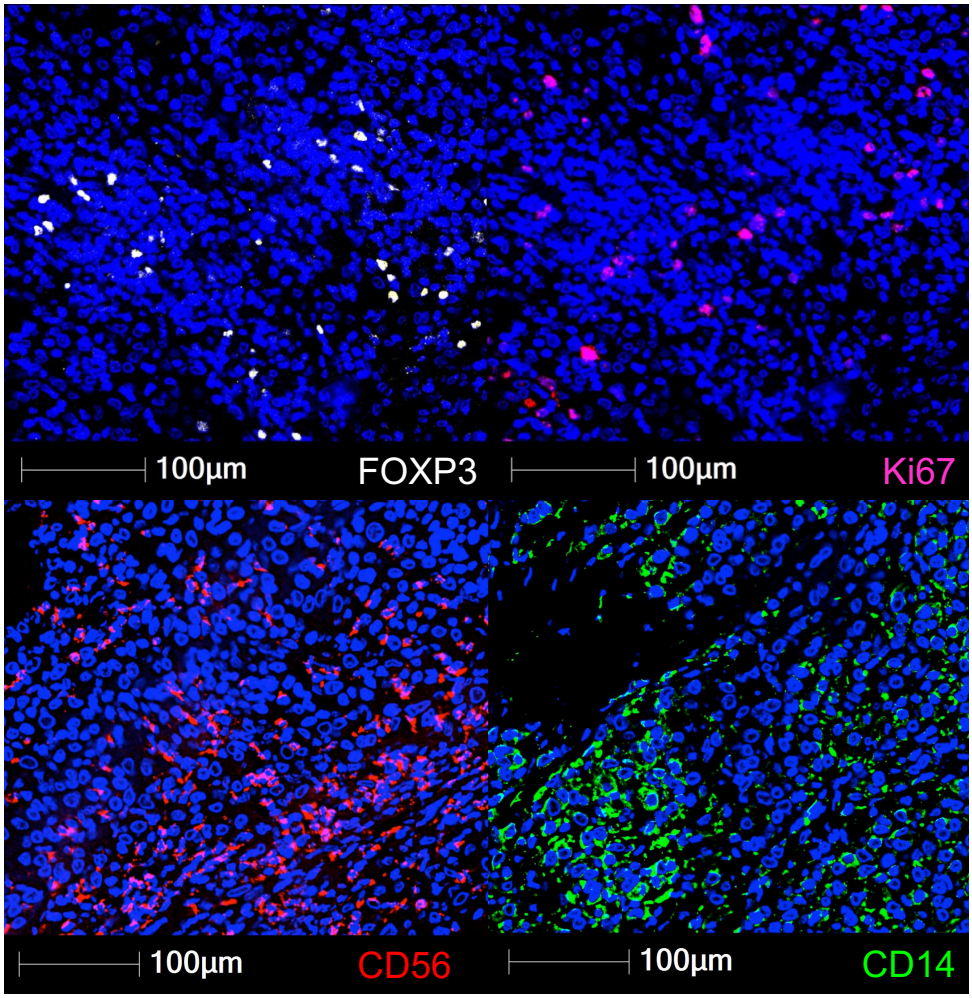

Figure 39 – *Representative images for 3 antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

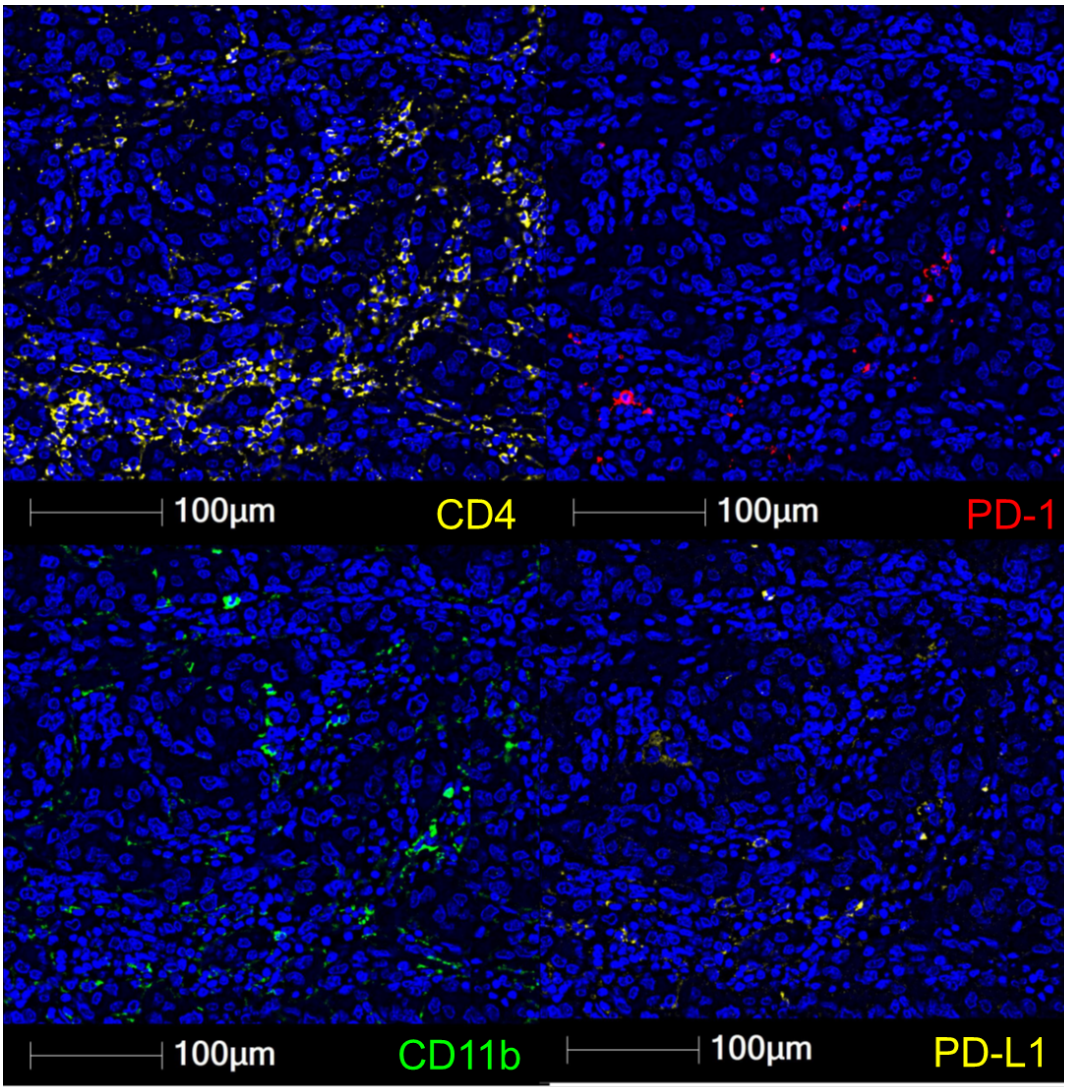

Figure 40 – *Representative images for 3 antibodies included in the panel. Images were obtained in a melanoma tissue. DAPI was used as nuclear marker and it is showed in blue.*

5.2.3 Preliminary characterization of CD73 expression within melanoma lesions

Tissues from 10 patients were included in the analysis and specifically, 5 tissues were skin metastases while the other 5 were metastases from lymph nodes, and each tissue was analyzed in triplicate. Importantly, CD73 was detected in all the cores analyzed.

The percentage of CD73+ subpopulations were calculated on the number of total DAPI positive cells.

Looking at the skin metastases, among the phenotypes analyzed, the CD73+ population most abundant was represented by tumor cells with an invasive phenotype (mean percentage 26.04%), while CD8+ T cells were the less abundant (mean percentage: 0.44%). The same trend was observed in the lymph node metastases, where CD73+ tumor cells with an invasive phenotype were present with a percentage of was 7.94. The less abundant CD73+ subpopulations in lymph nodes were CD8+ T cells (mean percentage: 0.35%) and CD31+ endothelial cells (mean percentage: 0.23). The percentage of each CD73+ subpopulation is summarized in table 14, while the expression of CD73+ populations for each patient is reported in figure 40.

Table 14 – *Summary of abundance of CD73+ subpopulations*

	Metastases from skin	**Metastases from lymph nodes**
	(Mean percentage)	
T cells	7.38	1.51
CD8+ T cells	0.44	0.35
CD4+ T cells	6.95	10.75
CD31+ endothelial cells	0.70	0.23
NK cells	4.77	4.66
Tumor cells Proliferative phenotype	4.79	5.44
Tumor cells Invasive phenotype	26.04	7.94

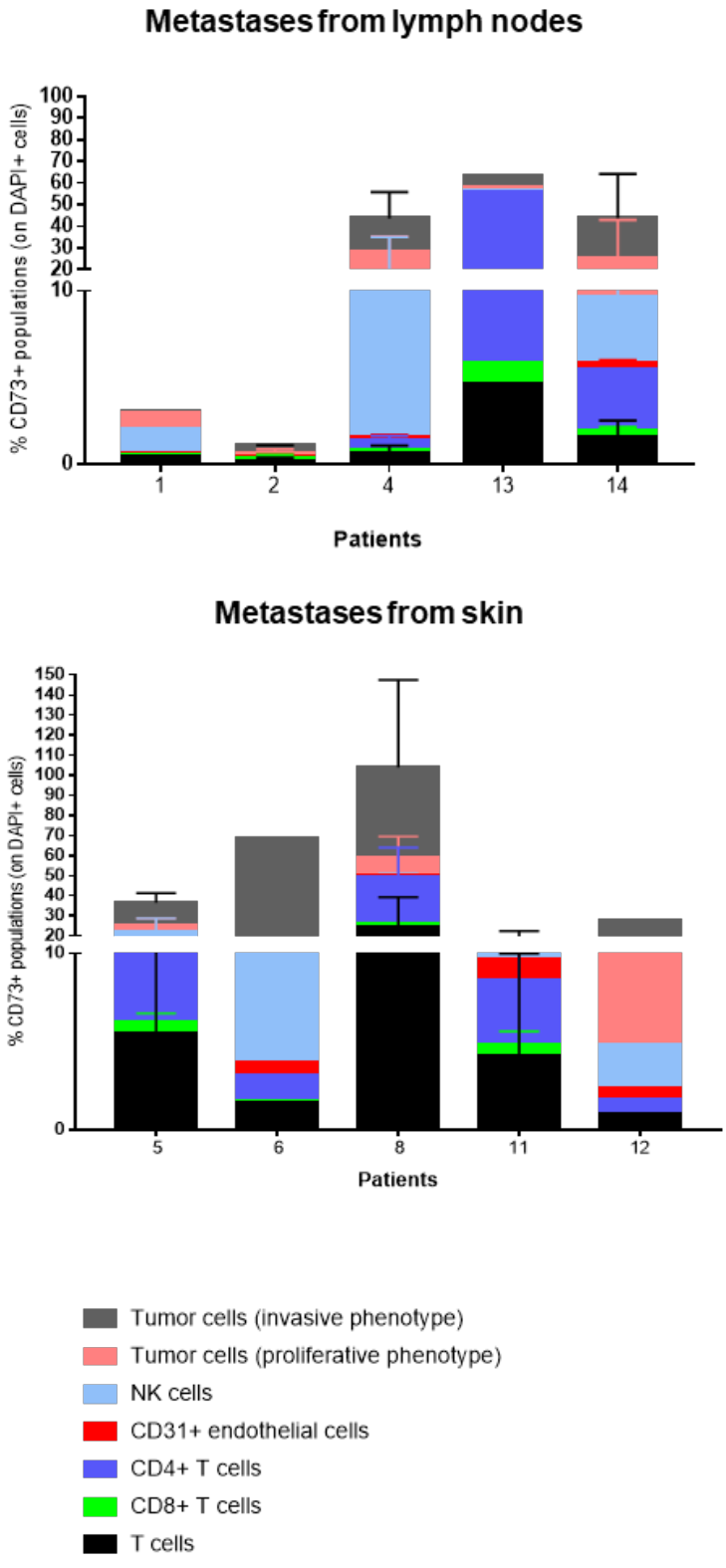

Figure 40 – *Percentage of CD73+ subpopulations in each patient. Mean±SD is shown. SD is not reported for the patients for whom the tissue was not available in triplicate.*

No further analyses were performed, due to the small number of cases analyzed. However, more tissues will be analyzed in the near future and more phenotypes will be included in the analyses, in order to obtain a complete overview of the CD73+ cells populating the tumor lesion. For this part of the project the result achieved was the setup of a procedure that will be used for further analyses.

5.3 Future directions

The CODEX® technology using the PhenoCycler™ instrument has been recently developed and optimized, showing a clear great potential as tool to perform advanced tissue imaging analyses. However, considered its novelty, some aspects still need improvements.

Indeed, if on one hand PhenoCycler™ enables analyses that cannot be performed using other experimental approaches, on the other hand the presence of high noise in the acquired images requires more efforts in terms of image processing and normalization, in order to eliminate all the sources of noise and to perform correct analyses.

The most common sources of noise include high background signal, low intensity signal, image processing artifacts, autofluorescence of tissue, incorrect cell segmentation.

The research group that hosted me to carry out this part of the thesis is currently working on methods to improve downstream data analysis, including not only phenotype classification but also spatial analysis. The project on the characterization of CD73 expression within tumor lesion will continue and we propose to identify populations that express this ectonucleotidase and that may be associated with the response of patients undergoing immune checkpoint inhibitor therapy.

6. Discussion

Immune checkpoint inhibitors, including anti PD-1 and anti CTLA-4 agents, improved the survival of patients with advanced melanoma, representing a revolution for the treatment of this aggressive and deadly type of cancer. However, given the high percentage of patients not responding to therapy or developing resistance during treatment, the individuation of reliable biomarker of response and the development of new therapeutic strategies are an urgent need and a great challenge for research.

While biopsies are a powerful tool to have a picture of the tumor microenvironment, providing information about cell phenotypes populating the lesion and indicating the presence or absence of factors that may lead to a favorable response to immune checkpoint blockade, the heterogeneity and the dynamic nature of tumor lesions, as well as the presence of multiple tumor sites may lead to a bias when evaluating the anti-tumor immune response (Callea et al., 2015). Furthermore, in case of advanced melanoma the presence of multiple tumor lesions is common, and the analysis of biopsies may be challenging, and it would require invasive procedure that could impact on the compliance of patients. The individuation of reliable prognostic factors in the bloodstream may represent a quick and easy tool to guide clinical choices.

A great number of studies highlighted the involvement of adenosine pathway in impairing anti-tumor immune response and the effectiveness of immune checkpoint inhibitors. Being the main enzyme responsible for extracellular adenosine production, CD73 has been addressed as potential therapeutic target and prognostic factor.

In this context, my work was focused on investigating the role of CD73 in patients with advanced melanoma treated with anti PD-1 agents. To this aim, I studied the circulating forms of this enzyme and its expression in tumor lesion.

In the first part of this thesis, I analyzed the circulating CD8+PD-1+ lymphocytes in patients with advanced melanoma prior to the start of therapy with nivolumab, focusing on the proportion of cells positive to CD73.

Different subpopulations of tumor infiltrating T cells or circulating T cells have been studied, by others, in association to outcomes of patients receiving immune checkpoint inhibitors and the expression of PD-1 has been investigated as marker of reactive T cells, that could indicate responsiveness to anti-PD-1 agents in melanoma patients. Specifically, the presence of CD8+PD-1+ T cells in melanoma lesion has been associated with effectiveness of anti PD-1 agents (Tumeh et al., 2014;Ngiow et al., 2015;Daud et al., 2016). A simultaneous high expression of PD-1 and CTLA-4 on tumor infiltrating CD8+ T cells strongly correlated with response to therapy to nivolumab or pembrolizumab and longer progression-free survival in melanoma patients (Daud et al., 2016).

A study from Manjarrez-Orduño and colleagues demonstrated that the frequency of circulating naïve T cells in patients with melanoma or NSCLC is significantly reduced, compared with healthy donors (Manjarrez-Orduño et al., 2018). Furthermore, the authors observed an association between high circulating memory T cell / effector T cell ratios and inflammation in tumor lesions, which corresponds with longer progression free survival, in NSCLC patients receiving nivolumab (Manjarrez-Orduño et al., 2018).

The results obtained in the first part of this project indicated that high levels of circulating CD8+PD-1+CD73+ lymphocytes at baseline are associated with worse survival and poor clinical response to nivolumab. The abundance of this lymphocyte population resulted particularly relevant in impacting clinical response in patients receiving nivolumab as first-line treatment. Therefore, it is possible to hypothesize that previous treatments may influence CD73 expression in lymphocytes.

Analyses using blood samples collected after the start of treatment were not performed, due to the lack of sample availability, given the retrospective nature of the study. Therefore, further investigations are required to clarify whether the frequency of this population may be prognostic or predictive also in patients undergoing treatment.

Another limitation of the first part of the thesis is the lack of experiments exploring the mechanisms by which CD8+PD-1+CD73+ lymphocytes may impair the antitumor immune response in melanoma patients receiving nivolumab. On this aspect, it is possible to hypothesize that the high frequency of CD8+PD-1+CD73+ T cells found in non-responder patients may indicate the establishment of an immunosuppressive mechanism mediated by CD73-derived adenosine, which makes these cells dysfunctional.

Prospectively, the analysis of CD73 on circulating T cells at baseline could integrate other markers and could be useful to identify melanoma patients that may benefit from treatment with nivolumab.

In the second part of this PhD project, I investigated the role of non-cell bound CD73, following preliminary data obtained by Morello's group, that highlighted the potential to measure the AMPase activity in serum of melanoma patients as factor able to predict response to anti-PD-1 therapy (Morello et al., 2017). The results obtained for this PhD project confirmed the preliminary findings, in a larger, multicentric and multinational cohort of patients and strongly confirmed the association of CD73 activity in serum of melanoma patients with clinical response to anti-PD-1 therapy.

Pretreatment activity and expression of CD73 in serum of melanoma patients are higher than in healthy donors. However, the enzymatic activity of CD73 better stratifies patients from healthy donors than the expression does. Given that, I focused all the analyses on the enzymatic activity of CD73.

Patients with high baseline activity of CD73 showed shorter overall survival, than patients with low baseline activity. This result supports the notion that soluble CD73 activity in serum is a factor of poor prognosis in patients with melanoma that could be useful in clinical practice, complementing the prognostic value of other factors.

Although no significant differences were found in the baseline CD73 activity levels according to the clinic-pathological characteristics of patients, interesting associations were found when analyzing survival in subgroups of patients.

Among patients receiving anti-PD-1 monotherapy, patients with both high serum CD73 activity and high serum LDH showed an extremely poor prognosis. Similar results were obtained in patients with both high CD73 activity and bone metastasis or in patients with *BRAF* mutation and high CD73 activity. A shorter OS was also found in patients aged <65 years with high CD73, and in females with high CD73 activity.

Further investigations are required to fully clarify the biological mechanisms behind these results, while in this thesis only a discussion based on literature evidence is possible.

In the case of the patients' subgroups with brain or bone metastases or with high serum LDH, who have already poor survival outcomes (Larkin et al., 2019), an high enzymatic activity of CD73 may be an additional factor worsening the effectiveness of anti-PD-1 agents and reducing survival.

It is already known that BRAF inhibitors can reduce the expression of CD73 within melanoma lesion (Reinhardt et al., 2017). However, to date, no study investigating the relation between *BRAF* status and CD73 activity has been published, therefore further investigations are required to understand the reason behind the result here obtained in the group of patients with *BRAF* mutation.

Regarding the impact of patients age on therapy effectiveness, it is known that patients over the age of 60 years respond better to treatment with the anti-PD-1 agents than younger patients, in whom immune escape mechanisms are more likely to occur (Kugel et al., 2018;Li et al., 2018;Wu et al., 2019). About this interesting aspect, a recent study showed a negative correlation between the expression of CD73 on CD8+ T cells and the age of healthy donors and patients with head and neck squamous cell carcinoma (Jeske et al., 2020). Furthermore, it has been reported that CD73 expression levels decrease with advancing of age (Crooke et al., 2017).

Given these results, it is possible to hypothesize that an elevated activity of CD73 may contribute to impair the immune response mostly in younger patients, worsening the clinical outcomes to anti-PD-1 therapy.

Importantly, the multivariate analysis from the patients' receiving anti-PD-1 monotherapy confirmed that the serum activity of CD73 is an independent predictor, besides serum LDH levels and the presence of brain metastasis, for both OS and PFS. These analyses were not performed in patients treated with nivolumab plus ipilimumab, in whom the serum CD73 activity was not significantly associated with OS. At this regard, it is important to note that the HR values are very similar between the group treated with anti-PD-1 monotherapy (1.36) and the group receiving nivolumab plus ipilimumab (1.41), but most likely because of the small number of patients in the subgroup receiving the combination, the statistical significance is not reached. Therefore, further analyses in a larger cohort of patients are required to make any reasonable conclusion.

The results obtained for this thesis showed that baseline activity of CD73 is strongly associated with response to anti-PD-1 agents, especially in patients receiving anti-PD-1 monotherapy as first line. In this latter group of patients, PFS was significantly associated with serum CD73 activity, suggesting the potential relevance of CD73 as biomarker of response in patients treated with anti-PD-1 agents especially as first-line setting.

Then, I analyzed serum CD73 activity in samples collected during treatment with anti-PD-1 agents. It is to note that I had availability of samples collected only after 3 months from the start of therapy and it was not possible to perform analyses at more time points. It emerged that the CD73 activity does not change after 3 months of treatment, compared with baseline levels, and importantly, non-responder patients still have higher levels of CD73 activity compared with responders. This suggests that the activity of CD73 in the peripheral blood does not change on-treatment, at least not between the two time points I considered for my thesis, but it remains higher in patients who do not benefit from treatment. Further investigations are required to clarify whether CD73 activity may change at different time points.

Taken together, the results obtained on serum CD73 strongly reinforce the potential of this enzymes both as therapeutic target and as prognostic factor to be used in clinical practice.

Elevated levels of CD73 in serum may reflect an elevated expression of CD73 in the tumor lesion, but unfortunately, for this retrospective study, the matched comparison between tissues and sera was not possible. Further investigations are required to verify whether there is a correlation between the expression of CD73 in the tissue and its expression or activity in the bloodstream. Another point that warrants to be explored is the individuation of the source of circulating non-cell bound CD73 and the understanding of the mechanisms that lead to increased levels of this form.

The shedding of CD73 from the membrane is mediated by the activity of metalloproteases or phospholipases (Yegutkin, 2008), but still less is known about the stimuli that enhance the cleavage of the protein.

The soluble form of CD73 is increased in cancer patients compared to healthy subjects. Already in 1989, Lal and coworkers observed that serum CD73 was significantly higher in patients with head and neck cancer, compared to the controls (Lal et al., 1989). Enzymatic activity resulted increased with the advancement in the stage of cancer (Lal et al., 1989).

CD73 activity is higher in prostate cancer patients, than in healthy donors, and patients who received radiotherapy showed higher AMPase activity than patients who did not receive radiotherapy (Gardani et al., 2019). In line with this, Wennerberg and colleagues observed that radiotherapy enhances the levels of soluble CD73 in cancer patients (Wennerberg et al., 2020).

A recent work published by Schneider and collaborators showed that that human T cells release enzymatically active CD73, upon activation (Schneider et al., 2021). The hydrolysis of AMP in supernatants of activated CD8 resulted mostly mediated by CD73-expressing extracellular vesicles, while the contribution of soluble CD73 or other soluble enzymes is negligible (Schneider et al., 2021). It is still unknown if these results may be true also in context of cancer and further investigations are required.

Here, I observed that levels of serum CD73, both in terms of expression and activity, are higher in melanoma patients than in healthy donors. Among patients, pretreatment activity of CD73 in serum was higher in non-responders group than in responders and it associates with survival of patients.

However, the results obtained for the second part of this project do not clarify whether circulating vesicles contribute to adenosine production in the bloodstream. Thus, in third part I focused on the role of circulating exosomes in impairing clinical response to anti-PD-1 treatment in melanoma patients.

To do this, the first challenge to solve was the setting up of a protocol to isolate intact exosomes from serum samples with the less amount of contaminants. As described in the Methods section, in chapter 4, I successfully adapted a protocol already published by others, based on the use of size exclusion chromatography and ultrafiltration (Hong et al., 2017). The choice between a technique or another to isolate exosomes may affect the experimental results. The first publications on exosomes were based on the use of ultracentrifugation, however the need to optimize new procedures became soon clear. Indeed, even if faster and cheaper than other

approaches, the use of ultracentrifugation do not exclude the presence of soluble contaminants, including cytokines, in samples.

When I started to work on this part of the project I compared the protocol based on ultracentrifugation (Théry et al., 2006), and the protocol based on size exclusion chromatography (Hong et al., 2017). I observed the presence of so many contaminants in samples obtained by ultracentrifugation that I decided to optimize the protocol described by Hong and colleagues. Importantly, recent studies demonstrate the combining centrifugation and filtration with SEC and with ultrafiltration of MWCO 100kDa is the only strategy to separate as much as possible exosomes from the presence of soluble factors (La Shu et al., 2020).

After optimizing the procedure to purify intact exosomes, it was finally possible to analyze the expression and the activity of exosomal CD73.

In line with other studies, I observed that exosomes isolated from serum of melanoma patients express CD73, which maintains its enzymatic activity. Importantly, in presence of AMP, exosomes isolated from patients produce more adenosine than the ones isolated from healthy donors. Then I analyzed CD73 expression in patients, at baseline and during treatment with nivolumab or pembrolizumab, accordingly to clinical outcomes. No differences in exosomal CD73 expression were observed between healthy donors and melanoma patients, at baseline. Among patients, pretreatment exosomal CD73 expression does not differ between responders and non-responders patients.

In these analyses, I also considered the expression of exosomal PD-L1, based on recent evidence indicating circulating PD-L1 as prognostic factors in many cancer types (Bailly et al., 2021). Interesting results about exosomal PD-L1 were published by Chen and collaborators (Chen et al., 2018). In this study, the authors showed that the exosomal form of PD-L1 is associated with clinical response to pembrolizumab in patients with melanoma, and specifically, early on treatment, exosomal PD-L1 is increased in responders patients, via IFN-

γ signaling. In line with these findings, I observed increased levels of exosomal PD-L1, early on treatment (week 4), in melanoma patients who responded to therapy with anti-PD-1 monotherapy. In this group of patients, the expression of exosomal CD73 at week 4 remains stable, compared with baseline. Conversely, exosomal CD73 significantly increased at week 4 in patients who did not respond to therapy. These findings are in line with what I observed *in vitro*. Serum-derived exosomes are able to produce adenosine in a CD73-dependent manner, suppressing in turn the production of IFN-γ in T cells. The production of adenosine by exosomes may represent a mechanism of immune evasion which leads to a lack of response to anti-PD-1 agents. These drugs exert their therapeutical effects by restoring the effector functions of T cells, which activation corresponds to an increased production of IFN- γ (Grasso et al., 2020). This cytokine is in turn responsible of the upregulation of PD-L1 on tumor cells. Exosomal PD-L1 resulted higher in patients who responded to therapy with anti-PD-1, compared with non-responders (Chen et al., 2018). The results of this thesis are in line with findings from Chen and colleagues, suggesting in addition that the lack of effector functions in T cells may be caused by exosome-derived adenosine.

The last part of the Project was focused on the study of the expression of CD73 within melanoma lesions and it was carried out in the laboratory of Prof. Dr. med Michael Hölzel, at Institute of Experimental Oncology, University Clinic Bonn (Germany).

As described in the introduction section, CD73 resulted upregulated in many types of human cancer and its expression has often been related to poor prognosis and short survival of patients, as the case of advanced melanoma (Monteiro et al., 2018), lymphoma (Wang et al., 2019), breast cancer (Loi et al., 2013;Turcotte et al., 2017;Buisseret et al., 2018;Jiang et al., 2018), head and neck cancer (Ren et al., 2016;Mandapathil et al., 2018), gastric cancer (Lu et al., 2013), non-small-cell lung cancer (Inoue et al., 2017), thyroid carcinoma (Bertoni et al., 2019),

ovarian cancer (Turcotte et al., 2015;Jiang et al., 2018), colorectal cancer (Wu et al., 2012), pancreatic cancer (Chen et al., 2020).

Recently, Yu and colleagues reported that CD73 is highly expressed on cancer-associated fibroblasts in human colorectal cancers, and high CAF frequency in cancer tissues correlates with poor prognosis of patients (Yu et al., 2020). In sarcoma and breast cancer, a population of tumor-infiltrating NK cells has been described and, additionally, the frequency of CD73+ NK population in the tumor microenvironment resulted to be correlated with larger tumor size in patients with breast cancer (Neo et al., 2020).

Other studies reported that the expression of CD73 in tumor lesions is on the contrary associated with good prognosis of patients, revealing a controversial role of CD73 as prognostic factor. It is the case of two studies focused on bladder cancer, that showed that CD73 expression is associated with better survival of patients (Koivisto et al., 2018). Results obtained by Koivisto and coworker showed that CD73 negative epithelial cells significantly associated with poor survival both in patients with non–muscle-invasive bladder cancer and muscle-invasive bladder cancer, while CD73 expression in stromal fibroblasts or lymphocytes had no predictive power (Koivisto et al., 2018). Being obtained in a single small retrospective study, further investigation and validation are required, but at the same time, these findings interestingly highlight the importance to analyze the CD73 expression in each cell types within the tumor microenvironment. The authors of this study suggest that their results may be related to the role of endothelial CD73 in regulating the permeability of the blood vessels and the extravasation of leukocytes. A different scenario has been described in prostate cancer by Leclerc and colleagues, who observed that the high CD73 expression in normal adjacent prostate epithelium was significantly associated with shorter biochemical recurrence–free survival of patients, while high levels of CD73 in the tumor stroma were associated with longer biochemical recurrence–free survival (Leclerc et al., 2016).

Endometrial carcinoma is another type of cancer in which CD73 appeared to be related to a better prognosis of patients, as described by Bowser and coworkers (Bowser et al., 2016). In this study, the authors observed a decreased expression of CD73 in carcinoma cells of poorly differentiated and advanced-stage disease, compared with normal endometrium and low-grade tumors, suggesting an important protective role of CD73-derived adenosine in maintaining epithelial integrity in normal endometrium (Bowser et al., 2016). Based on these findings, it is possible to hypothesize that the loss of CD73 disrupts the integrity of endothelium, promoting the tumor progression.

Taken together, this evidence clearly indicates that the prognostic value of CD73 needs to be further investigated and clarified. As indicated by others, the analysis of the different populations expressing this enzyme is crucial to have a better understanding of the role of CD73 within tumor tissue.

Additionally, in a comprehensive evaluation of the prognostic value of CD73 in different human cancers, Jiang and colleagues highlighted that the discrepancy of results between studies may be due to differences in the methods chosen to measure the expression (Jiang et al., 2018). Indeed, it is important to note that the gene expression level is not always related to the corresponding protein expression level and this could be behind differences observed between studies evaluating the gene expression and those evaluating tissue imaging.

An approach based on multiplexed tissue imaging technology could be useful to clarify the controversial role of CD73 in tumor lesion as prognostic factor. This kind of approach allows to analyze many phenotypes by using up to 60 markers per staining and it enables spatial analysis, including cell-cell interactions and environmental context; all information that would be lost when using other technologies, as flow cytometry or mass cytometry.

Aimed to obtain a complete overview of CD73 expression within melanoma lesions, during the last part of this PhD project, I worked to set up a panel of antibodies to be used with the

PhenoCycler™ instrument, which exploits the CODEX® technology, a multiplex tissue imaging approach that has been recently developed.

The panel was set up including markers enabling to identify the several immune subpopulations, the stroma compartment and the tumor cells. The antibodies here used have been validated in melanoma tissues, after screening several clones for each target and after testing different experimental conditions.

After validating the antibodies, a preliminary characterization of CD73 expression has been performed and the results have been included in this thesis. The tissues analyzed included metastases from skin or lymph nodes and all of them were positive for CD73 expression. Tumor cells with an invasive phenotype and positive to CD73 expression represented the 26 % of the total cells within lesions from skin and the 7% in metastases from lymph nodes, being the CD73+ population most abundant among the phenotypes included in the analysis. These results are in line with what observed in a study by Reinhardt and colleagues, showing that CD73 has a variable expression in human melanoma, while the analysis of gene signature indicated that its expression associated with a nascent and fully established invasive melanoma phenotype (Reinhardt et al., 2017). It is to note that the preliminary results obtained for this thesis, showing that CD73 is mostly expressed by melanoma cells with an invasive phenotype, represent the first in situ proof of what observed by gene analyses in the paper published by Reinhardt and coworkers. In the same study, the authors observed an intense CD73 expression around necrotic areas in melanoma lesions, both human and murine, which is in line with the already known notion that hypoxia induce the expression of this ectonucleotidase. Furthermore, hypoxia promotes the switching to an invasive phenotype in melanoma cells (O'Connell et al., 2013;Widmer et al., 2013).

This part of the project needs to be further developed and it will be object of future studies. Most of the time spent abroad was dedicated to the setting up of the procedure and the validation

of antibodies, as well as to the study of tissue imaging technology and related analyses, which represented a new challenging field.

6.1 Conclusions

To conclude, the results obtained for this PhD project are in line with other evidence indicating that CD73-derived adenosine impairs the antitumor immunity, limiting in turn the efficacy of anti-PD-1 agents. This thesis strongly supports the notion to use CD73 as prognostic factor and therapeutical target to be blocked in combination with anti-PD-1 agents in patients with advanced melanoma.

To sum up, regarding the potential of use CD73 as prognostic factor, the results of this thesis clearly indicate that all the forms of CD73 are associated to poor response to anti PD-1 therapy in melanoma patients. Specifically, high pretreatment frequency of CD8+PD-1+CD73+ T cells and high pretreatment activity of serum CD73 associates with reduced overall survival are associated with poor outcomes and worse overall survival, compared with a low percentage. Furthermore, exosomal CD73 increases early on treatment with anti PD-1 agents in non-responder patients, highlighting an important role for exosomes in inducing immune evasion. Importantly, a mechanism behind this evidence has also been proposed.

Taken together, the results discussed in this thesis indicate that the measurement of CD73 activity and/or expression could be informative to identify a suppressive mechanism that influence the anti-tumor immune response, impairing the therapeutic effectiveness of immune checkpoint inhibitors.

Prospectively, the measurement of CD73 activity and/or expression may represent a useful tool to be added to other prognostic factors to guide therapeutic choices in patients with advanced melanoma. In addition, measurement of CD73 may be also useful to identify patients who are

more likely to benefit from treatment with CD73-blocking agents, that in combination with immune checkpoint inhibitors could further improve the clinical response.

7. Bibliography

Acurio, J., Herlitz, K., Troncoso, F., Aguayo, C., Bertoglia, P., and Escudero, C.J.P.S. (2017). Adenosine A2A receptor regulates expression of vascular endothelial growth factor in feto-placental endothelium from normal and late-onset pre-eclamptic pregnancies. 13**,** 51-60.

Allard, B., Allard, D., Buisseret, L., and Stagg, J. (2020). The adenosine pathway in immuno-oncology. *Nature Reviews Clinical Oncology***,** 611-629.

Allard, B., Cousineau, I., Allard, D., Buisseret, L., Pommey, S., Chrobak, P., and Stagg, J. (2019). Adenosine A2a receptor promotes lymphangiogenesis and lymph node metastasis. *Oncoimmunology* 8**,** 1601481.

Allard, B., Pommey, S., Smyth, M.J., and Stagg, J. (2013). Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. *Clin Cancer Res* 19**,** 5626-5635.

Allard, B., Turcotte, M., Spring, K., Pommey, S., Royal, I., and Stagg, J. (2014a). Anti-CD73 therapy impairs tumor angiogenesis. 134**,** 1466-1473.

Allard, B., Turcotte, M., Spring, K., Pommey, S., Royal, I., and Stagg, J. (2014b). Anti-CD73 therapy impairs tumor angiogenesis. *Int J Cancer* 134**,** 1466-1473.

Antonioli, L., Fornai, M., Pellegrini, C., D'antongiovanni, V., Turiello, R., Morello, S., Haskó, G., and Blandizzi, C. (2021). Adenosine Signaling in the Tumor Microenvironment. *Adv Exp Med Biol* 1270**,** 145-167.

Antonioli, L., Pacher, P., Vizi, E.S., and Hasko, G. (2013). CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 19**,** 355-367.

Antonioli, L., Yegutkin, G.G., Pacher, P., Blandizzi, C., and Haskó, G. (2016). Anti-CD73 in cancer immunotherapy: awakening new opportunities. *Trends Cancer* 2**,** 95-109.

Arozarena, I., and Wellbrock, C. (2019). Phenotype plasticity as enabler of melanoma progression and therapy resistance. *Nat Rev Cancer* 19**,** 377-391.

Ascierto, P.A., Dummer, R., Gogas, H.J., Flaherty, K.T., Arance, A., Mandala, M., Liszkay, G., Garbe, C., Schadendorf, D., Krajsova, I., Gutzmer, R., De Groot, J.W.B., Loquai, C., Gollerkeri, A., Pickard, M.D., and Robert, C. (2020). Update on tolerability and overall survival in COLUMBUS: landmark analysis of a randomised phase 3 trial of encorafenib plus binimetinib vs vemurafenib or encorafenib in patients with BRAF V600-mutant melanoma. *Eur J Cancer* 126**,** 33-44.

Bailly, C., Thuru, X., and Quesnel, B. (2021). Soluble Programmed Death Ligand-1 (sPD-L1): A Pool of Circulating Proteins Implicated in Health and Diseases. *Cancers (Basel)* 13.

Balch, C.M., Gershenwald, J.E., Soong, S.-J., Thompson, J.F., Atkins, M.B., Byrd, D.R., Buzaid, A.C., Cochran, A.J., Coit, D.G., Ding, S., Eggermont, A.M., Flaherty, K.T., Gimotty, P.A., Kirkwood, J.M., Mcmasters, K.M., Mihm, M.C., Jr., Morton, D.L., Ross, M.I., Sober, A.J., and Sondak, V.K. (2009). Final version of 2009 AJCC melanoma staging and classification. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27**,** 6199-6206.

Beavis, P.A., Divisekera, U., Paget, C., Chow, M.T., John, L.B., Devaud, C., Dwyer, K., Stagg, J., Smyth, M.J., and Darcy, P.K. (2013). Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. *Proc Natl Acad Sci U S A* 110**,** 14711-14716.

Beavis, P.A., Milenkovski, N., Henderson, M.A., John, L.B., Allard, B., Loi, S., Kershaw, M.H., Stagg, J., and Darcy, P.K. (2015). Adenosine Receptor 2A Blockade Increases the Efficacy of Anti-PD-1 through Enhanced Antitumor T-cell Responses. *Cancer Immunol Res* 3**,** 506-517.

Bendell, J.C., Lorusso, P., Overman, M.J., Noonan, A.M., Kim, D.-W., Strickler, J., Kim, S.-W., Clarke, S.J., George, T.J., Grimison, P.S., Barve, M.A., Amin, M.A., Desai, J., Wise-Draper, T., Cooper, Z., Elgeioushi, N., Mueller, N.K., Kumar, R., Wu, K.Y., and Patel, S.P. (2021). Safety and efficacy of the anti-CD73 monoclonal antibody (mAb) oleclumab ± durvalumab in patients (pts) with advanced colorectal cancer (CRC), pancreatic ductal adenocarcinoma (PDAC), or EGFR-mutant non-small cell lung cancer (EGFRm NSCLC). 39**,** 9047-9047.

Bertoni, A.P.S., Bracco, P.A., De Campos, R.P., Lutz, B.S., Assis-Brasil, B.M., Meyer, E.L.S., Saffi, J., Braganhol, E., Furlanetto, T.W., and Wink, M.R. (2019). Activity of ecto-5'-nucleotidase

(NT5E/CD73) is increased in papillary thyroid carcinoma and its expression is associated with metastatic lymph nodes. *Mol Cell Endocrinol* 479**,** 54-60.

Black, S., Phillips, D., Hickey, J.W., Kennedy-Darling, J., Venkataraaman, V.G., Samusik, N., Goltsev, Y., Schürch, C.M., and Nolan, G.P. (2021). CODEX multiplexed tissue imaging with DNA-conjugated antibodies. *Nat Protoc* 16**,** 3802-3835.

Boison, D., and Yegutkin, G.G. (2019). Adenosine Metabolism: Emerging Concepts for Cancer Therapy. *Cancer Cell* 36**,** 582-596.

Bouma, M.G., Wildenberg, F.a.V.D., and Buurman, W.A. (1996). Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells. 270**,** C522-C529.

Bowser, J.L., Blackburn, M.R., Shipley, G.L., Molina, J.G., Dunner, K., Jr., and Broaddus, R.R. (2016). Loss of CD73-mediated actin polymerization promotes endometrial tumor progression. *The Journal of clinical investigation* 126**,** 220-238.

Buisseret, L., Pommey, S., Allard, B., Garaud, S., Bergeron, M., Cousineau, I., Ameye, L., Bareche, Y., Paesmans, M., Crown, J.P.A., Di Leo, A., Loi, S., Piccart-Gebhart, M., Willard-Gallo, K., Sotiriou, C., and Stagg, J. (2018). Clinical significance of CD73 in triple-negative breast cancer: multiplex analysis of a phase III clinical trial. *Ann Oncol* 29**,** 1056-1062.

Callea, M., Albiges, L., Gupta, M., Cheng, S.C., Genega, E.M., Fay, A.P., Song, J., Carvo, I., Bhatt, R.S., Atkins, M.B., Hodi, F.S., Choueiri, T.K., Mcdermott, D.F., Freeman, G.J., and Signoretti, S. (2015). Differential Expression of PD-L1 between Primary and Metastatic Sites in Clear-Cell Renal Cell Carcinoma. *Cancer Immunol Res* 3**,** 1158-1164.

Canale, F.P., Ramello, M.C., Núñez, N., Furlan, C.L.A., Bossio, S.N., Serrán, M.G., Boari, J.T., Del Castillo, A., Ledesma, M., Sedlik, C., Piaggio, E., Gruppi, A., Rodríguez, E.V.A., and Montes, C.L. (2018). CD39 Expression Defines Cell Exhaustion in Tumor-Infiltrating CD8⁺ T Cells. 78**,** 115-128.

Carlino, M.S., Larkin, J., and Long, G.V. (2021). Immune checkpoint inhibitors in melanoma. *The Lancet* 398**,** 1002-1014.

Chen, G., Huang, A.C., Zhang, W., Zhang, G., Wu, M., Xu, W., Yu, Z., Yang, J., Wang, B., Sun, H., Xia, H., Man, Q., Zhong, W., Antelo, L.F., Wu, B., Xiong, X., Liu, X., Guan, L., Li, T., Liu, S., Yang, R., Lu, Y., Dong, L., Mcgettigan, S., Somasundaram, R., Radhakrishnan, R., Mills, G., Lu, Y., Kim, J., Chen, Y.H., Dong, H., Zhao, Y., Karakousis, G.C., Mitchell, T.C., Schuchter, L.M., Herlyn, M., Wherry, E.J., Xu, X., and Guo, W. (2018). Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature* 560**,** 382-386.

Chen, Q., Pu, N., Yin, H., Zhang, J., Zhao, G., Lou, W., and Wu, W. (2020). CD73 acts as a prognostic biomarker and promotes progression and immune escape in pancreatic cancer. *J Cell Mol Med* 24(15)**,** 8674–8686.

Colgan, S.P., Eltzschig, H.K., Eckle, T., and Thompson, L.F. (2006). Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal* 2**,** 351-360.

Crooke, A., Martínez-Henández, J., Martínez-López, J., Cruz-Jentoft, A., Huete-Toral, F., and Pintor, J. (2017). Low expression of CD39 and CD73 genes in centenarians compared with octogenarians. *Immunity & Ageing* 14**,** 11.

Csoka, B., Selmeczy, Z., Koscso, B., Nemeth, Z.H., Pacher, P., Murray, P.J., Kepka-Lenhart, D., Morris, S.M., Jr., Gause, W.C., Leibovich, S.J., and Hasko, G. (2012). Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *Faseb j* 26**,** 376-386.

Curran, M.A., Montalvo, W., Yagita, H., and Allison, J.P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. 107**,** 4275-4280.

Czarnecka, A.M., Bartnik, E., Fiedorowicz, M., and Rutkowski, P. (2020). Targeted Therapy in Melanoma and Mechanisms of Resistance. *Int J Mol Sci* 21.

D'angelo, S.P., Larkin, J., Sosman, J.A., Lebbé, C., Brady, B., Neyns, B., Schmidt, H., Hassel, J.C., Hodi, F.S., Lorigan, P., Savage, K.J., Miller, W.H., Jr., Mohr, P., Marquez-Rodas, I., Charles, J., Kaatz, M., Sznol, M., Weber, J.S., Shoushtari, A.N., Ruisi, M., Jiang, J., and Wolchok, J.D. (2017).

Efficacy and Safety of Nivolumab Alone or in Combination With Ipilimumab in Patients With Mucosal Melanoma: A Pooled Analysis. *J Clin Oncol* 35**,** 226-235.

Daud, A.I., Loo, K., Pauli, M.L., Sanchez-Rodriguez, R., Sandoval, P.M., Taravati, K., Tsai, K., Nosrati, A., Nardo, L., Alvarado, M.D., Algazi, A.P., Pampaloni, M.H., Lobach, I.V., Hwang, J., Pierce, R.H., Gratz, I.K., Krummel, M.F., and Rosenblum, M.D. (2016). Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. *J Clin Invest* 126**,** 3447-3452.

De Lourdes Mora-García, M., López-Cisneros, S., Gutiérrez-Serrano, V., García-Rocha, R., Weiss-Steider, B., Hernández-Montes, J., Sánchez-Peña, H.I., Ávila-Ibarra, L.R., Don-López, C.A., Muñóz-Godínez, R., Pineda, D.B.T., Chacón-Salinas, R., Vallejo-Castillo, L., Pérez-Tapia, S.M., and Monroy-García, A. (2019). HPV-16 Infection Is Associated with a High Content of CD39 and CD73 Ectonucleotidases in Cervical Samples from Patients with CIN-1. *Mediators Inflamm* 2019**,** 4651627.

Di Virgilio, F., Sarti, A.C., Falzoni, S., De Marchi, E., and Adinolfi, E. (2018). Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. *Nature Reviews Cancer* 18**,** 601-618.

Eisenhauer, E.A., Therasse, P., Bogaerts, J., Schwartz, L.H., Sargent, D., Ford, R., Dancey, J., Arbuck, S., Gwyther, S., Mooney, M., Rubinstein, L., Shankar, L., Dodd, L., Kaplan, R., Lacombe, D., and Verweij, J. (2009). New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 45**,** 228-247.

Fausther, M., Sheung, N., Saiman, Y., Bansal, M.B., and Dranoff, J.A. (2012). Activated hepatic stellate cells upregulate transcription of ecto-5'-nucleotidase/CD73 via specific SP1 and SMAD promoter elements. *Am J Physiol Gastrointest Liver Physiol* 303**,** G904-914.

Feoktistov, I., Ryzhov, S., Zhong, H., Goldstein, A.E., Matafonov, A., Zeng, D., and Biaggioni, I. (2004). Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. *Hypertension* 44**,** 649-654.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136**,** E359-386.

Ferretti, E., Horenstein, A.L., Canzonetta, C., Costa, F., and Morandi, F. (2019). Canonical and non-canonical adenosinergic pathways. *Immunol Lett* 205**,** 25-30.

Forte, G., Sorrentino, R., Montinaro, A., Luciano, A., Adcock, I.M., Maiolino, P., Arra, C., Cicala, C., Pinto, A., and Morello, S. (2012). Inhibition of CD73 improves B cell-mediated anti-tumor immunity in a mouse model of melanoma. *J Immunol* 189**,** 2226-2233.

Gaestel, M. (2006). MAPKAP kinases — MKs — two's company, three's a crowd. *Nature Reviews Molecular Cell Biology* 7**,** 120-130.

Gardani, C.F.F., Cappellari, A.R., De Souza, J.B., Da Silva, B.T., Engroff, P., Moritz, C.E.J., Scholl, J.N., Battastini, A.M.O., Figueiró, F., and Morrone, F.B. (2019). Hydrolysis of ATP, ADP, and AMP is increased in blood plasma of prostate cancer patients. *Purinergic Signal* 15**,** 95-105.

Goltsev, Y., Samusik, N., Kennedy-Darling, J., Bhate, S., Hale, M., Vazquez, G., Black, S., and Nolan, G.P. (2018). Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging. *Cell* 174**,** 968-981.e915.

Govindarajan, B., Bai, X., Cohen, C., Zhong, H., Kilroy, S., Louis, G., Moses, M., and Arbiser, J.L. (2003). Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. *J Biol Chem* 278**,** 9790-9795.

Grant, M.B., Tarnuzzer, R.W., Caballero, S., Ozeck, M.J., Davis, M.I., Spoerri, P.E., Feoktistov, I., Biaggioni, I., Shryock, J.C., and Belardinelli, L. (1999). Adenosine Receptor Activation Induces Vascular Endothelial Growth Factor in Human Retinal Endothelial Cells. 85**,** 699-706.

Grasso, C.S., Tsoi, J., Onyshchenko, M., Abril-Rodriguez, G., Ross-Macdonald, P., Wind-Rotolo, M., Champhekar, A., Medina, E., Torrejon, D.Y., Shin, D.S., Tran, P., Kim, Y.J., Puig-Saus, C., Campbell, K., Vega-Crespo, A., Quist, M., Martignier, C., Luke, J.J., Wolchok, J.D., Johnson, D.B., Chmielowski, B., Hodi, F.S., Bhatia, S., Sharfman, W., Urba, W.J., Slingluff, C.L., Jr., Diab, A., Haanen, J., Algarra, S.M., Pardoll, D.M., Anagnostou, V., Topalian, S.L., Velculescu, V.E., Speiser,

D.E., Kalbasi, A., and Ribas, A. (2020). Conserved Interferon-γ Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. *Cancer Cell* 38**,** 500-515.e503.

Grünewald, J.K.G., and Ridley, A.J. (2010). CD73 represses pro-inflammatory responses in human endothelial cells. *Journal of Inflammation* 7**,** 10.

Grzywa, T.M., Paskal, W., and Włodarski, P.K. (2017). Intratumor and Intertumor Heterogeneity in Melanoma. *Transl Oncol* 10**,** 956-975.

Guy, G.P., Jr., Thomas, C.C., Thompson, T., Watson, M., Massetti, G.M., and Richardson, L.C. (2015). Vital signs: melanoma incidence and mortality trends and projections - United States, 1982-2030. *MMWR Morb Mortal Wkly Rep* 64**,** 591-596.

Henttinen, T., Jalkanen, S., and Yegutkin, G.G. (2003). Adherent Leukocytes Prevent Adenosine Formation and Impair Endothelial Barrier Function by Ecto-5'-nucleotidase/CD73-dependent Mechanism. 278**,** 24888-24895.

Heuts, D.P.H.M., Weissenborn, M.J., Olkhov, R.V., Shaw, A.M., Gummadova, J., Levy, C., and Scrutton, N.S. (2012). Crystal Structure of a Soluble Form of Human CD73 with Ecto-5'-Nucleotidase Activity. 13**,** 2384-2391.

Hodi, F.S., O'day, S.J., Mcdermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., Akerley, W., Van Den Eertwegh, A.J., Lutzky, J., Lorigan, P., Vaubel, J.M., Linette, G.P., Hogg, D., Ottensmeier, C.H., Lebbé, C., Peschel, C., Quirt, I., Clark, J.I., Wolchok, J.D., Weber, J.S., Tian, J., Yellin, M.J., Nichol, G.M., Hoos, A., and Urba, W.J. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363**,** 711-723.

Hoek, K.S., Eichhoff, O.M., Schlegel, N.C., Döbbeling, U., Kobert, N., Schaerer, L., Hemmi, S., and Dummer, R. (2008). In vivo switching of human melanoma cells between proliferative and invasive states. *Cancer Res* 68**,** 650-656.

Hong, C.S., Funk, S., and Whiteside, T.L. (2017). Isolation of Biologically Active Exosomes from Plasma of Patients with Cancer. *Methods Mol Biol* 1633**,** 257-265.

Horenstein, A.L., Chillemi, A., Zaccarello, G., Bruzzone, S., Quarona, V., Zito, A., Serra, S., and Malavasi, F. (2013). A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology* 2**,** e26246.

Huang, Q., Durham, N.M., Sult, E., Wu, Y., Liu, J., Holoweckyj, N., Iciek, L., Hollingsworth, R., Hall, B., Herbst, R., Leow, C.C., and Sachsenmeier, K. (2015). Abstract 1538: Levels and enzyme activity of CD73 in primary samples from cancer patients. 75**,** 1538-1538.

Hugo, W., Shi, H., Sun, L., Piva, M., Song, C., Kong, X., Moriceau, G., Hong, A., Dahlman, K.B., Johnson, D.B., Sosman, J.A., Ribas, A., and Lo, R.S. (2015). Non-genomic and Immune Evolution of Melanoma Acquiring MAPKi Resistance. *Cell* 162**,** 1271-1285.

Iannone, R., Miele, L., Maiolino, P., Pinto, A., and Morello, S. (2013). Blockade of A2b adenosine receptor reduces tumor growth and immune suppression mediated by myeloid-derived suppressor cells in a mouse model of melanoma. *Neoplasia* 15**,** 1400-1409.

Iannone, R., Miele, L., Maiolino, P., Pinto, A., and Morello, S. (2014). Adenosine limits the therapeutic effectiveness of anti-CTLA4 mAb in a mouse melanoma model. *Am J Cancer Res* 4**,** 172-181.

Ilmonen, S., Jahkola, T., Turunen, J.P., Muhonen, T., and Asko-Seljavaara, S. (2004). Tenascin-C in primary malignant melanoma of the skin. *Histopathology* 45**,** 405-411.

Inoue, Y., Yoshimura, K., Kurabe, N., Kahyo, T., Kawase, A., Tanahashi, M., Ogawa, H., Inui, N., Funai, K., Shinmura, K., Niwa, H., Suda, T., and Sugimura, H. (2017). Prognostic impact of CD73 and A2A adenosine receptor expression in non-small-cell lung cancer. *Oncotarget* 8**,** 8738-8751.

Jeske, S.S., Schuler, P.J., Doescher, J., Theodoraki, M.N., Laban, S., Brunner, C., Hoffmann, T.K., and Wigand, M.C. (2020). Age-related changes in T lymphocytes of patients with head and neck squamous cell carcinoma. *Immunity & Ageing* 17**,** 3.

Jiang, T., Xu, X., Qiao, M., Li, X., Zhao, C., Zhou, F., Gao, G., Wu, F., Chen, X., Su, C., Ren, S., Zhai, C., and Zhou, C. (2018). Comprehensive evaluation of NT5E/CD73 expression and its prognostic significance in distinct types of cancers. *BMC Cancer* 18**,** 267.

Jin, R., Liu, L., Xing, Y., Meng, T., Ma, L., Pei, J., Cong, Y., Zhang, X., Ren, Z., Wang, X., Shen, J., and Yu, K. (2020). Dual mechanisms of novel CD73-targeted antibody and antibody-drug conjugate in inhibiting lung tumor growth and promoting antitumor immune-effector function. *Mol Cancer Ther*.

Kääriäinen, E., Nummela, P., Soikkeli, J., Yin, M., Lukk, M., Jahkola, T., Virolainen, S., Ora, A., Ukkonen, E., Saksela, O., and Hölttä, E. (2006). Switch to an invasive growth phase in melanoma is associated with tenascin-C, fibronectin, and procollagen-I forming specific channel structures for invasion. 210**,** 181-191.

Kalluri, R. (2016). The biology and function of exosomes in cancer. *J Clin Invest* 126**,** 1208-1215.

Katamura, K., Shintaku, N., Yamauchi, Y., Fukui, T., Ohshima, Y., Mayumi, M., and Furusho, K. (1995). Prostaglandin E2 at priming of naive CD4+ T cells inhibits acquisition of ability to produce IFN-gamma and IL-2, but not IL-4 and IL-5. 155**,** 4604-4612.

Kennedy-Darling, J., Bhate, S.S., Hickey, J.W., Black, S., Barlow, G.L., Vazquez, G., Venkataraaman, V.G., Samusik, N., Goltsev, Y., Schürch, C.M., and Nolan, G.P. (2021). Highly multiplexed tissue imaging using repeated oligonucleotide exchange reaction. *Eur J Immunol* 51**,** 1262-1277.

Keung, E.Z., and Gershenwald, J.E. (2018). The eighth edition American Joint Committee on Cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. *Expert review of anticancer therapy* 18**,** 775-784.

Khoa, N.D., Montesinos, M.C., Williams, A.J., Kelly, M., and Cronstein, B.N. (2003). Th1 Cytokines Regulate Adenosine Receptors and Their Downstream Signaling Elements in Human Microvascular Endothelial Cells. 171**,** 3991-3998.

Khoja, L., Day, D., Wei-Wu Chen, T., Siu, L.L., and Hansen, A.R. (2017). Tumour- and class-specific patterns of immune-related adverse events of immune checkpoint inhibitors: a systematic review. *Ann Oncol* 28**,** 2377-2385.

Knapp, K., Zebisch, M., Pippel, J., El-Tayeb, A., Müller, C.E., and Sträter, N. (2012). Crystal structure of the human ecto-5'-nucleotidase (CD73): insights into the regulation of purinergic signaling. *Structure* 20**,** 2161-2173.

Koivisto, M.K., Tervahartiala, M., Kenessey, I., Jalkanen, S., Boström, P.J., and Salmi, M. (2018). Cell-type-specific CD73 expression is an independent prognostic factor in bladder cancer. *Carcinogenesis* 40**,** 84-92.

Korn, E.L., Liu, P.-Y., Lee, S.J., Chapman, J.-a.W., Niedzwiecki, D., Suman, V.J., Moon, J., Sondak, V.K., Atkins, M.B., Eisenhauer, E.A., Parulekar, W., Markovic, S.N., Saxman, S., and Kirkwood, J.M. (2008). Meta-Analysis of Phase II Cooperative Group Trials in Metastatic Stage IV Melanoma to Determine Progression-Free and Overall Survival Benchmarks for Future Phase II Trials. 26**,** 527-534.

Kugel, C.H., 3rd, Douglass, S.M., Webster, M.R., Kaur, A., Liu, Q., Yin, X., Weiss, S.A., Darvishian, F., Al-Rohil, R.N., Ndoye, A., Behera, R., Alicea, G.M., Ecker, B.L., Fane, M., Allegrezza, M.J., Svoronos, N., Kumar, V., Wang, D.Y., Somasundaram, R., Hu-Lieskovan, S., Ozgun, A., Herlyn, M., Conejo-Garcia, J.R., Gabrilovich, D., Stone, E.L., Nowicki, T.S., Sosman, J., Rai, R., Carlino, M.S., Long, G.V., Marais, R., Ribas, A., Eroglu, Z., Davies, M.A., Schilling, B., Schadendorf, D., Xu, W., Amaravadi, R.K., Menzies, A.M., Mcquade, J.L., Johnson, D.B., Osman, I., and Weeraratna, A.T. (2018). Age Correlates with Response to Anti-PD1, Reflecting Age-Related Differences in Intratumoral Effector and Regulatory T-Cell Populations. *Clinical cancer research : an official journal of the American Association for Cancer Research* 24**,** 5347-5356.

Kumar, V., Chaudhary, N., Garg, M., Floudas, C.S., Soni, P., and Chandra, A.B. (2017). Current Diagnosis and Management of Immune Related Adverse Events (irAEs) Induced by Immune Checkpoint Inhibitor Therapy. *Front Pharmacol* 8**,** 49.

La Shu, S., Yang, Y., Allen, C.L., Hurley, E., Tung, K.H., Minderman, H., Wu, Y., and Ernstoff, M.S. (2020). Purity and yield of melanoma exosomes are dependent on isolation method. 9**,** 1692401.

Lal, H., Kumar, L., Kohli, G.S., Sharma, A., and Goel, H. (1989). Serum enzymes in head and neck cancer. IV: 5-nucleotidase. *The Journal of Laryngology & Otology* 103**,** 200-202.

Larkin, J., Chiarion-Sileni, V., Gonzalez, R., Grob, J.-J., Rutkowski, P., Lao, C.D., Cowey, C.L., Schadendorf, D., Wagstaff, J., Dummer, R., Ferrucci, P.F., Smylie, M., Hogg, D., Hill, A., Márquez-Rodas, I., Haanen, J., Guidoboni, M., Maio, M., Schöffski, P., Carlino, M.S., Lebbé, C., Mcarthur, G., Ascierto, P.A., Daniels, G.A., Long, G.V., Bastholt, L., Rizzo, J.I., Balogh, A., Moshyk, A., Hodi, F.S., and Wolchok, J.D. (2019). Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. 381**,** 1535-1546.

Lawrence, M.S., Stojanov, P., Polak, P., Kryukov, G.V., Cibulskis, K., Sivachenko, A., Carter, S.L., Stewart, C., Mermel, C.H., Roberts, S.A., Kiezun, A., Hammerman, P.S., Mckenna, A., Drier, Y., Zou, L., Ramos, A.H., Pugh, T.J., Stransky, N., Helman, E., Kim, J., Sougnez, C., Ambrogio, L., Nickerson, E., Shefler, E., Cortés, M.L., Auclair, D., Saksena, G., Voet, D., Noble, M., Dicara, D., Lin, P., Lichtenstein, L., Heiman, D.I., Fennell, T., Imielinski, M., Hernandez, B., Hodis, E., Baca, S., Dulak, A.M., Lohr, J., Landau, D.A., Wu, C.J., Melendez-Zajgla, J., Hidalgo-Miranda, A., Koren, A., Mccarroll, S.A., Mora, J., Crompton, B., Onofrio, R., Parkin, M., Winckler, W., Ardlie, K., Gabriel, S.B., Roberts, C.W.M., Biegel, J.A., Stegmaier, K., Bass, A.J., Garraway, L.A., Meyerson, M., Golub, T.R., Gordenin, D.A., Sunyaev, S., Lander, E.S., and Getz, G. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 499**,** 214-218.

Leclerc, B.G., Charlebois, R., Chouinard, G., Allard, B., Pommey, S., Saad, F., and Stagg, J. (2016). CD73 Expression Is an Independent Prognostic Factor in Prostate Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 22**,** 158-166.

Leone, R.D., and Emens, L.A. (2018). Targeting adenosine for cancer immunotherapy. *J Immunother Cancer* 6**,** 57.

Leone, R.D., Sun, I.M., Oh, M.H., Sun, I.H., Wen, J., Englert, J., and Powell, J.D. (2018). Inhibition of the adenosine A2a receptor modulates expression of T cell coinhibitory receptors and improves effector function for enhanced checkpoint blockade and ACT in murine cancer models. *Cancer Immunol Immunother* 67**,** 1271-1284.

Li, P., Yang, X., Feng, Y., Wu, L., Ma, W., Ding, G., Wei, Y., and Sun, L. 2018. The impact of immunosenescence on the efficacy of immune checkpoint inhibitors in melanoma patients: a meta-analysis. *OncoTargets and therapy* [Online], 11. [Accessed 2018].

Logozzi, M., De Milito, A., Lugini, L., Borghi, M., Calabrò, L., Spada, M., Perdicchio, M., Marino, M.L., Federici, C., Iessi, E., Brambilla, D., Venturi, G., Lozupone, F., Santinami, M., Huber, V., Maio, M., Rivoltini, L., and Fais, S. (2009). High Levels of Exosomes Expressing CD63 and Caveolin-1 in Plasma of Melanoma Patients. *PLOS ONE* 4**,** e5219.

Loi, S., Pommey, S., Haibe-Kains, B., Beavis, P.A., Darcy, P.K., Smyth, M.J., and Stagg, J. (2013). CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *PNAS* 110**,** 11091-11096.

Lu, X.-X., Chen, Y.-T., Feng, B., Mao, X.-B., Yu, B., and Chu, X.-Y. (2013). Expression and clinical significance of CD73 and hypoxia-inducible factor-1α in gastric carcinoma. *World journal of gastroenterology* 19**,** 1912-1918.

Ludwig, N., Yerneni, S.S., Azambuja, J.H., Gillespie, D.G., Menshikova, E.V., Jackson, E.K., and Whiteside, T.L. (2020). Tumor-derived exosomes promote angiogenesis via adenosine A2B receptor signaling. *Angiogenesis* 23**,** 599–610.

Luke, J.J., Powderly, J.D., Merchan, J.R., Barve, M.A., Hotson, A.N., Mobasher, M., Kwei, L., Luciano, G., Buggy, J.J., Piccione, E., and Miller, R.A. (2019). Immunobiology, preliminary safety, and efficacy of CPI-006, an anti-CD73 antibody with immune modulating activity, in a phase 1 trial in advanced cancers. 37**,** 2505-2505.

Maksimow, M., Kyhälä, L., Nieminen, A., Kylänpää, L., Aalto, K., Elima, K., Mentula, P., Lehti, M., Puolakkainen, P., Yegutkin, G.G., Jalkanen, S., Repo, H., and Salmi, M. (2014). Early prediction of persistent organ failure by soluble CD73 in patients with acute pancreatitis*. *Crit Care Med* 42**,** 2556-2564.

Mandapathil, M., Boduc, M., Netzer, C., Güldner, C., Roessler, M., Wallicek-Dworschak, U., Jahns, E., and Stuck, B. (2018). CD73 expression in lymph node metastases in patients with head and neck cancer. *Acta Otolaryngol* 138**,** 180-184.

Manjarrez-Orduño, N., Menard, L.C., Kansal, S., Fischer, P., Kakrecha, B., Jiang, C., Cunningham, M., Greenawalt, D., Patel, V., Yang, M., Golhar, R., Carman, J.A., Lezhnin, S., Dai, H., Kayne, P.S., Suchard, S.J., Bernstein, S.H., and Nadler, S.G. (2018). Circulating T Cell Subpopulations Correlate With Immune Responses at the Tumor Site and Clinical Response to PD1 Inhibition in Non-Small Cell Lung Cancer. *Front Immunol* 9**,** 1613.

Messaoudi, N., Cousineau, I., Arslanian, E., Henault, D., Stephen, D., Vandenbroucke-Menu, F., Dagenais, M., Létourneau, R., Plasse, M., Roy, A., Lapointe, R., Ysebaert, D., Trudel, D., Soucy, G., Stagg, J., and Turcotte, S. (2020). Prognostic value of CD73 expression in resected colorectal cancer liver metastasis. *Oncoimmunology* 9**,** 1746138.

Mittal, D., Young, A., Stannard, K., Yong, M., Teng, M.W.L., Allard, B., Stagg, J., and Smyth, M.J. (2014). Antimetastatic Effects of Blocking PD-1 and the Adenosine A2A Receptor. 74**,** 3652-3658.

Moesta, A.K., Li, X.-Y., and Smyth, M.J. (2020). Targeting CD39 in cancer. *Nature Reviews Immunology* 20**,** 739-755.

Monteiro, I., Vigano, S., Faouzi, M., Treilleux, I., Michielin, O., Ménétrier-Caux, C., Caux, C., Romero, P., and De Leval, L. (2018). CD73 expression and clinical significance in human metastatic melanoma. *Oncotarget* 9**,** 26659-26669.

Morello, S., Capone, M., Sorrentino, C., Giannarelli, D., Madonna, G., Mallardo, D., Grimaldi, A.M., Pinto, A., and Ascierto, P.A. (2017). Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. *Journal of translational medicine* 15**,** 244-244.

Morello, S., Turiello, R., Madonna, G., Pinto, A., Ascierto, P.A., and Capone, M. (2019). Enzyme activity of circulating CD73 in human serum. *Methods Enzymol* 629**,** 257-267.

Nakamura, Y., Namikawa, K., Yoshino, K., Yoshikawa, S., Uchi, H., Goto, K., Nakamura, Y., Fukushima, S., Kiniwa, Y., Takenouchi, T., Uhara, H., Kawai, T., Hatta, N., Funakoshi, T., Teramoto, Y., Otsuka, A., Doi, H., Ogata, D., Matsushita, S., Isei, T., Hayashi, T., Shibayama, Y., and Yamazaki, N. (2020). Anti-PD1 checkpoint inhibitor therapy in acral melanoma: a multicenter study of 193 Japanese patients. *Ann Oncol* 31**,** 1198-1206.

Narravula, S., Lennon, P.F., Mueller, B.U., and Colgan, S.P. (2000). Regulation of Endothelial CD73 by Adenosine: Paracrine Pathway for Enhanced Endothelial Barrier Function. 165**,** 5262-5268.

Nct02503774 *MEDI9447 Alone and in Combination With MEDI4736 in Adult Subjects With Select Advanced Solid Tumors* [Online]. Available: https://ClinicalTrials.gov/show/NCT02503774 [Accessed].

Nct03207867 *A Phase 2 Study of NIR178 in Combination With PDR001 in Patients With Solid Tumors and Non-Hodgkin Lymphoma* [Online]. Available: https://ClinicalTrials.gov/show/NCT03207867 [Accessed].

Nct03454451 "CPI-006 Alone and in Combination With Ciforadenant and With Pembrolizumab for Patients With Advanced Cancers".).

Nct03629756 *A Study to Evaluate the Safety and Tolerability of Immunotherapy Combinations in Participants With Advanced Malignancies* [Online]. Available: https://ClinicalTrials.gov/show/NCT03629756 [Accessed].

Nct04261075 *IPH5201 as Monotherapy or in Combination With Durvalumab +/- Oleclumab in Subjects With Advanced Solid Tumors* [Online]. Available: https://ClinicalTrials.gov/show/NCT04261075 [Accessed].

Nct04306900 *TTX-030 in Combination With Immunotherapy and/or Chemotherapy in Subjects With Advanced Cancers* [Online]. Available: https://ClinicalTrials.gov/show/NCT04306900 [Accessed].

Neo, S.Y., Yang, Y., Record, J., Ma, R., Chen, X., Chen, Z., Tobin, N.P., Blake, E., Seitz, C., Thomas, R., Wagner, A.K., Andersson, J., De Boniface, J., Bergh, J., Murray, S., Alici, E., Childs, R., Johansson, M., Westerberg, L.S., Haglund, F., Hartman, J., and Lundqvist, A. (2020). CD73 immune checkpoint defines regulatory NK cells within the tumor microenvironment. *The Journal of Clinical Investigation* 130**,** 1185-1198.

Ngiow, S.F., Young, A., Jacquelot, N., Yamazaki, T., Enot, D., Zitvogel, L., and Smyth, M.J. (2015). A Threshold Level of Intratumor CD8+ T-cell PD1 Expression Dictates Therapeutic Response to Anti-PD1. *Cancer Res* 75**,** 3800-3811.

O'connell, M.P., Marchbank, K., Webster, M.R., Valiga, A.A., Kaur, A., Vultur, A., Li, L., Herlyn, M., Villanueva, J., Liu, Q., Yin, X., Widura, S., Nelson, J., Ruiz, N., Camilli, T.C., Indig, F.E., Flaherty, K.T., Wargo, J.A., Frederick, D.T., Cooper, Z.A., Nair, S., Amaravadi, R.K., Schuchter, L.M., Karakousis, G.C., Xu, W., Xu, X., and Weeraratna, A.T. (2013). Hypoxia induces phenotypic plasticity and therapy resistance in melanoma via the tyrosine kinase receptors ROR1 and ROR2. *Cancer Discov* 3**,** 1378-1393.

Ohta, A., Gorelik, E., Prasad, S.J., Ronchese, F., Lukashev, D., Wong, M.K.K., Huang, X., Caldwell, S., Liu, K., Smith, P., Chen, J.-F., Jackson, E.K., Apasov, S., Abrams, S., and Sitkovsky, M. (2006). A2A adenosine receptor protects tumors from antitumor T cells. *Proceedings of the National Academy of Sciences of the United States of America* 103**,** 13132-13137.

Ohta, A., and Sitkovsky, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414**,** 916-920.

Palmieri, G., Ombra, M., Colombino, M., Casula, M., Sini, M., Manca, A., Paliogiannis, P., Ascierto, P.A., and Cossu, A. (2015). Multiple Molecular Pathways in Melanomagenesis: Characterization of Therapeutic Targets. *Front Oncol* 5**,** 183.

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., García-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B.A., Callahan, M.K., Yuan, J., Martins, V.R., Skog, J., Kaplan, R.N., Brady, M.S., Wolchok, J.D., Chapman, P.B., Kang, Y., Bromberg, J., and Lyden, D. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 18**,** 883-891.

Persson, J.N., Baird, C.H., Tong, S., Urban, T.T., Klawitter, J., Wischmeyer, P.E., and Davidson, J.A. (2018). Infant cardiopulmonary bypass: CD73 kinetics, association with clinical outcomes, and influence on serum adenosine production capacity. *Pediatric Research* 83**,** 858-865.

Pfeffer, S.R., Grossmann, K.F., Cassidy, P.B., Yang, C.H., Fan, M., Kopelovich, L., Leachman, S.A., and Pfeffer, L.M. (2015). Detection of Exosomal miRNAs in the Plasma of Melanoma Patients. 4**,** 2012-2027.

Pires Da Silva, I., Wang, K.Y.X., Wilmott, J.S., Holst, J., Carlino, M.S., Park, J.J., Quek, C., Wongchenko, M., Yan, Y., Mann, G., Johnson, D.B., Mcquade, J.L., Rai, R., Kefford, R.F., Rizos, H., Scolyer, R.A., Yang, J.Y.H., Long, G.V., and Menzies, A.M. (2019). Distinct Molecular Profiles and Immunotherapy Treatment Outcomes of V600E and V600K BRAF-Mutant Melanoma. *Clin Cancer Res* 25**,** 1272-1279.

Postow, M.A., Sidlow, R., and Hellmann, M.D. (2018). Immune-Related Adverse Events Associated with Immune Checkpoint Blockade. *N Engl J Med* 378**,** 158-168.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 200**,** 373-383.

Reinhardt, J., Landsberg, J., Schmid-Burgk, J.L., Ramis, B.B., Bald, T., Glodde, N., Lopez-Ramos, D., Young, A., Ngiow, S.F., Nettersheim, D., Schorle, H., Quast, T., Kolanus, W., Schadendorf, D., Long, G.V., Madore, J., Scolyer, R.A., Ribas, A., Smyth, M.J., Tumeh, P.C., Tüting, T., and Hölzel, M. (2017). MAPK Signaling and Inflammation Link Melanoma Phenotype Switching to Induction of CD73 during Immunotherapy. *Cancer Res* 77**,** 4697-4709.

Ren, Z.-H., Lin, C.-Z., Cao, W., Yang, R., Lu, W., Liu, Z.-Q., Chen, Y.-M., Yang, X., Tian, Z., Wang, L.-Z., Li, J., Wang, X., Chen, W.-T., Ji, T., and Zhang, C.-P. (2016). CD73 is associated with poor prognosis in HNSCC. *Oncotarget* 7**,** 61690-61702.

Ridolfi, L., De Rosa, F., Petracci, E., Tanda, E.T., Marra, E., Pigozzo, J., Marconcini, R., Guida, M., Cappellini, G.C.A., Gallizzi, G., Occelli, M., Pala, L., Gambale, E., Bersanelli, M., Galdo, G., Cortellini, A., Morgese, F., Zoratto, F., Stucci, L.S., Strippoli, S., and Guidoboni, M. (2020). Anti-PD1 antibodies in patients aged ≥ 75 years with metastatic melanoma: A retrospective multicentre study. *J Geriatr Oncol* 11**,** 515-522.

Rigel, D.S., and Carucci, J.A. (2000). Malignant melanoma: prevention, early detection, and treatment in the 21st century. *CA Cancer J Clin* 50**,** 215-236; quiz 237-240.

Robert, C., Grob, J.J., Stroyakovskiy, D., Karaszewska, B., Hauschild, A., Levchenko, E., Chiarion Sileni, V., Schachter, J., Garbe, C., Bondarenko, I., Gogas, H., Mandalá, M., Haanen, J.B.a.G., Lebbé, C., Mackiewicz, A., Rutkowski, P., Nathan, P.D., Ribas, A., Davies, M.A., Flaherty, K.T., Burgess, P., Tan, M., Gasal, E., Voi, M., Schadendorf, D., and Long, G.V. (2019a). Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma. 381**,** 626-636.

Robert, C., Ribas, A., Schachter, J., Arance, A., Grob, J.J., Mortier, L., Daud, A., Carlino, M.S., Mcneil, C.M., Lotem, M., Larkin, J.M.G., Lorigan, P., Neyns, B., Blank, C.U., Petrella, T.M., Hamid, O., Su, S.C., Krepler, C., Ibrahim, N., and Long, G.V. (2019b). Pembrolizumab versus ipilimumab in advanced melanoma (KEYNOTE-006): post-hoc 5-year results from an open-label, multicentre, randomised, controlled, phase 3 study. *Lancet Oncol* 20**,** 1239-1251.

Ryzhov, S., Novitskiy, S.V., Zaynagetdinov, R., Goldstein, A.E., Carbone, D.P., Biaggioni, I., Dikov, M.M., and Feoktistov, I. (2008). Host A(2B) adenosine receptors promote carcinoma growth. *Neoplasia (New York, N.Y.)* 10**,** 987-995.

Sade-Feldman, M., Yizhak, K., Bjorgaard, S.L., Ray, J.P., De Boer, C.G., Jenkins, R.W., Lieb, D.J., Chen, J.H., Frederick, D.T., Barzily-Rokni, M., Freeman, S.S., Reuben, A., Hoover, P.J., Villani, A.C., Ivanova, E., Portell, A., Lizotte, P.H., Aref, A.R., Eliane, J.P., Hammond, M.R., Vitzthum, H., Blackmon, S.M., Li, B., Gopalakrishnan, V., Reddy, S.M., Cooper, Z.A., Paweletz, C.P., Barbie, D.A., Stemmer-Rachamimov, A., Flaherty, K.T., Wargo, J.A., Boland, G.M., Sullivan, R.J., Getz, G., and Hacohen, N. (2019). Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* 176**,** 404.

Sadej, R., and Skladanowski, A.C. (2012). Dual, enzymatic and non-enzymatic, function of ecto-5'-nucleotidase (eN, CD73) in migration and invasion of A375 melanoma cells. *Acta Biochim Pol* 59**,** 647-652.

Sansom, D.M. (2000). CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* 101**,** 169-177.

Schäkel, L., Schmies, C.C., Idris, R.M., Luo, X., Lee, S.Y., Lopez, V., Mirza, S., Vu, T.H., Pelletier, J., Sévigny, J., Namasivayam, V., and Müller, C.E. (2020). Nucleotide Analog ARL67156 as a Lead Structure for the Development of CD39 and Dual CD39/CD73 Ectonucleotidase Inhibitors. *Front Pharmacol* 11**,** 1294.

Schneider, E., Rissiek, A., Winzer, R., Puig, B., Rissiek, B., Haag, F., Mittrücker, H.W., Magnus, T., and Tolosa, E. (2019). Generation and Function of Non-cell-bound CD73 in Inflammation. *Front Immunol* 10**,** 1729.

Schneider, E., Winzer, R., Rissiek, A., Ricklefs, I., Meyer-Schwesinger, C., Ricklefs, F.L., Bauche, A., Behrends, J., Reimer, R., Brenna, S., Wasielewski, H., Lauten, M., Rissiek, B., Puig, B., Cortesi, F., Magnus, T., Fliegert, R., Müller, C.E., Gagliani, N., and Tolosa, E. (2021). CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression. *Nature Communications* 12**,** 5911.

Selby, M., Engelhardt, J., Lu, L.-S., Quigley, M., Wang, C., Chen, B., and Korman, A.J. (2013). Antitumor activity of concurrent blockade of immune checkpoint molecules CTLA-4 and PD-1 in preclinical models. 31**,** 3061-3061.

Sharma, P., Diergaarde, B., Ferrone, S., Kirkwood, J.M., and Whiteside, T.L. (2020). Melanoma cell-derived exosomes in plasma of melanoma patients suppress functions of immune effector cells. *Scientific Reports* 10**,** 92.

Sharma, P., Ludwig, S., Muller, L., Hong, C.S., Kirkwood, J.M., Ferrone, S., and Whiteside, T.L. (2018). Immunoaffinity-based isolation of melanoma cell-derived exosomes from plasma of patients with melanoma. *J Extracell Vesicles* 7**,** 1435138.

Sharpe, A.H., Wherry, E.J., Ahmed, R., and Freeman, G.J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8**,** 239-245.

Sheppard, K.A., Fitz, L.J., Lee, J.M., Benander, C., George, J.A., Wooters, J., Qiu, Y., Jussif, J.M., Carter, L.L., Wood, C.R., and Chaudhary, D. (2004). PD-1 inhibits T-cell receptor induced

phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett* 574**,** 37-41.

Siegel, R.L., Miller, K.D., Fuchs, H.E., and Jemal, A. (2021). Cancer Statistics, 2021. 71**,** 7-33.

Sorrentino, C., Miele, L., Porta, A., Pinto, A., and Morello, S. (2015). Myeloid-derived suppressor cells contribute to A2B adenosine receptor-induced VEGF production and angiogenesis in a mouse melanoma model. *Oncotarget* 6**,** 27478-27489.

Sorrentino, C., Miele, L., Porta, A., Pinto, A., and Morello, S. (2016). Activation of the A2B adenosine receptor in B16 melanomas induces CXCL12 expression in FAP-positive tumor stromal cells, enhancing tumor progression. *Oncotarget* 7**,** 64274-64288.

Sorrentino, C., and Morello, S. (2017). Role of adenosine in tumor progression: focus on A_{2B} receptor as potential therapeutic target. *Journal of Cancer Metastasis and Treatment* 3**,** 127-138.

Stagg, J., Divisekera, U., Duret, H., Sparwasser, T., Teng, M.W.L., Darcy, P.K., and Smyth, M.J. (2011). CD73-Deficient Mice Have Increased Antitumor Immunity and Are Resistant to Experimental Metastasis. 71**,** 2892-2900.

Stagg, J., Divisekera, U., Mclaughlin, N., Sharkey, J., Pommey, S., Denoyer, D., Dwyer, K.M., and Smyth, M.J. (2010). Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A* 107**,** 1547-1552.

Sträter, N. (2006). Ecto-5'-nucleotidase: Structure function relationships. *Purinergic Signal* 2**,** 343-350.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71**,** 209-249.

Synnestvedt, K., Furuta, G.T., Comerford, K.M., Louis, N., Karhausen, J., Eltzschig, H.K., Hansen, K.R., Thompson, L.F., and Colgan, S.P. (2002). Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J Clin Invest* 110**,** 993-1002.

Thecancergenomeatlasnetwork (2015). Genomic Classification of Cutaneous Melanoma. *Cell* 161**,** 1681-1696.

Theodoraki, M.N., Hoffmann, T.K., Jackson, E.K., and Whiteside, T.L. (2018). Exosomes in HNSCC plasma as surrogate markers of tumour progression and immune competence. *Clinical and experimental immunology* 194**,** 67-78.

Théry, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* Chapter 3**,** Unit 3.22.

Thompson, E.A., and Powell, J.D. (2020). Inhibition of the Adenosine Pathway to Potentiate Cancer Immunotherapy: Potential for Combinatorial Approaches. *Annu Rev Med* 72**,** 6.1-6.18.

Tumeh, P.C., Harview, C.L., Yearley, J.H., Shintaku, I.P., Taylor, E.J., Robert, L., Chmielowski, B., Spasic, M., Henry, G., Ciobanu, V., West, A.N., Carmona, M., Kivork, C., Seja, E., Cherry, G., Gutierrez, A.J., Grogan, T.R., Mateus, C., Tomasic, G., Glaspy, J.A., Emerson, R.O., Robins, H., Pierce, R.H., Elashoff, D.A., Robert, C., and Ribas, A. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 515**,** 568-571.

Turcotte, M., Allard, D., Mittal, D., Bareche, Y., Buisseret, L., Jose, V., Pommey, S., Delisle, V., Loi, S., Joensuu, H., Kellokumpu-Lehtinen, P.L., Sotiriou, C., Smyth, M.J., and Stagg, J. (2017). CD73 Promotes Resistance to HER2/ErbB2 Antibody Therapy. *Cancer Res* 77**,** 5652-5663.

Turcotte, M., Spring, K., Pommey, S., Chouinard, G., Cousineau, I., George, J., Chen, G.M., Gendoo, D.M.A., Haibe-Kains, B., Karn, T., Rahimi, K., Le Page, C., Provencher, D., Mes-Masson, A.-M., and Stagg, J. (2015). CD73 Is Associated with Poor Prognosis in High-Grade Serous Ovarian Cancer. *Cancer Res* 75**,** 4494-4503.

Van Wilpe, S., Koornstra, R., Den Brok, M., De Groot, J.W., Blank, C., De Vries, J., Gerritsen, W., and Mehra, N. (2020). Lactate dehydrogenase: a marker of diminished antitumor immunity. *Oncoimmunology* 9**,** 1731942.

Vijayan, D., Young, A., Teng, M.W.L., and Smyth, M.J. (2017). Targeting immunosuppressive adenosine in cancer. *Nat Rev Cancer* 17**,** 709-724.

Walker, G., Langheinrich, A.C., Dennhauser, E., Bohle, R.M., Dreyer, T., Kreuzer, J., Tillmanns, H., Braun-Dullaeus, R.C., and Haberbosch, W. (1999). 3-Deazaadenosine Prevents Adhesion Molecule Expression and Atherosclerotic Lesion Formation in the Aortas of C57BL/6J Mice. 19**,** 2673-2679.

Wang, L., Fan, J., Thompson, L.F., Zhang, Y., Shin, T., Curiel, T.J., and Zhang, B. (2011). CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. *The Journal of Clinical Investigation* 121**,** 2371-2382.

Wang, L., Tang, S., Wang, Y., Xu, S., Yu, J., Zhi, X., Ou, Z., Yang, J., Zhou, P., and Shao, Z. (2013). Ecto-5'-nucleotidase (CD73) promotes tumor angiogenesis. *Clin Exp Metastasis* 30**,** 671-680.

Wang, X., Zhang, T., Song, Z., Li, L., Zhang, X., Liu, J., Liu, X., Qiu, L., Qian, Z., Zhou, S., Feng, L., Hu, G., Meng, B., Zhai, Q., Ren, X., Fu, K., Li, L., Wang, P., and Zhang, H. (2019). Tumor CD73/A2aR adenosine immunosuppressive axis and tumor-infiltrating lymphocytes in diffuse large B-cell lymphoma: correlations with clinicopathological characteristics and clinical outcome. *Int J Cancer* 145**,** 1414-1422.

Wennerberg, E., Spada, S., Rudqvist, N.-P., Lhuillier, C., Gruber, S., Chen, Q., Zhang, F., Zhou, X.K., Gross, S.S., Formenti, S.C., and Demaria, S. (2020). CD73 blockade promotes dendritic cell infiltration of irradiated tumors and tumor rejection. *CAncer Immunol Res* 8(4)**,** 465-478.

Widmer, D.S., Hoek, K.S., Cheng, P.F., Eichhoff, O.M., Biedermann, T., Raaijmakers, M.I.G., Hemmi, S., Dummer, R., and Levesque, M.P. (2013). Hypoxia contributes to melanoma heterogeneity by triggering HIF1α-dependent phenotype switching. *J Invest Dermatol* 133**,** 2436-2443.

Wolchok, J.D., Kluger, H., Callahan, M.K., Postow, M.A., Rizvi, N.A., Lesokhin, A.M., Segal, N.H., Ariyan, C.E., Gordon, R.-A., Reed, K., Burke, M.M., Caldwell, A., Kronenberg, S.A., Agunwamba, B.U., Zhang, X., Lowy, I., Inzunza, H.D., Feely, W., Horak, C.E., Hong, Q., Korman, A.J., Wigginton, J.M., Gupta, A., and Sznol, M. (2013). Nivolumab plus Ipilimumab in Advanced Melanoma. 369**,** 122-133.

Wu, Q., Wang, Q., Tang, X., Xu, R., Zhang, L., Chen, X., Xue, Q., Wang, Z., Shi, R., Wang, F., Ju, F., Zhang, B., and Zhou, Y.L. (2019). Correlation between patients' age and cancer immunotherapy efficacy. *Oncoimmunology* 8**,** e1568810.

Wu, X.R., He, X.S., Chen, Y.F., Yuan, R.X., Zeng, Y., Lian, L., Zou, Y.F., Lan, N., Wu, X.J., and Lan, P. (2012). High expression of CD73 as a poor prognostic biomarker in human colorectal cancer. *J Surg Oncol* 106**,** 130-137.

Yegutkin, G.G. (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783**,** 673-694.

Yu, M., Guo, G., Huang, L., Deng, L., Chang, C.-S., Achyut, B.R., Canning, M., Xu, N., Arbab, A.S., Bollag, R.J., Rodriguez, P.C., Mellor, A.L., Shi, H., Munn, D.H., and Cui, Y. (2020). CD73 on cancer-associated fibroblasts enhanced by the A2B-mediated feedforward circuit enforces an immune checkpoint. *Nature Communications* 11**,** 515.

Zanin, R.F., Braganhol, E., Bergamin, L.S., Campesato, L.F., Filho, A.Z., Moreira, J.C., Morrone, F.B., Sévigny, J., Schetinger, M.R., De Souza Wyse, A.T., and Battastini, A.M. (2012). Differential macrophage activation alters the expression profile of NTPDase and ecto-5'-nucleotidase. *PLoS One* 7**,** e31205.

8. Summary of the research activities of the candidate

8.1 Publications

2022 **Turiello R**, Capone M, Morretta E, et al. Exosomal CD73 from serum of melanoma patients suppresses lymphocyte functions and is associated with therapy resistance to anti-PD1 agents. *Journal for ImmunoTherapy of Cancer* 2022*; 10:e004043*

2022 De Marchi E, Pegoraro A, **Turiello R**, Di Virgilio F, Morello S and Adinolfi E. A2A Receptor contributes to tumor progression in P2X7 null mice. Frontiers Cell and Developmental Biology, 2022, accepted.

2021 Antonioli L, Fornai M, Pellegrini C, D'Antongiovanni V, **Turiello R**, Morello S, Haskó G, Blandizzi C. Adenosine signaling in the tumor microenvironment. *Advances in Experimental Medicine and Biology*, 2021, 1270, pp. 145–167

2021 Morello S, Caiazzo E, **Turiello R**, Cicala C. Thrombo-Inflammation: A Focus on NTPDase1/CD39. *Cells* 2021, 10, 2223.

2020 **Turiello R**, Capone M, Giannarelli D, et al. Serum CD73 is a prognostic factor in patients with metastatic melanoma and is associated with response to anti-PD-1 therapy. *Journal for ImmunoTherapy of Cancer* 2020;8:e001689

2020 **Turiello R**, Pinto A, Morello S. CD73: A Promising Biomarker in Cancer Patients. *Frontiers in Pharmacology* 2020;11:609931

2020 Capone M, Fratangelo F, Giannarelli D, Sorrentino C, **Turiello R**, Zanotta S, Galati D, Madonna G, Tuffanelli M, Scarpato L, Grimaldi AM, Esposito A, Azzaro R, Pinto A, Cavalcanti E, Pinto A, Morello S, Ascierto PA. Frequency of circulating CD8+CD73+T cells is associated with survival in nivolumab-treated melanoma patients. *J Transl Med*. 2020 Mar 11;18(1):121

2020 Caiazzo E, Cerqua I, Riemma M.A, **Turiello R**, Ialenti A, Schrader J, Fiume G, Caiazza C, Roviezzo F, Morello S, Cicala C. Exacerbation of Allergic Airway Inflammation in Mice Lacking ECTO-5′-Nucleotidase (CD73). *Frontiers in Pharmacology* 2020;11:589343

2022 De Marchi E, Pegoraro A, Turiello R, Di Virgilio F, Morello S and Adinolfi E. A2A Receptor contributes to tumor progression in P2X7 null mice

8.2 Participation in congresses

9th -10th November 2021

Cluster Science Days

Bonn (Germany)

"Analysis of CD73 expression in human melanoma by multiplexed tissue imaging". Poster presentation

4th – 5th March 2021

Annual Meeting of the Italian Purine Club

Virtual meeting

"Characterization and analysis of circulating CD39 and CD73 in melanoma"

Oral communication

27th January 2020

Annual Meeting of the Italian Purine Club

Ferrara (Italy)

"*Characterization of CD73 in peripheral blood of melanoma patients*".

Oral communication

19th-23rd November 2019

39th Congress of Italian Society of Pharmacology

Firenze (Italy)

"CD73 expression and activity in serum of melanoma patients and healthy donors"

4th – 6th September 2019

1st European Purine Meeting

Santiago de Compostela (Spain)

"*Analysis of CD73 in serum of cancer patients and healthy donors*"

Poster presentation

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