SAPIENZA
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Immune profile of cancer patients to improve selection and efficacy of immunotherapeutic strategies

PhD School in Innovation in immuno-mediated and hematological disorders
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Abstract

Over the years, the clinical outcome of cancer patients has remarkably improved with the introduction of immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs), that by targeting immune system restore an efficient anti-tumor immune response. Despite the potential of immunotherapy as cancer leading treatment, initial response rates with ICIs are however limited and depend on the host pre-existing anti-cancer immunity and the degree of immunosuppression present in the patient. Most of patients fail to respond and to increase the number of responder patients is necessary a more in-depth understanding of the underlying immunity and the identification of biomarkers. In this research project we investigate and characterize the immune system of cancer patients (mRCC, NSCLC, HNSCC, UM) before and during treatment with TKIs or ICIs, in order to investigate the relation between circulating immune profile, tumor microenvironment (TME), the gut microbiome and clinical outcome. The aim was the identification of possible biomarkers/immune profile able to select patients and improve clinical outcome. We assessed immunological analysis to evaluate exhausted/activated circulating T cells by cytofluorimetric assay, 14 immune checkpoint-related proteins and 20 inflammation cytokines/chemokines using Luminex assay. The immunological profile was correlated with survival (PFS and OS), clinical parameters and response to treatment. Gut microbiota composition was evaluated through metagenomic analysis and immunohistochemistry was used to characterize tumor microenvironment.

Our results demonstrated that TKIs and ICIs modulate immune system. We observed a decrease of soluble immune checkpoint molecules in serum of mRCC (sPDL2, sHVEM, sPD1, sGITR) and NSCLC patients (sPD1, sPDL2) during treatment ($p<0.05$). In particular, the decrease of sPD1 and sPDL2 resulted associated with response to treatments ($p=0.03$ and $p=0.01$, respectively). Moreover, the immune profile of responder (R) patients was characterized by low levels of soluble protein (sCTLA4, sPD-L1 in mRCC vs sCD137, sTIM3, sPDL1, sPDL2 in NSCLC) ($p<0.05$) and by a high proportion of eubiosis-associated gut metabolites. These data resulted also associated with better clinical status, PS=0 (performance status).

Profiling circulating immune cells in patients undergoing ICI treatment (anti-PD1) we identified $CD3^+CD137^+$ and $CD3^+CD8^+CD137^+$ T cells that correlate with improved response to therapy. The percentage of both $CD3^+CD137^+$ and $CD3^+CD8^+CD137^+$ T population was higher in R patients

(p=0.03 and p=0.02) and correlated to a better survival in terms of PFS and OS ($p<0.05$). Moreover, R patients had higher levels of $CD3^+CD137^+PD1^+$ and of $CD8^+CD137^+PD1^+$ lymphocytes (p=0.02 and p=0.01), but only $CD3^+CD137^+PD1^+$ resulted associated with a low number of metastasis (p=0.01) and longer survival (OS)(p=0.015). Instead, the high concentration of the immunosuppressive sCD137 in the serum was associated with a lower PFS (p=0.038) and OS (p=0.012). In tumor microenvironment, patients with a complete pathological response showed a high percentage of $CD137^+$ and $CD8^+$ T cells. Results were validated in an independent cohort of metastatic cancer patients.

These results identified immunological parameters that, independently from tumor setting and administered therapy, predict clinical outcome of cancer patients, monitor immune response and help clinicians in the decision-making.

Introduction

Tumor microenvironment as a hallmark of cancer

Cancer is the second leading cause of death worldwide with a high rate of incidence [1]. However, the development of new treatments, progression in cancer screening and prevention, are improving survival rates for many types of tumors.

Cancer is a multi-step process driven by the so called *"hallmarks"* of cancer (Figure 1), a set of functional capabilities acquired by human cells, crucial for their ability to form tumors[2].

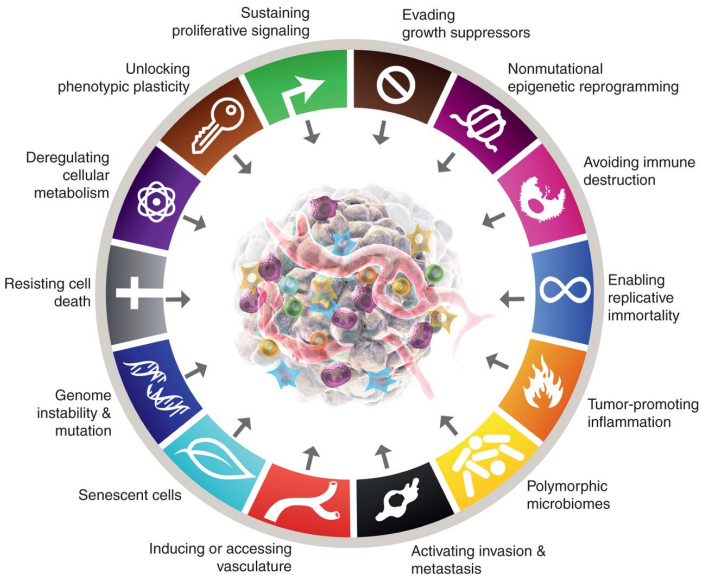

Figure 1: Hallmarks of cancer[2]

Hallmarks and enabling characteristics to explain the complexity of cancer phenotype and genotype

Genetic insults that involve oncogenes and tumor suppressor genes are part of several factors that causes heterogeneity within and between tumors and are known to deregulate the pathways that govern the cell's physiological process like metabolism, proliferation, cellular growth and death [3] [4]. For example, mutation affecting the structure of the oncogene B-Raf, PI3K, Her2, K-RAS and c-Myc as well as that striking the most known tumor suppressor genes, p53 and Rb confer to cancer cells the ability to deregulate the growth-promoting signals required to the cell growth - and-division cycle, resulting in sustained proliferation.

The tumorigenesis process is not solely regulated by genetic alterations within tumor cells but is also critically regulated by the surrounding niche, known as "*tumor microenvironment" (TME),* an heterogenous environment in which several cells interact among them promoting cancer progression. TME is characterized by the presence of several cellular components. Apart from tumor cells it is composed by stromal cells, fibroblasts, tumor endothelial cells, immune cells, and it also includes surrounding blood vessels and the extracellular matrix (ECM). [9,10] In particular, TME resulted infiltrated by immune cells that have a dual role either suppressing tumor growth or promoting it (Figure 2).

All these cells and component interact with each other by a complex networks of cytokines , chemokines, growth factor but even by others mechanism of interaction like circulating tumor cells, exosome or apoptotic bodies that deliver information to distant target. [11]

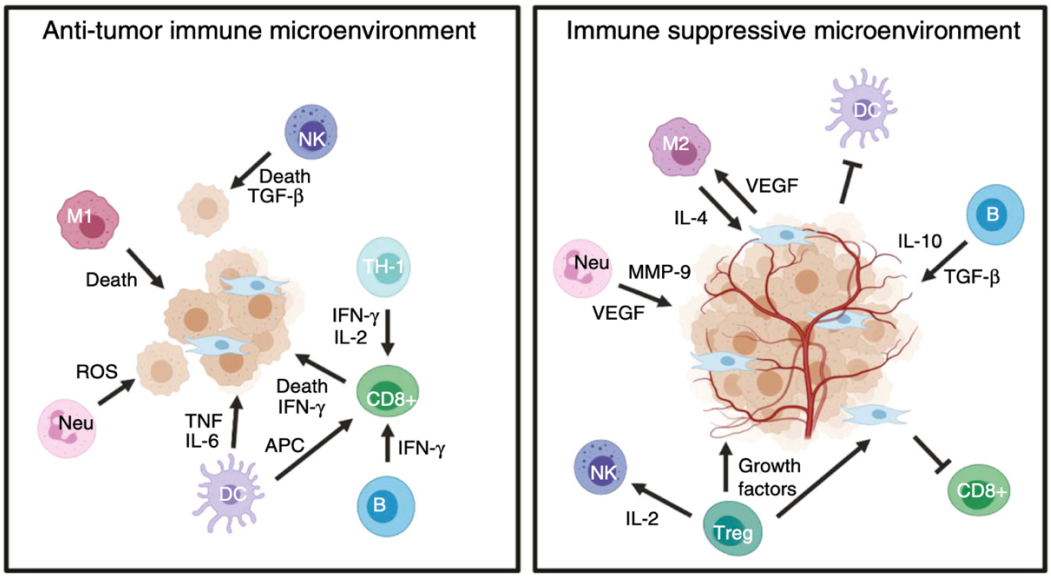

Figure 2. Impact of immune cells within tumor microenvironment [10]

The role of immune cells in the TME can be to either suppress tumor formation or promote tumorigenesis. Depending on context and tumor type, immune cells can be either pro- or anti-tumorigenic. M1=TAM M1; M2= TAM M2; Neu= neutrophils; DC= dendritic cells; NK= natural killer; T_{reg}= T regulatory cells;

Stomal cells and fibroblasts can secrete in TME growth factors like FGF, HGF and some cytokines like CXCL12 both promoting growth and survival of cancerous cells both stimulating the recruiting of other cells into TME[12].

Tumor cells can release several soluble factors like transforming growth factor (TGFβ) or tumor necrosis factor (TNF-α) that remodel the Extracellular matrix. In turn, TGFβ stimulates the

differentiation of fibroblast in cancer associated fibroblast (CAFs), the most abundant cells in the stroma, that by secreting many types of cytokines like vascular endothelial growth factors (VEGFA), CXCL12, IL-6, they produce ECM protein and as a result, they contribute to a tumor-friendly microenvironment that influences tumor growth and progression, especially invasion and metastasis.

TME is also conditioned by physiological changes such as hypoxia, low pH, nutrient deprivation, and production of immunosuppressive metabolites that could also impede anti-tumor immunity[15].

Tumor promotes angiogenesis to restore oxygen and nutrient supply and remove metabolic waste. Low oxygen tension (pO2) leads to the activation of hypoxia-inducible factor (HIF1) that stimulates endothelial cells to overproduce VEGF, that together with other molecules like Angiopoietin 1 and angiopoietin 2 (Ang 1/2) plays a pivotal role in promoting angiogenesis. Angiogenic factors stimulate endothelial cells to produce matrix metalloproteinases (MMP) that degrade the extracellular matrix (ECM) allows endothelial cells to migrate, proliferate and form new blood vessels.

Besides hypoxic conditions, VEGF is produced and secreted by tumor cells and surrounding stroma and is associated with tumor progression, effects on vascular permeability, invasiveness, and metastasis. It orchestrates the proliferation and spread of endothelial cells through the interaction with the tyrosine kinase receptors VEGFR-1 and VEGFR-2 (vascular endothelial growth factor receptor) expressed on endothelial cells. This binding is followed by receptors dimerization and phosphorylation, and allows the recruitment of specific downstream mediators promoting tumor angiogenesis. [8]

In addition to angiogenesis, VEGF exerts immunosuppressive functions. It creates a pro-tumor microenvironment by increasing the number and enhancing the suppressive functions of T_{reg} and TAMs. VEGF inhibits DCs maturation and antigens presentation, as well as inhibits CTL trafficking, proliferation, and effector function[16,17]. (Fig. 3)

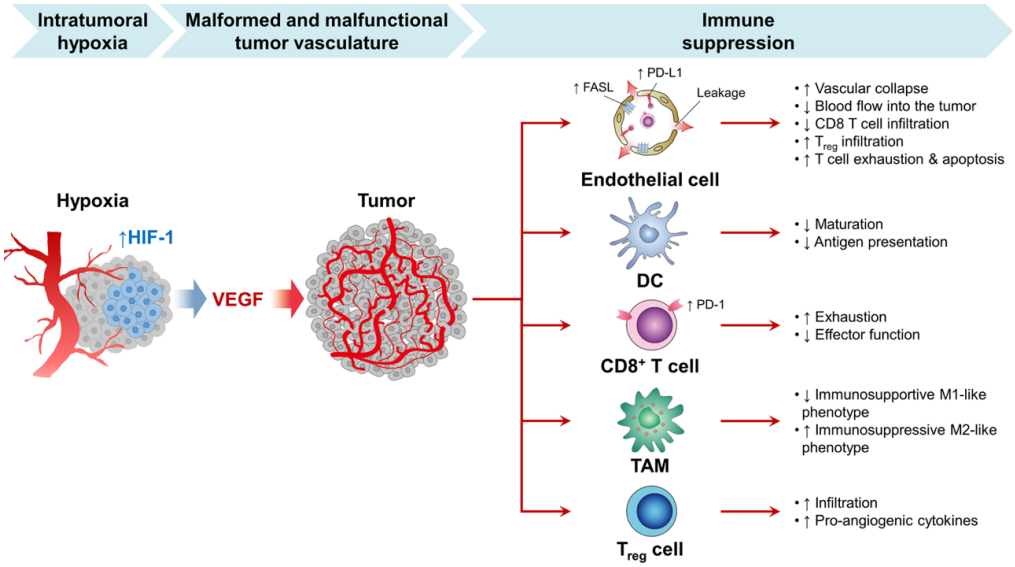

Figure 3. The abnormal tumor vasculature elicits immune suppression in the tumor microenvironment[17]

Hypoxia and acidosis in TME promote immune suppressive mechanism. Hypoxia stimulates HIF-1 thereby upregulates VEGF that in turn induces tumor angiogenesis and modulates the functions of innate and adaptative immune cells towards immunosuppression.

Furthermore, recent evidences have pointed out the role in tumor growth and spread of an unexpected player: the *gut microbiota*, recently added as an hallmarks of cancer (Figure 1) [2].

Its complexity and behavior deserve the definition of *tissue organ,* an important immunological organ that influences different pathways of whole metabolism.

The entire human body (skin, gut, or other mucosa) is colonized by trillions of different microbes which interact with the host maintaining its physiology and health and influencing basic function, such as metabolism, nutrition, immunomodulation, and pathogen resistance.

Regarding the immunity, it plays a fundamental role in development, function, maintenance of the host immune system. It shapes immune system, and these changes lately affect the immune system it-self. For example, gut microbiota is crucial in reducing neutrophils number. Moreover, the inosine, a gut microbiota metabolite, promotes the differentiation of TH1 cells as well as the short chain fatty acids (SCFA) activate DC cells and macrophages (Figure 4).

In addition to pathogen defense, to maintenance of intestinal ecosystem, to immunity and others physiological processes, gut microbiota plays a pivotal role in the pathogenesis of disease [19].

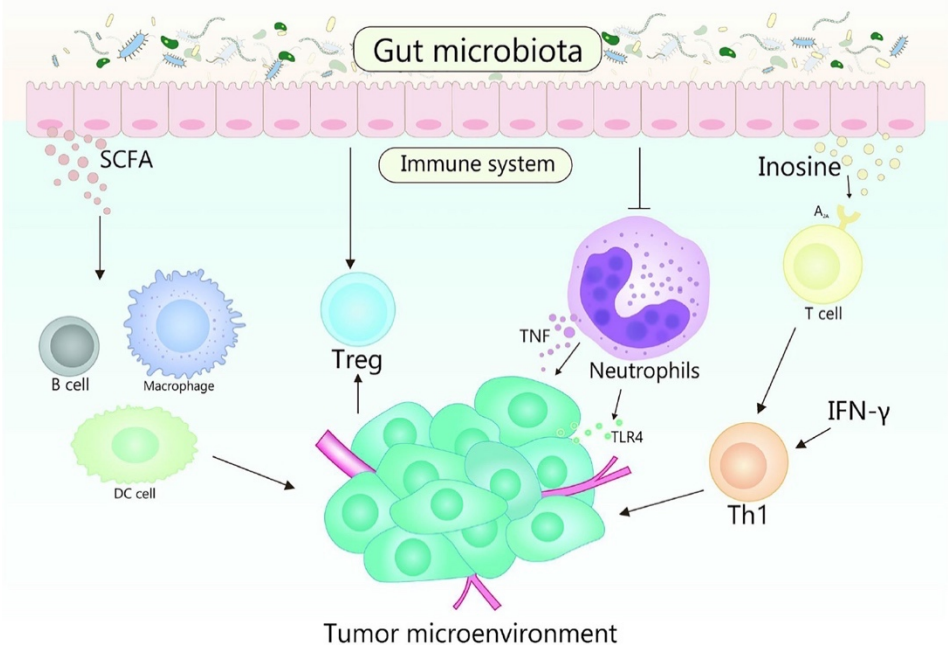

Figure 4: Gut microbiota and its metabolite act on the host immune system [18]

Gut microbiota and its metabolites influence the shaping of the TME. TLR4 signaling in tumor cells can recruit neutrophils, while TNF released by neutrophils induce metastasis of tumor cells. Gut microbiota reduces the number of neutrophils, which plays a promoting role in the treatment of tumors. Gut microbiota metabolite inosine significantly promotes the differentiation of Th1 cells, while SCFA can regulate the production of cytokines, activate DC cells and macrophages, and affect the differentiation of memory T cells, which also plays an important role in cancer therapy.

Alterations in composition and function of gut microbiota are referred to as dysbiosis and may increase metabolic disorders and the abundance of inflammation-inducing bacteria, which can induce carcinogenesis. Indeed, a broad effect of polymorphic microbiomes involves the modulation of the adaptive and innate immune systems through several routes. Bacteria produce immunomodulatory factors that activate damage sensors on epithelial or resident immune cells. Moreover, can intact protective biofilm and the mucus lining the colonic epithelia, disrupting the epithelial cell-cell tight junctions that maintain the integrity of the physical barrier that normally compartmentalizes the intestinal microbiome. Once invaded the stroma, bacteria can trigger both innate and adaptative immune response that secrete a repertoire of cytokines and chemokines.

One manifestation can be the creation of tumor-promoting or tumor-suppressing immune microenvironments, consequently protecting against or facilitating tumorigenesis.[2]

The complex network among all these factors orchestrates tumor progression.

Immune system and cancer: cell cycle immunity and immunoediting

Immune system involves a complex network of chemical and molecular signals in which specialized cells and tissue have the potential to control the homeostasis and to defend the host from chemical, traumatic, and infectious insults, such as bacteria, viruses but also transformed cells. To fight foreign pathogens and tumor cells, immune system has two arms: innate and adaptative immunity. (Figure 5)

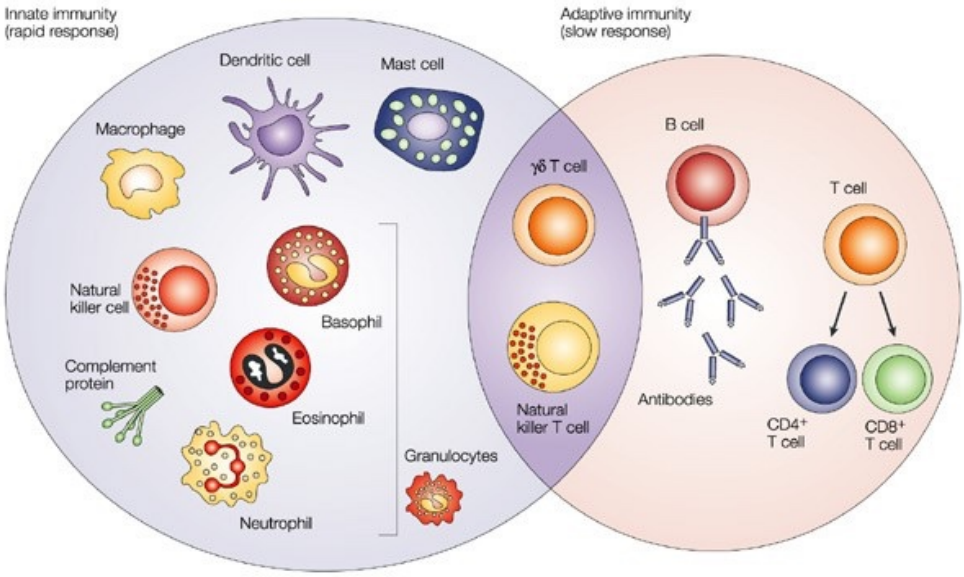

Figure 5: The innate and adaptative immune response[20]

The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and several cellular components including granulocytes, macrophages, dendritic cells, and natural killer cells. The adaptive immune response is slower to develop but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes.

During tumor development and progression, the host' immune system reacts by generating a series of stepwise anti-cancer immune mechanisms termed as the "cancer-immunity" cycle. [22] (Figure 6)

It is defined as a series of functional steps needed to provide control of cancer growth and generate a protective anti-cancer response by the immune system.

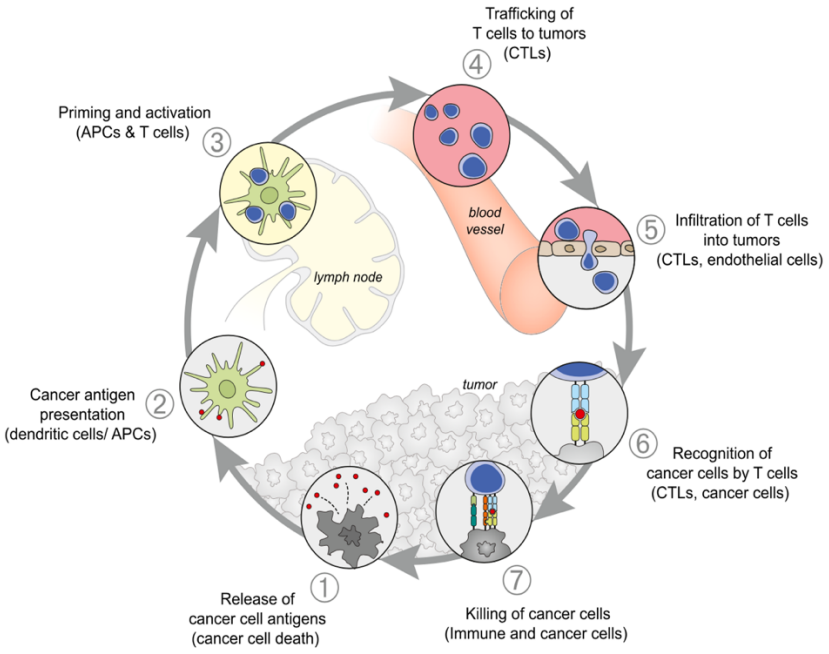

Figure 6: Cancer-immunity cycle[22]

The generation cancer immunity is a cyclic process that is characterized by immune-stimulatory factors that should amplify T cell responses but also inhibitory factors that lead to immune regulatory feedback mechanisms, which can limit the immunity. This cycle can be divided into seven major steps, starting with the release of antigens from the cancer cell and ending with the killing of cancer cells.

The process initiates with the production of neoantigens generated by genomic instability (I). This tumor-associated antigens (TAAs) are captured by dendritic cells and presented on MHC I or MHC II molecule (II). DCs migrate away from TME and go towards lymph nodes. Here tumor-specific cytolytic $CD8^+$ T cells recognize TAAs presented on MHC-I through their TCR (III). Once activated, T cells migrate and infiltrate TME (IV-V) where can recognize the specific antigen and eliminate tumor cells. (VI-VII). After killing, tumor cells release additional tumor-associated antigens amplifying the subsequent round of cancer immunity cycle. [22]

Adaptative immune cells, together with cells of innate immunity are predominant in TME and act by identifying and eliminating mutated or abnormal cells thanks to a process called "*immunosurveillance*"[26].

Immune cells had a role in protecting the host from microbial pathogens, and although is still controversial, it has been recognized that immune system can also control tumor.

So, in this way, to describe the dual function of immune system both in protecting and remodeling tumor, the term immunosurveillance loses its effectiveness. The concept evolves into that of

immunoediting, a dynamic process consisting of three phases: elimination, equilibrium, escape such are termed the "three Es of cancer immunoediting" (Figure 7) [26].

Elimination is a consequence of immunosurveillance. During this phase malignant or potentially malignant cells can be identified and eliminated by cells of both innate and adaptative immune system [27]. Cells present in TME together with tumor cells produce and release pro-inflammatory cytokines like IL-12, IFN-γ, IFN-αβ necessary for recruit and activate immune system cells such as T lymphocytes, NKT, NK and DCs in tumor site. These immune cells exert several effector mechanisms to eliminate tumor cells, releasing IFN-γ that controls tumor growth and amplifies immune responses through the production of chemokines. In return, chemokines attract more immune cells to the tumor site.

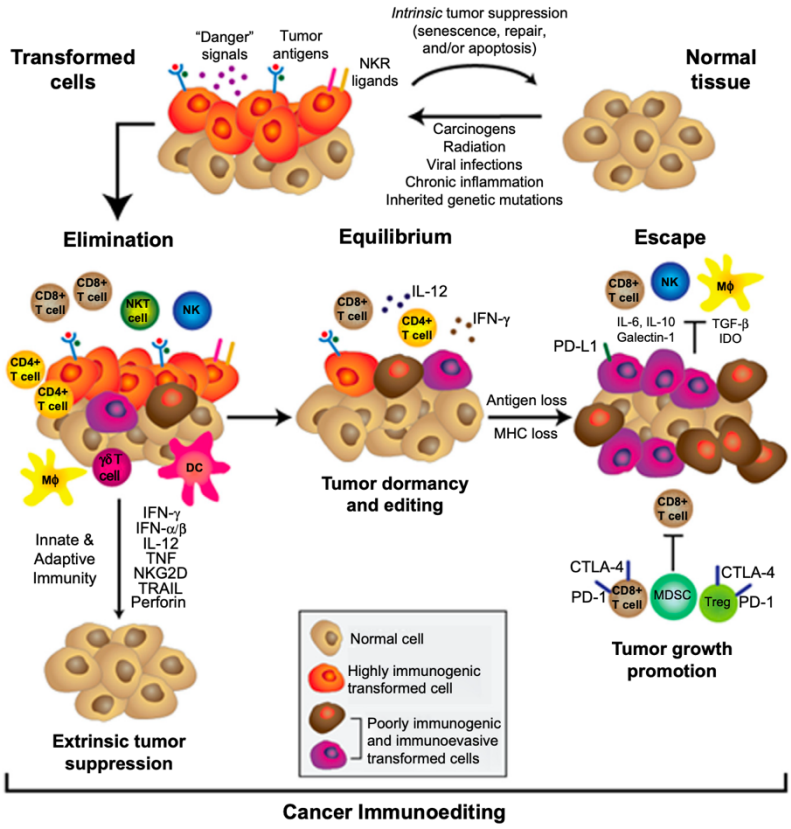

Figure 7: Cancer immunoediting [26]

Cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape.

Although the elimination phase contributes to destruction of transformed cells, there are cells with reduced immunogenicity that escape control and enter a period of latency that constitutes the

equilibrium phase [28]. During this second phase the elimination of tumor cells occurs accompanied by the simultaneous production of new tumor variants with an increased resistance.

This is the longest of three phases and may occur over a period of many years. Tumor cells that cannot be recognized and eliminated by immune system progress in the third and last phase, the *escape,* which is acknowledge as one of hallmarks of cancer. Further mutations occur in malignant cells and lead to progression of tumor that grows and becomes clinically detectable.

Several studies demonstrated that tumors avoid the immune system through direct or/and indirect mechanism like lack of costimulatory molecules, defects developed by tumor cells in antigen processing and presentation or tolerance of T cells to tumor antigens, presence of immunosuppressive cells (Treg, MDSCs), suppression of T cells caused by tumor-derived factors (TGF-β, IL-10). Another fundamental immune escape mechanism used by cancer cells is the ability to exploit immune checkpoint (ICs) molecules expressed on T cells. Immune checkpoint are cell surface inhibitory molecules that inhibit T cells hyperactivation, maintaining self-tolerance and preventing autoimmunity. The most famous are programmed cell death-1 (PD1) and cytotoxic T – Lymphocyte antigen – 4 (CTLA4). In particular, PD1 recognizes its ligand PDL1 or PDL2 expressed on APCs, while CTLA-4 binds the ligands CD80 and CD86, thereby inducing T lymphocyte anergy and exhaustion both in the lymphoid tissue and in the periphery [25]. Tumor cells, by expressing immune checkpoint ligands prevent T cells activation and avoid anti-tumor immune response (Figure 8). Besides ICs with inhibitory functions, T cells express on their surface also costimulatory receptors that play a central role in T-cell priming and activation as well as in modulating T-cell differentiation, proliferation, and effector function. The most known is CD137 (4-1BB, TNFRSF), a member of tumor necrosis factor receptors (TNFR) family. It has costimulatory function expressed by $CD8^+$ and $CD4^+$ T cells upon activation, by NKs, DCs, eosinophils and vascular endothelium cell. The engagement with its ligand, CD137L, expresses on APC, is capable of increasing T-cell survival, proliferation, and cytokine production. Within cancer landscape, CD137 has generated great interest because it identifies antigen-specific T cells that kill tumor cell upon activation. Tumor cells by expressing CD137L, also in this case block T cells activation, avoiding anti-tumor immune response.

The interplay between host immune system, tumor cells and tumor microenvironment have a key role in cancer progression. An in-depth understanding of this dynamic process has allowed the development of appropriate therapeutic strategies.

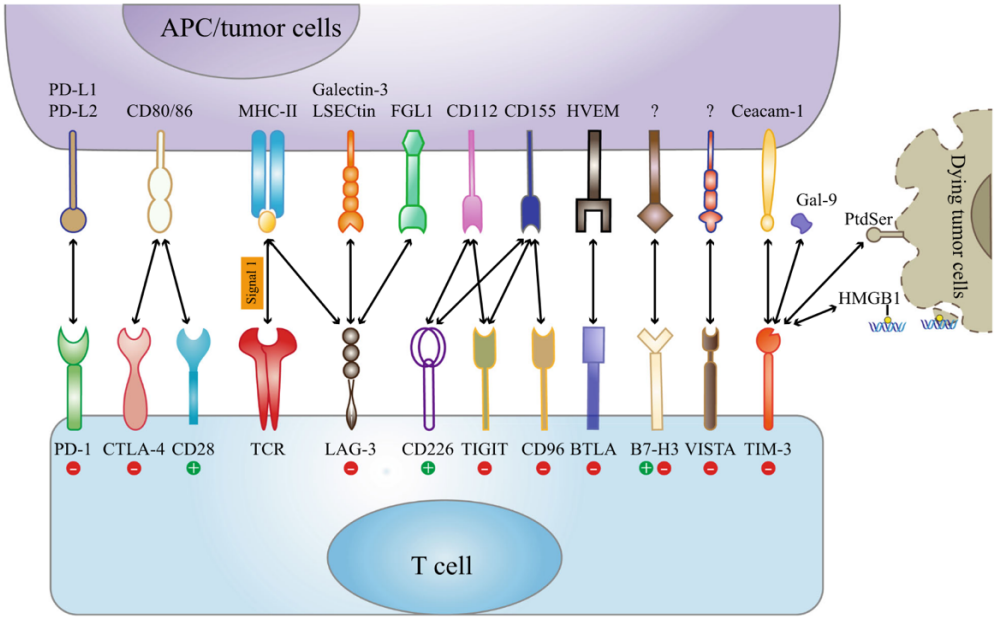

Figure 8. Current and emerging immune checkpoint receptors and their respective ligands[25]

Various immune checkpoint molecules expressed on T cells were shown with their ligands. Immune checkpoints such as PD-1, CTLA-4, LAG-3, TIM-3, TIGIT bound with their respective ligands on APCs and/or tumor cells, triggering a negative or positive signal to T cells response

The evolving landscape of cancer treatment: immunotherapy and target therapy

The backbone of cancer treatment has traditionally included surgery, radiotherapy, and chemotherapy that are still part of current therapeutic approaches.

They are designed to kill cancer cells by compromising cellular integrity during division, inhibiting the growth and proliferation of tumor cells but, despite the anti-tumor function, they also display side effect. In particular, chemotherapy has the inability to distinguish cancer cells from normal cells inducing toxicities and adverse reactions. [29]

Anyway, over the years thanks to a better understanding of tumor biology, to a comprehension of tumor microenvironment and to the contribution of immune system, therapeutic landscape has been drastically evolved.

The scenario shifted from cytotoxic drugs to targeted drugs that differently from the first one can specifically target tumor cells and save normal cells, hence having high efficacy and low toxicity.

Targeted drugs can be classified in small molecules and immunotherapy, both interact with specific protein inhibiting cancer cells proliferation and progression, and with the aim to re-activate anti-cancer immunity. *Small molecules*, because of their small size, target extracellular receptors as well as intracellular proteins involved in transducing downstream signaling in the pathway of tumor growth and metastasis proliferation. For instance, Palbociclib and ribociclib are two cyclin – dependent kinase inhibitors (CDKi) used in metastatic breast cancer therapy that block protein involved in cell cycle, thereby inhibiting cancer cells proliferation and tumor progression. [30] Pazopanib and Sunitinib are tyrosine kinase inhibitors used in treatment of metastatic renal cell carcinoma which inhibit the tyrosine kinases receptors associated with the molecular pathways of VEGF and PDGF, both implicated in angiogenesis and tumor growth.[31] (Figure 9)

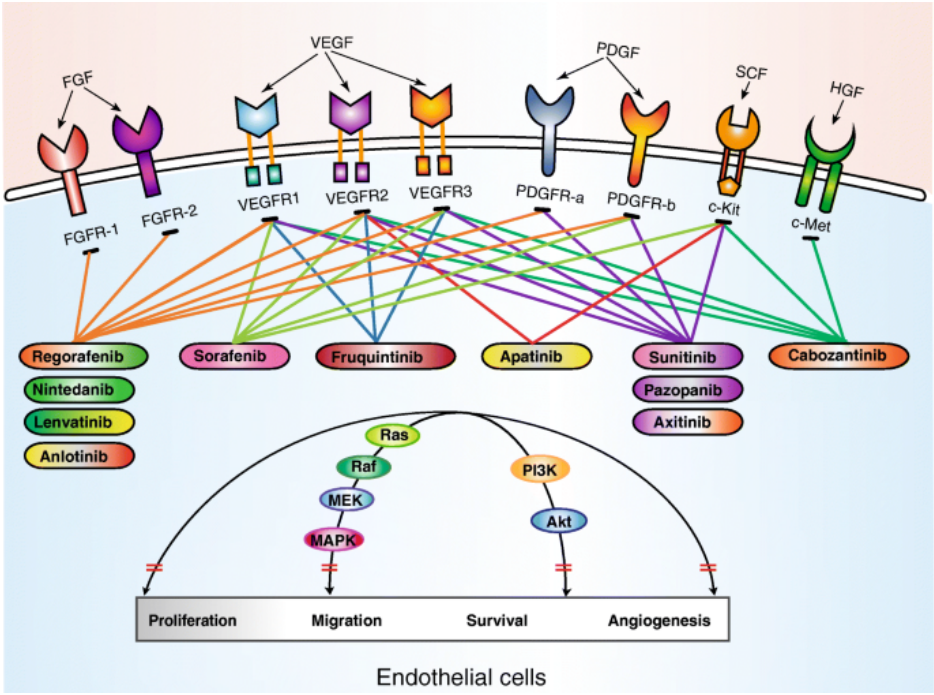

Figure 9: Main targets of approved tyrosine kinase inhibitors (TKIs)[32]

Anti-angiogenic TKIs can target multiple receptor sites simultaneously. The main targets included vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), c-Kit, and c-Met. Anti-angiogenic TKIs block the kinase activity of receptor and transduction of downstream signal involved in the proliferation, migration, and survival

Taken together, small molecules can inhibit proliferation, survival, progression in the cell cycle, angiogenesis, and migration of tumor cells.[32]

Immunotherapy includes approaches that overcome tumors by using immune system. Examples include checkpoint blockade, cancer vaccines, adoptive T cell therapies (ATC). In ATC therapy TILs are isolated from a cancer biopsy, expanded, and re-inoculate into patients. Immune checkpoint inhibitors (ICI) instead are monoclonal antibodies that target the immune checkpoints like PD1 and CTLA-4, express on T cells impeding the binding with their ligands, respectively PD-L1 and CD80/CD86 express on tumor cells. Abrogating this negative regulation, they restore T cells effector function and proliferation. Several ICI are approved by FDA and the most know are directed to PD1/PD-L1 axis or CTLA4/CD80-CD86 axis (Figure 10).

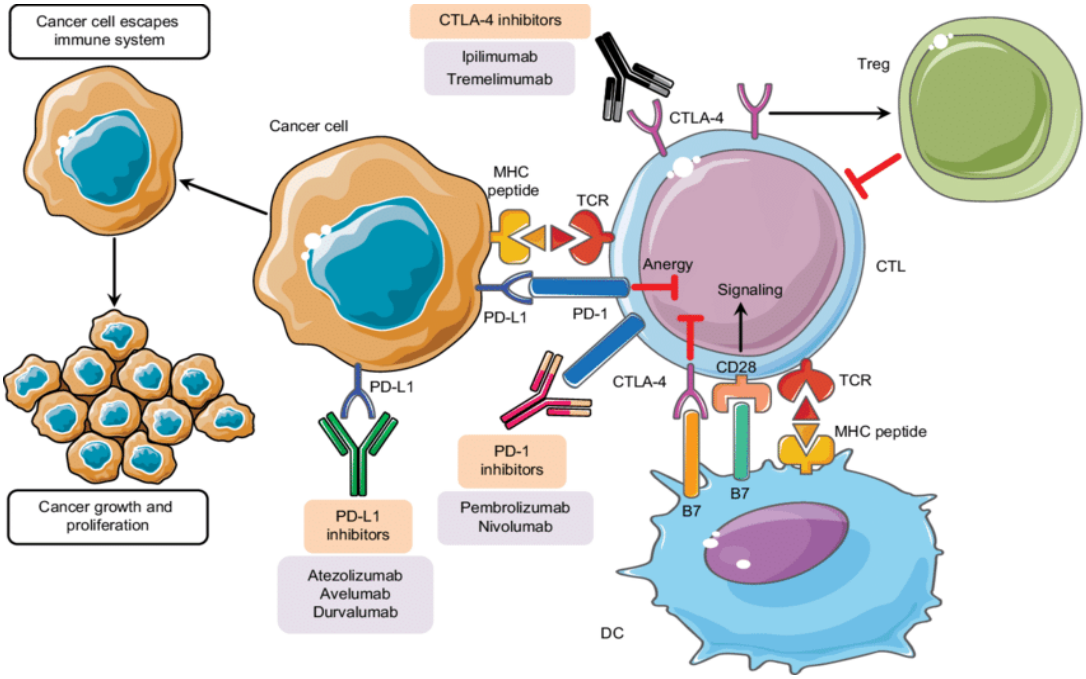

Figure 10: Immune checkpoint inhibitors in cancer treatment [33]

Inability to activate CTLs in tumor microenvironment through the immune checkpoints allows cancer cells to escape immune attack, survive, and grow. Pharmacological inhibition of immune checkpoints with monoclonal antibodies restores CTL antitumor activity and relieves immunosuppression

Pembrolizumab and Nivolumab are anti-PD1 antibodies and was approved for non-small cell lung cancer (NSCLC), renal cell cancer and head and neck cancer. Ipilimumab, an anti-CTLA4 is used in metastatic melanoma treatment.

Although immunotherapy has notably improved patients' survival, not all patients benefit from therapy and most experienced immune related adverse events. [24] The synergy between tumor cells and immune cells contributes to mechanisms of tumor evasion and progression, inducing resistance to therapy in individual patients. To overcome these limitations there is a need to search for predictive biomarkers to stratify patients into responders and non-responders and to determine the outcome of a therapy before starting it. In addition, these biomarkers can indicate where and when a patient may benefit from monotherapy or combination therapy, while also reducing side effects [34].

Biomarkers and the importance of immune profiling

Despite the encouraging progresses made in cancer treatment, a large part of patients does not benefit from therapy. The interplay between tumor, TME and immune cells has been widely explored with the purpose to identify immune biomarkers capable of predicting clinical response. These biomarkers are biomolecules produced either by tumor cells and by other cells in response to tumor and they could be a tool of diagnosis, prognosis, and prediction.

To date tumor mutation burden (TMB) and PD-L1 expression on immune and tumor cells are validated factors useful for the qualification of cancer patients for immunotherapy.

The high number of mutations in somatic cells (TMB) causes an elevated numbers of neoantigens, which translates into increased immunogenicity of tumors. Several studies demonstrated that tumor types with high TMB are associated with a better PFS or OS. [35] Similarly, neoantigens presented on the surface of tumor cells by MHC and then recognized by T cells may be considered a useful biomarker to predict patient response to cancer immunotherapy. [36]

PD-L1 expression on numerous tumor cells is a strategy to evade immune response and may play an important role in suppress T cell. The anti-PD1 antibody inhibits the PD1/PD-L1 axis stimulating tumor reactive T lymphocytes to kill cancer cells. Several studies reported better results in patients with a positive PD-L1 expression. For instance, in a multi tumor study (melanoma, NSCLC, RCC, colorectal and prostate cancer), patients with PD-L1 positive tumor exhibited a significant objective response compared to PD-L1 negative tumors. Similarly, in NSCLC patients with a PD-L1 expression >50% was registered a better efficacy of Pembrolizumab treatment[37].

However, other studies reported opposite results, showing a clinical benefit to anti-PD1 therapy in patients with low or negative expression of PD-L1[38]. This controversy could be explained by a spatial and temporal heterogeneity of PD-L1 expression, it varies substantially across different anatomic sites and during clinical course. Moreover, it may depend also on different definition of PD-L1 expression evaluated either on tumor than immune cells. [36]

In TME the presence of tumor infiltrating lymphocytes (TILs), a mixture of cytotoxic T cells and helper T cells, as well as B cells, macrophages, NK and DCs) provides a successful control of tumor progression. This concept is reflected in three different phenotypes that influence therapy efficacy: immune-desert, immune-excluded, and immune-inflamed phenotypes[39] (Figure 11). The immune-

desert is a non-inflamed tumor characterized by the absence of TILs, a poor expression of PD-L1 and a low mutational burden. It is a no reactive TME permissive to tumor progression and unresponsive to immunotherapy. Immune-excluded phenotype is always part of non-inflamed tumor but there is a mild infiltrate of T cells that remained confined surrounding the tumor mass. In the immune-inflamed, the scenario radically changes in favor of a more conspicuous number of TILs in the parenchyma, a high mutational load, several cytokines (like IFN, IL-12, IL1β) which provide a more favorable environment for T cells activation and expansion. Moreover, T lymphocytes express on their surface several immune checkpoints including PD-L1, LAG-3 and Tim-3 that leads to T cells exhaustion. This profile suggests that an anti-tumor immune response is present but is hampered by the tumor and it's necessary reactivate it.

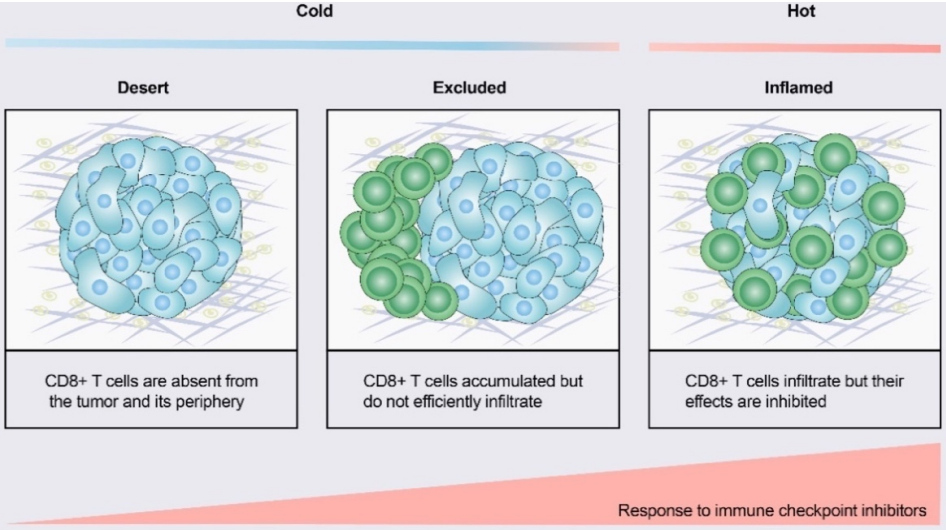

Figure 11: Tumor immune phenotypes [40]

These phenotypes expand the concept of "cold" vs "hot" tumors and reflect the infiltration status of immune cells, predominantly T lymphocytes Based on the spatial distribution of $CD8^+$ T lymphocytes in the tumor microenvironment (TME), a gradient of three immunophenotypes is observed: the immune-desert, immune-excluded and immune-inflamed phenotypes. The three different phenotypes have different response rates to immune checkpoint inhibitors.

TILs role as prognostic biomarkers has been largely studied. The presence of $CD8^+$ T cells in TME is associated with a better prognosis in melanoma or NSCLC [41]. A study conducted on patients with advanced melanoma showed that before starting Pembrolizumab therapy responder patients had higher $CD8^+$ T cells densities at both the invasive margin and tumor center respect to non-responder. In addition , an increase in the intratumoral $CD8^+$ densities during therapy was

associated with a reduction in tumor size, indicating that pre-existing immune response ameliorate treatment efficacy.[42]

Quantify the $CD3^+$ and $CD8^+$ expression within tumor and its invasive margin, provides the introduction of *Immunoscore* as a valid biomarker to predict treatment response and prognosis.

Biomarker's identification in tumor site requires invasive procedure like tumor tissue biopsy or surgical removal. Sometimes the amount of tissue is not sufficient because of difficult to access due to the tumor's anatomical site. To overcome this problem, peripheral blood marker can be used as with multiple advantages, limited costs, non-invasive procedures that monitor not only biochemical changes, but also variation in frequencies of immune cells during treatment.

Peripheral blood samples evaluate different blood components such as lymphocytes, neutrophils, monocytes, eosinophils, myelogenous suppressor cells. High lymphocyte and low neutrophil counts were associated with a good prognosis in cancer patients [43,44]. Consequently, the high neutrophil-to-lymphocyte ratio (NLR) has been shown to be associated with poor response in advanced cancers.

In the blood could be detected also the immunosuppressives population *Tregs and MDSCs* as well as it could be evaluated the expression of inhibitory receptors, such as PD1, CTLA4, Tim3, on T lymphocytes. It has been reported that a high percentage of this markers characterized an exhausted T lymphocytes with reduced proliferation and effector functions.

Immune checkpoint receptors could exist as soluble form. These soluble proteins can be produced by a proteolytic cleavage of membrane receptors, as an alternative variants of mRNA splicing that lack the transmembrane domain, or they can be included on exosome or microvesicles and released in circulation[45]. Their functions are not completely elucidated, but mounting evidence demonstrated that they seem to play a crucial role in immune regulation[46]. It was demonstrated that high serum levels of several IC proteins correlate with resistance to immunotherapy in melanoma patients[47]. A study conducted on mRCC patients showed that high baseline value of sPDL1 and sPD1 predict response with Nivolumab treatment[48]. sICs could be considered as prognostic biomarker for tumour progression or predictive for response to therapy[46].

In the variety of biomarkers should be included those host-related such as gender, age, body fat distribution, but also intestinal commensal microbiota[49].

The microbiome plays an important role in the maintenance of host metabolism and immune system and its role in cancer has attracted much interest. Metagenome analysis showed a significant difference in the gut microbiota composition between healthy donors and cancer patients. In patients with colorectal cancer there was a decrease in microbial diversity and an increase in the presence of fusobacterium nucleatum, a common resident in oral microbiome , but rarely found in healthy gut[50]. A high abundance of this bacterium is associated with regional lymph node metastasis and shorter survival[51].

Biomarker's landscape is widespread but their predictive and prognostic abilities have not yet been validated (Figure 12). To dissect and predict anti-cancer immune response, it's crucial to not only monitor TME, the frequency and phenotype of tumor-infiltrating cells, but also monitor circulating immune cells as well as soluble immune checkpoint molecules and cytokines or any other molecules involved in anti-tumor immune response correlating them with survival and clinical parameters. Moreover, using a single biomarker could not be an appropriate strategy due to heterogeneity of tumor, TME and immune system. Therefore, a comprehensive and dynamic assessment of tumor immunity is essential for a successful anti-cancer therapy.

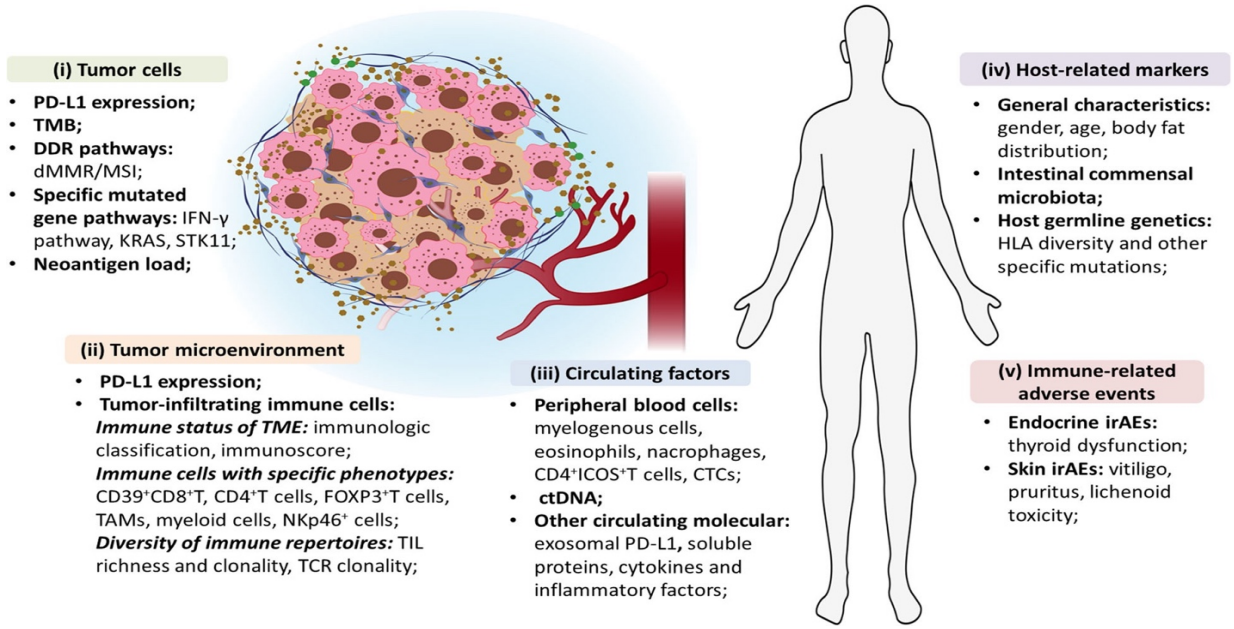

Figure 12: An overview of predictive biomarkers for immune checkpoint inhibitors efficacy.[49]
Key elements in predictive biomarker development for the efficacy of immune checkpoint inhibitors therapy are briefly described in the figure, including tumor cells-related biomarkers, tumor immune microenvironment phenotype biomarkers, circulating factors, host-related factors, and immune-related adverse events

Aim of Project

In this project we characterized patients' immune profile to investigate the role of immune system before and during immunotherapeutic interventions (TKIs or ICIs) and to identify possible prognostic and predictive biomarkers able to improve patients' selection.

Consequently, this project has involved immunological analysis on peripheral blood of cancer patients evaluating exhausted/activated circulating T cells, immune checkpoint-related proteins and inflammation cytokines/chemokines and correlating the immunological status of patients with different clinical parameters.

Material and methods

Patients' enrollment

This study prospectively enrolled 129 patients with a confirmed diagnosis of no squamous cell lung carcinoma (NSCLC) (51 patients), metastatic renal cell carcinoma (mRCC) (28 patients), Head and neck squamous cell carcinoma (HNSCC) (36 patients) and uveal melanoma (UM) (14 patients) at the Medical Oncology Department of Policlinico Umberto I Hospital, Azienda Ospedaliera S. Andrea and Fondazione Policlinico Universitario Agostino Gemelli IRCCS between April 2017 and August 2021.

20 out of 28 mRCC patients were treated in first line with TKIs. The remaining 109 out of 129 patients underwent treatment with single-agent anti-PD1 as the first or second line of treatment. Patients were treated according to the tumor type with standard dose of TKI (sunitinib or pazopanib every 3 weeks) or Immunotherapy (Nivolumab or Pembrolizumab every 2 weeks) and scheduled until disease progression or unacceptable toxicity. Toxicity was reported according to Common Terminology Criteria for Adverse Events (version 4.0) and was evaluated on day 1 of every cycle until the end of treatment. Criteria of inclusion were: age > 18 years, histologically-documented diagnosis of NSCLS, RCC, HNSCC and UM, adequate cardiac, pulmonary, renal, liver and bone marrow function, EOCG Performance Status (PS) scored between 0-2. Criteria of exclusion were: autoimmune disease, systemic immunosuppression and any significant comorbidity.

Performance status (PS) defines the functional status of a patient. Patients scored as PS=0 are fully active, able to carry on all pre-disease performance without restriction. Patients with PS=1 are restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature. Patients scored as PS=2 are ambulatory and capable of all self-care patients, but are unable to carry out any work activities; up and about more than 50% of waking hours [52].

PFS, OS and clinical response rate were evaluated. PFS was defined as the time from the start of therapy (TKI or immunotherapy) until the first documented tumor progression or death from any cause. OS was defined as the interval between the beginning of treatments (immunotherapy or TKi) (OS) or tumor diagnosis (OStot) to death for any case. The response was assessed every 4 weeks until disease progression using immune-related Response Evaluation Criteria in Solid

Tumors (i-RECIST) and classified as an incomplete and partial response, stable and progressive disease. The Clinical Response Rate was used to classified patients in Responders (patients with a complete, partial response and stable disease) and not-responders (progressors) after 6 months of therapy. The study was conducted in accordance with the Declaration of Helsinki and with good clinical practice guidelines. All patients signed informed consent. The Institutional Ethics Committee of the three involved institutions agreed to the final version of the protocol (RIF.CE: 4181).

Isolation of PBMCs (peripheral blood mononuclear cells) and serum collection

Peripheral blood samples of 129 cancer patients and 20 healthy donors were collected into EDTA anticoagulant tubes (BD vacutainer) and processed within 1 hour after blood sampling to collect PBMCs.

Blood samples, diluted with an equal volume of PBS were layered onto Ficoll-Hypaque gradient separation (1077 g/mL; Pharmacia LKB), centrifuged at 1800 rpm for 30 minutes without brake. PBMCs were collected and cryopreserved until use. Concurrently, serum of cancer patients was collected using BD Vacutainer Plus Plastic Serum tubes (Becton Dickinson, NJ, USA) after centrifugation at 1800 rpm for 10 min and stored at -80°C.

In cancer patients, blood samples were collected at baseline (T0) in 129 patients (51 NSCLC, 28 mRCC, 36 HNSCC and 14 UM patients) and in 42 patients at the first clinical evaluation (22 NSCLC, 20 mRCC,) performed after 3 months from the beginning of treatment (>T0) according to the research plan (Table 1).

Table 1: Samples collection and patient's therapy

Histotype	Therapy	Blood samples		Fecal samples
		T0	>T0	T0
mRCC	TKIs	20	20	/
	Anti-PD1	8	/	/
NSCLC	Anti-PD1	51	22	11
HNSCC	Anti-PD1	36	/	/
UM	Anti-PD1	14	/	/

Fecal samples collection

Fecal samples derived from 11 NSCLC were collected at baseline (T0) and stored at -20°C until use.

Immunophenotyping

Cell immunophenotype was carried out by cytofluorimetry using a multi-parametric analysis combining the following conjugated anti-human monoclonal antibodies (MoAbs): anti- CD3 BV510 (clone HIT3a), anti-CD8 APCH7 (clone SK1), anti- CD137 APC (clone 4B4-1), anti-KI67 Pecy7 (clone B56), anti-PD1 BB700 (clone EH12.1).

Cell autofluorescence and the fluorescence minus one (FMO) were used as negative controls. Flow cytometric acquisition was performed using FACSCantoII flow cytometer running FACS Diva data acquisition. FACS DIVA analysis software and FlowJo were used to analyze data (BD Biosciences).

All Abs were purchased by BD Biosciences.

Immunohistochemistry

Immunohistochemistry was performed on paraffin slides representative of each tumor with the Leica Bond 3 auto Stainer, using the primary antibodies to CD4 (4B12), CD8 (4B11), CD20 (L26), CD21 (2G9), CD23 (1B12), CD3 (LN10) all purchased by Leica Biosystems, (Wetzlar, Germany) and CD137 (ab197942, Abcam, Cambridge, UK). The signal was obtained with Bond Polymer Refine detection that contains peroxide block, post primary, polymer reagent, DAB chromogen (brown signal) and Hematoxylin counterstain. The section was dehydrated and mounted. Tertiary lymphoid structures (TLS) characterization was determined based on cellular marker composition (CD20, CD21, CD23 and CD3)[53,54].

Detection of inflammatory cytokines, chemokines, and soluble immune checkpoints in serum

Serum derived from all different cancer patients was used to evaluate the levels of 34 circulating immune molecules by multiplex assay.

The concentration of cytokines, chemokines and, soluble immune checkpoints was measured using the ProcartaPlex Human Inflammation Panel (20 Plex, catalog number EPX200-12185-901; sE-Selectin; GM-CSF; ICAM-1/CD54; IFN alpha; IFN gamma; IL-1 alpha; IL-1 beta; IL-4; IL-6; IL-8; IL-10; IL-12p70; IL-13; IL-17A/CTLA-8; IP-10/CXCL10; MCP-1/CCL2; MIP-1alpha/CCL3; MIP-1 beta/CCL4; sP-Selectin; TNF alpha) (eBioscence, Vienna, Austria) and the Human Immuno-Oncology Checkpoint 14-Plex ProcartaPlex Panel 1 (catalog number EPX14A-15803-901; BTLA; GITR; HVEM; IDO; LAG-3: 47; PD1; PD-L1; PD-L2; TIM-3; CD28; CD80; CD137; CD27; CD152) (eBioscence).

Samples were prepared according to manufacturer's instructions samples were prepared and measured using Luminex 200 platform (BioPlex; Bio-Rad, Bio-Rad, Hercules, CA, USA). Data, expressed in pg/mL of protein, were analyzed using Bio-Plex Manager Software (version 6.1, Bio-Rad).

Targeted Metagenomic on Fecal Microbiota

To extract DNA from stool samples a QIAmp Fast DNA Stool mini kit (Qiagen, Hilden, Germany) was used. The bacterial DNA library was obtained by the amplification of 16S rRNA variable region V3-V4 (~460 bp) following the MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, CA, USA). Then the pooled library was sequenced on an Illumina MiSeqTM platform. Obtained raw reads, after quality and length trimming and chimera checking, were analyzed by Qiime v1.8.(http: //qiime.org/1.4.0/) [55]. Operational Taxonomic Units (OTUs, Chicago, IL, USA) with a 97% clustering threshold of pairwise identity and representative sequences were aligned using PyNAST v.0.1. (https://biocore.github.io/pynast/) [56] and matched against Greengenes 13_08 database [57]. An OTU table was filtered, retaining all OTUs that had at least a 0.01% total abundance in the table and removing all OTUs present in less than 25% of samples.

Gut Microbiome Metabolomics Profiling

The gut metabolome profile was characterized for 11 NSCLC patients in order to analyze volatile and non-volatile metabolites. For volatile organic compound (VOC) detection, stool samples were

analyzed with gas chromatography-mass spectrometry (GC-MS) by using the carboxen-polydimethylsiloxane coated fiber (CAR-PDMS) (85 μm) and the manual solid-phase microextraction (SPME) holder (Supelco Inc., Bellefonte, PA, USA) [58]. Run conditions were previously reported by Botticelli et al. [59]. The chromatograms were integrated and identified compared to fragment pattern presents in the mass spectral NIST library (version 2.2, NIST 14MS database; National Institute of Standards and Technology, Rockville, MD, USA), with the literature [60] and also followed by manual visual inspection. Quantitative data compounds were obtained by interpolation of the relative areas vs. internal standard (IS) area expressed as ppm (mg/kg).

Determination of non-volatile metabolites was performed by nuclear magnetic resonance spectroscopy (NMR) analysis; the stools were processed to obtain fecal waters as described by Brasili et al.[61]. Subsequently, to sample collection, 2 out of 11 NSCLC samples were excluded for an inadequate sample amount. The pipelines and the NMR analyses were performed according to Brasili et al. [62] and Botticelli et al. [59]Moreover, the assignment was confirmed according to the Human Metabolome Data Base [63] and our own laboratory database. 1D 1H NMR spectra were processed and quantified (μmol/g) according to Botticelli et al. [59]

Statistical analysis

Descriptive statistics (median, range, and percentages) of clinical and biological characteristics of cancer patients were calculated. Student's t-test was used for comparing continuous variables between groups, whereas Fisher's exact test or $\chi 2$-test was used for categorical variables. The impact of clinicopathological variables on OS and PFS was analysed by both the univariate and multivariate analyses (UVA and MVA, respectively). With regards to UVA, patients' OStot or OS (from diagnosis or therapy, respectively) and PFS were analysed using the Kaplan–Meier method and log-rank tests. Prognostic clinic-pathological variables deemed of potential relevance in the univariate analysis (corresponding to a cutoff of $p < 0.10$) were included in the multivariate Cox proportional hazards regression analysis. A nomogram to predict 1- or 2-year OS probability was developed based on covariates retaining a statistically significant power ($p < 0.05$) in MVA. The nomogram was validated using the point assignment as follow: female/male: 0/28 points; PS=0,1/PS=2: 0/36 point; the levels of %CD137$^+$ T cells of 5 corresponded to 37.5 points.

Discrimination of nomogram was tested by Kaplan–Meier curves. A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using R-package software (Version 1.4.1106).

Results

This research project has involved immunological analysis on several setting of metastatic cancer patients treated with immunotherapy (Pembrolizumab or Nivolumab) or TKIs (Pazopanib and Sunitinib) as first or second line agent. Despite tumor heterogeneity the through line of project is to delineate patients' immune profile taking into account all the actors implicated as promoters or inhibitors of the anti-tumor immune response. The requisite for understanding why immunotherapeutic agents fail and promote tumor rejection is to understand the role of immune system and monitor the impact of therapies on its immunological components, considering also clinical feature i.e. microbiota or performance status. Hence, independently from tumor type or administered treatment, the fundamental thought is the idea of immune system as driving force in clinical outcome.

Patients' characteristics

In this research studies were enrolled 129 metastatic cancer patients belonging to several cancer setting (mRCC, NSCLC, HNSCC, UM). Their main characteristics were listed in Table 2 (mRCC patients treated with TKIs) and in Table 3 (patients treated with anti-PD1).

Table 2. Clinic-pathological characteristics and treatment of mRCC patients.

	All Patients N 20 (100%)
Age (years)	
Median Age (range)	56,5 (36-78)
Gender	
Male	15 (75)
Female	5 (25)
Risk Factors	
Smoking history (SH)	9 (45)
Histology	
Clear cell carcinoma	16 (80)
Other	4 (20)
Fuhrman grading	
G2	7 (35)
G3	9 (45)
unknown	4 (20)
Metastatic site at the diagnosis	
Liver	4 (20)
Nodal	8 (40)
Lung	12 (60)
Bone	5 (25)
Brain	3 (15)
Adrenal	1 (5)
IMDC score	
Poor risk	5 (25)
intermediate	10 (50)
good risk	5 (25)
I line treatment	20
Sunitinib	8 (40)
Pazopanib	12 (60)
II line treatment	10 (50)
Nivolumab	10 (100)
III line treatment	2
Cabozantinib	2

Table 3: clinical and pathological characteristics

Clinical parameters	Identification cohort	Validation cohort
Total	66	43
Tumor:		
NSCLC	34	17
RCC	8	0
HNSCC	10	26
UM	14	0
Age:		
< 64 year	32	17
≥ 64 year	34	26
Gender:		
female	27	8
male	39	35
Performance status before ICIs:		
0	33	21
1	25	14
2	8	8
3	0	0
4	0	0
N° metastasis before ICIs:		
≤2	46	34
>2	20	9
Toxicity:		
No	37	14
Yes	29	29
Previous therapies:		
No	18	38
Yes	48	5
Response to ICIs:		
No	32	18
Yes	34	15
NE	0	10
Biological parameters	**Median (range)**	
%CD3CD137 cells	1.2 (0.1-7.4)	1.07 (0.15-3.47)
%CD3CD8CD137 cells	0.8 (0.1-2.9)	0.53 (0.076- 1.91)
%CD3CD4CD137 cells	0.2 (0.04-6.1)	0.51 (0.04-2.03)
%PD1CD137 cells	1.85 (0.2-9.9)	0.69 (0.1-2)
soluble CD137	158 pg/mL (6-16636)	ND

ND: Not detected

Soluble immune profile and response to immunological treatment

TKIs and ICI modulate the release of sIC

Levels of circulating soluble immune checkpoint (sIC) were measured in serum of metastatic cancer patient in order to evaluate the effect of immunological treatment on the release of these soluble proteins both at baseline (T0) and during treatment (TKIs or ICIs) (>T0; after 3 months of therapy, at first clinical revaluation).

It was recently demonstrated that the soluble isoforms of the checkpoint receptors are involved in positive or negative immune regulation and that changes in their plasma levels affect the development, prognosis, and treatment of cancer [46]. Moreover, several evidence demonstrated that these molecules could contribute to immune regulation, representing a putative biomarker for tumor outcome [64].

Our results suggested that both TKIs and ICIs modulate the release of several sICs in mRCC patients and NSCLC patients respectively.

In particular, as shown in figure 13 the concentration of sPD1 and its ligand sPDL2 significantly decreased in NSCLC patients during anti-PD1 treatment (respectively p=0.02 and p=0.03) instead levels of sLAG3 were significantly increased (p=0.007). Correlating these results with clinical response, it was observed that the significant decrease of sPD1 occurs only in Responder (R) patients (p=0.03) and not in Non-Responder (NR) group. On the contrary, a significant increase of sLAG3 was ascribable only to NR patients (p=0.02). No correlation with clinical response was observed for sPDL2 decrease.

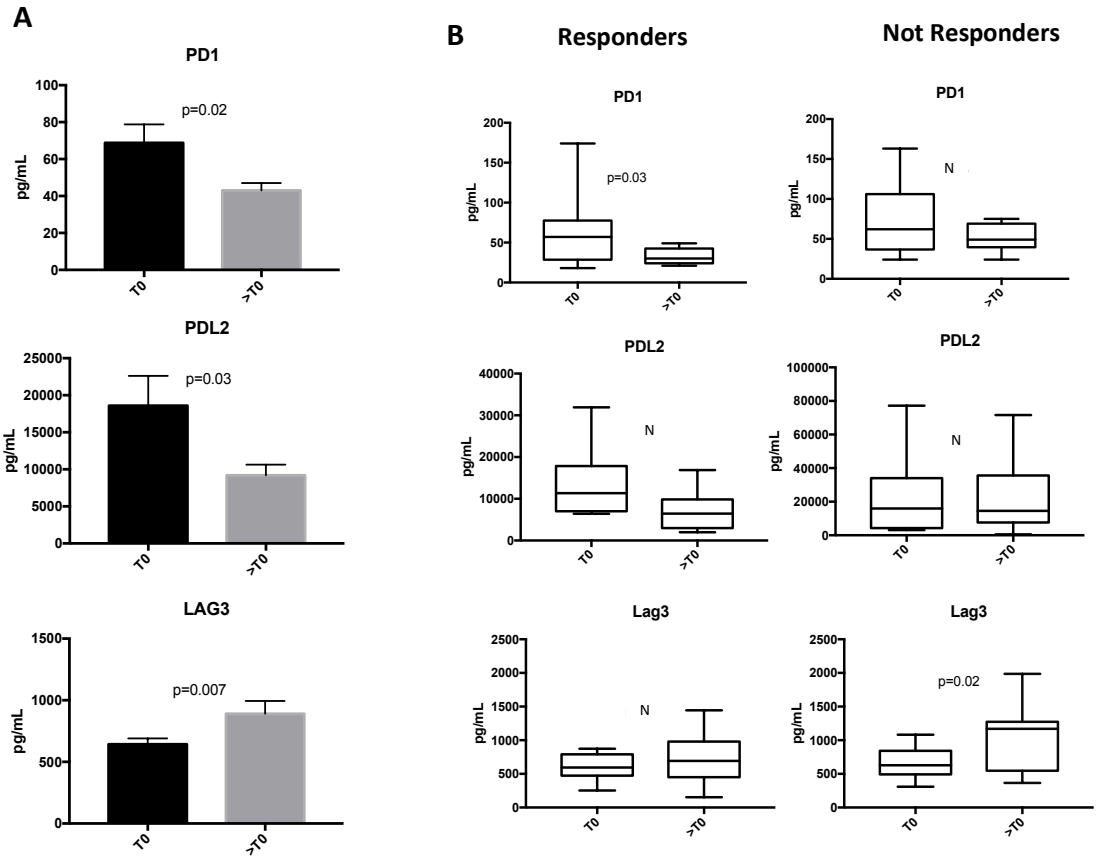

Figure 13: Changes in soluble immune checkpoints (sICI) in NSCLC patients during Nivolumab treatment. (**A**) Levels of soluble immune checkpoint-related protein evaluated in 22 NSCLC patients before the beginning of Nivolumab treatment (T0) and at first clinical evaluation (>T0). The proteins were analyzed by Luminex assay, and the results are reported as concentration (pg/mL) of sICs present in patient's sera. Histogram represent the concentration mean values ± SEM of sPD1 (T0 67 ± 10 pg/mL vs. >T0 41 ± 10 pg/mL), sPD-L2 (T0 18.6 ± 4 pg/mL vs >T0 8 ± 1.4 pg/mL), and sLAG3 (T0 627 ± 50 pg/mL vs >T0 859 ± 109 pg/mL), at baseline (T0, black histogram) and at first clinical evaluation (>T0, gray histogram). (**B**) Box plots of sPD1, sPDL2 and sLAG-3 in responder (R) and not-responder (NR) patients (respectively 11 R and 11 NR patients) between Nivolumab initiation (T0) and the first clinical evaluation (>T0). The line in the box shows the median values. The error bars represent the minimum and the maximum values of sICs concentration (pg/mL). A student's paired t-test was used to compare the differences between T0 and >T0. *p* values < 0.05 were considered significant. NS = not significant

Similarly, in mRCC patients, the release of several sICs were modulated by TKIs. Figure 14 Showed that the concentration of sPDL2 significantly decreased during TKI therapy (7842.5 ± 2865 pg/mL for T0 vs. 4989 ± 4462 pg/mL for >T0; p = 0.02). Similar results were observed for sHVEM (4085.5 ± 3388 pg/mL for T0 vs. 1777 ± 1578 pg/mL for >T0; p = 0.01). It was shown that the high concentration of sHVEM seems to contribute to tumor development and progression [65]. Moreover,

the results indicate that TKI treatment also affects the release of sPD1 and sGITR, decreasing the concentration of both molecules between T0 and >T0 (sPD1: 561.5 ± 431 pg/mL for T0 vs. 238 ± 176 pg/mL for >T0, p = 0.02; sGITR: 548 ± 425 pg/mL for T0 vs. 214 ± 212 pg/mL for >T0, p = 0.01).

Correlating the concentration of these soluble molecules with clinical response, sPDL2 resulted the unique sICs differently modulated during TKI treatment and, in particular, only in R patients (Figure 14B) (sPDL2: 8855 ± 3985 pg/mL for T0 vs. 5057 ± 4243 pg/mL for >T0, p = 0.01). This result is in line with literature, in fact in a recent study, conducted on ccRCC patients, it was demonstrated that high levels of sPDL2 were associated with an augmented risk of recurrence [66]. No significative difference was observed for NR patients.

These data demonstrate that immunological treatments positively modulate the release of soluble immune molecules, suggesting their possible role in the clinical outcome of cancer patients.

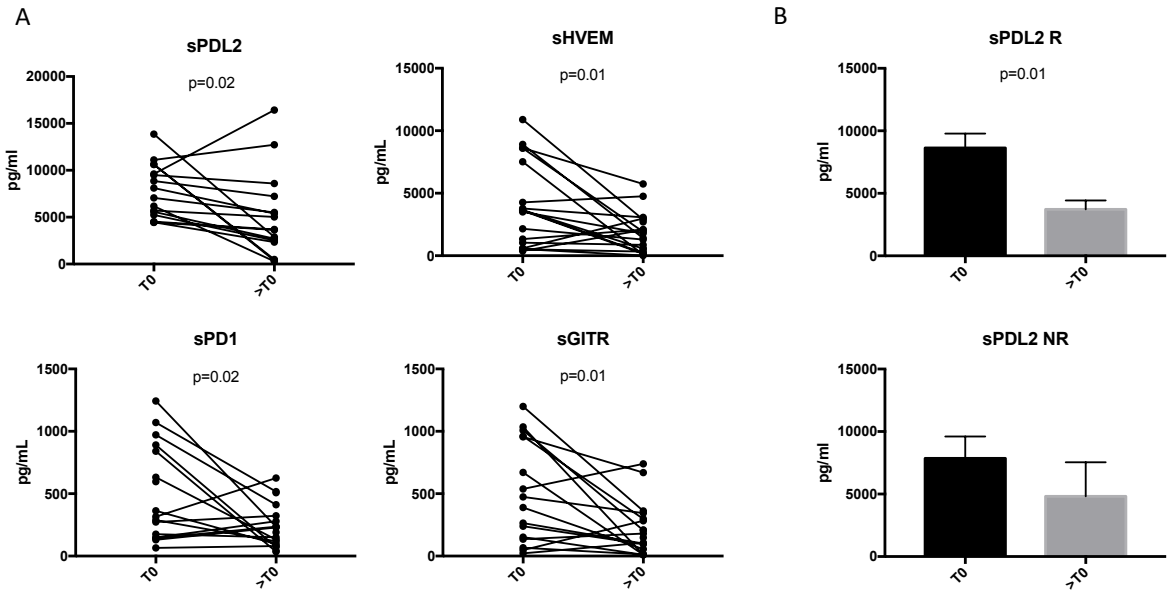

Figure 14 Changes in the soluble immune checkpoint--related proteins during TKI therapy in mRCC patients.

(**A**) Analysis of soluble immune checkpoint-related proteins levels (i.e. sPDL2, sHVEM, sPD1, and sGITR) in patients with mRCC at T0 and after 3-4 months of TKI treatment (>T0). The proteins were analyzed by Luminex multiplex assay and the results are reported as the concentration (pg/mL) of soluble checkpoint inhibitors present in the serum of mRCC patients. (**B**) sPDL2 levels in the serum of R and NR mRCC patients analyzed at T0 and >T0. sPDL2 resulted in the only significant molecule modulated associated with the response to TKI treatment. Statistical significance was determined by a student's paired *t-test*, and a *p*-value < 0.05 was considered statistically significant.

TKI Responsive Patients Have Low Levels of Serum IFNγ

To further analyze the contribution of TKI treatment to patient' response, cytokines were evaluated at T0 and at first clinical revaluation (>T0). Figure 15 showed that, at baseline, R patients had a significantly lower concentrations of IFNγ compared to NR patients (27.47 ± 8.5 pg/ml for R vs 515.8±210.6 pg/mL for NR p=0.007). The same significance difference was found at >T0 (48.74± 21.24 pg/mL for R vs 267.8±77.12 pg/mL for NR p=0.002). These data show that low levels of IFNγ correlates with response to TKI treatment.

IFNγ is a pleiotropic cytokine involved in anti-tumor immunity, that play a key role in the elimination phase of the immunoediting paradigm. More recent evidence demonstrated a dark side of this cytokines, as tumor promoting [70].

To determine whether IFNγ levels could contribute to mRCC patient' survival from TKI, survival curves were calculated. According to median value of IFNγ, patients were divided in those with high levels of IFNγ (>65 pg/ml) and those with low levels (< 65 pg/ml). Figure 15 shows that IFNγ predicts at baseline the duration of response to TKI treatment, in fact patients with IFNγ levels < 65 pg/ml had a longer duration of response to TKI therapy compared to high levels group (p=0.04). The average time of duration of response was undefined vs 7 months, and no significance correlation was observed during treatment (>T0) (median value of IFNγ equal to 59 pg/ml).

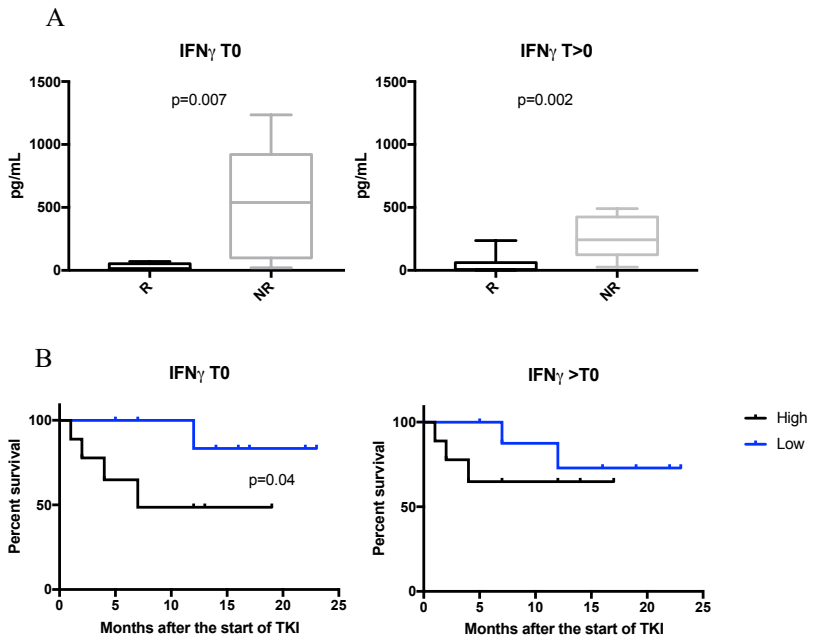

Figure 15: Levels of IFNγ in serum of R and NR patients evaluated at baseline (T0) and during TKI treatment (>T0). (A) the line in the boxes shows the median value. The error bars show the minimum and maximum value. **(B)** Survival curve analysis of mRCC patients at baseline and during TKI treatment according to the levels of IFNγ. According to median value of IFNγ, equal to 65 pg/mL at T0 and equal to 59 pg/mL at >T0, patients were dichotomized in those with high concentration and those with low concentration of IFNγ. A log-rank test was used to compare the survival between two groups. p-value < 0.05 was considered statistically significant.

Levels of sICs were associated with clinical response

To further understand the immune profile of cancer patients, we investigated differences between R and NR patients, by evaluating levels of soluble immune molecules in serum of NSCLC and mRCC patients at T0 and during treatment (>T0).

It's interesting to note that independently from tumor histotype, significant differences in the levels of sICs between R and NR patients were detected during therapy (>T0).

In fact, in mRCC responder patients sPDL1 and sCTLA4 resulted differently concentrated in serum of patients, with high levels in NR patient. This difference was statistically significant at >T0 where levels of sPDL1 and sCTLA4 were higher in NR compared to R patients (respectively 146.5 ± 122.3 pg/mL vs. 56.25 ± 36.5 pg/mL, p=0.03 616.4 ± 330.3; pg/mL vs 281.6 ± 133 pg/mL p=0.008) (Figure 16).

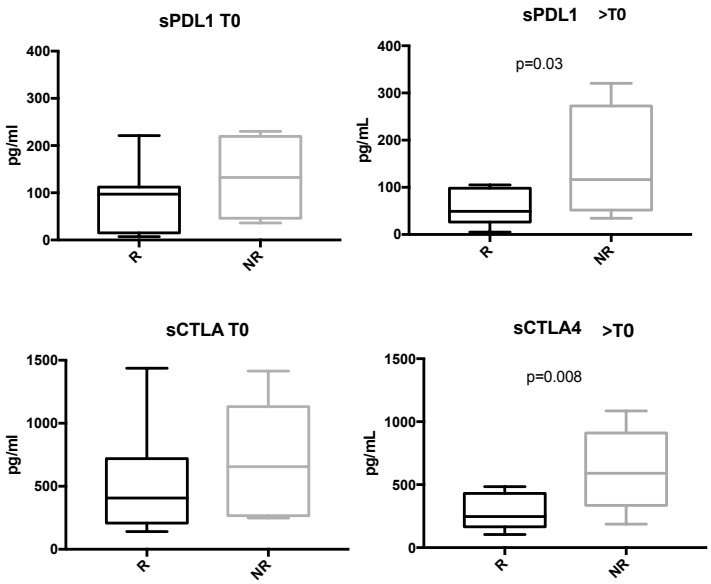

Figure 16: Profiling of levels of immune molecules at baseline and during TKI treatment in responder (R) and non-responder (NR) patients. Box plots of sPDL1 and sCTLA4 at T0 and after 3-4 months of therapy (>T0). A student's unpaired t-test was used to compare R vs NR patients, and a *p*-value < 0.05 was considered statistically significant

Likewise in NSCLC patients results (Figure 17A) showed that levels of sPD1, as well as its ligands sPDL1 and sPDL2 were significantly higher in serum of NR patients compared to R. As well as

concentrations of other sICs such as BTLA and its receptor HVEM, Tim3 and CTLA4, were higher in the serum of NR patients compared to R.

Interestingly, also the levels of the costimulatory molecules sCD137 is higher in NR patients. This protein acts as T cell activator in its membrane bound structure, but its soluble form inhibits CD137 – CD137L ligand , blocking T cells proliferation and antigen-presenting cells maturation [67].

Consequently, to better understand the significance of these experimental evidence and the relation with clinical outcome, the association between sICs and duration of response to therapy in NSCLC patients was studied. Median concentrations of sICs were calculated and based on these median value NSCLC patients were stratified in those with low and high levels of a certain molecules. According to this classification, patients with low levels of sPD1, sPDL2, sCD137, sTim3 or sBTLA performed a longer clinical response (Figure 17B) compared to patients with high levels of these factor, suggesting the possible role of these molecules as biomarkers of response to treatment.

These results strongly suggest that the response to immunotherapeutic agent is associated with low levels of sICs measured 3 months from the start of therapy

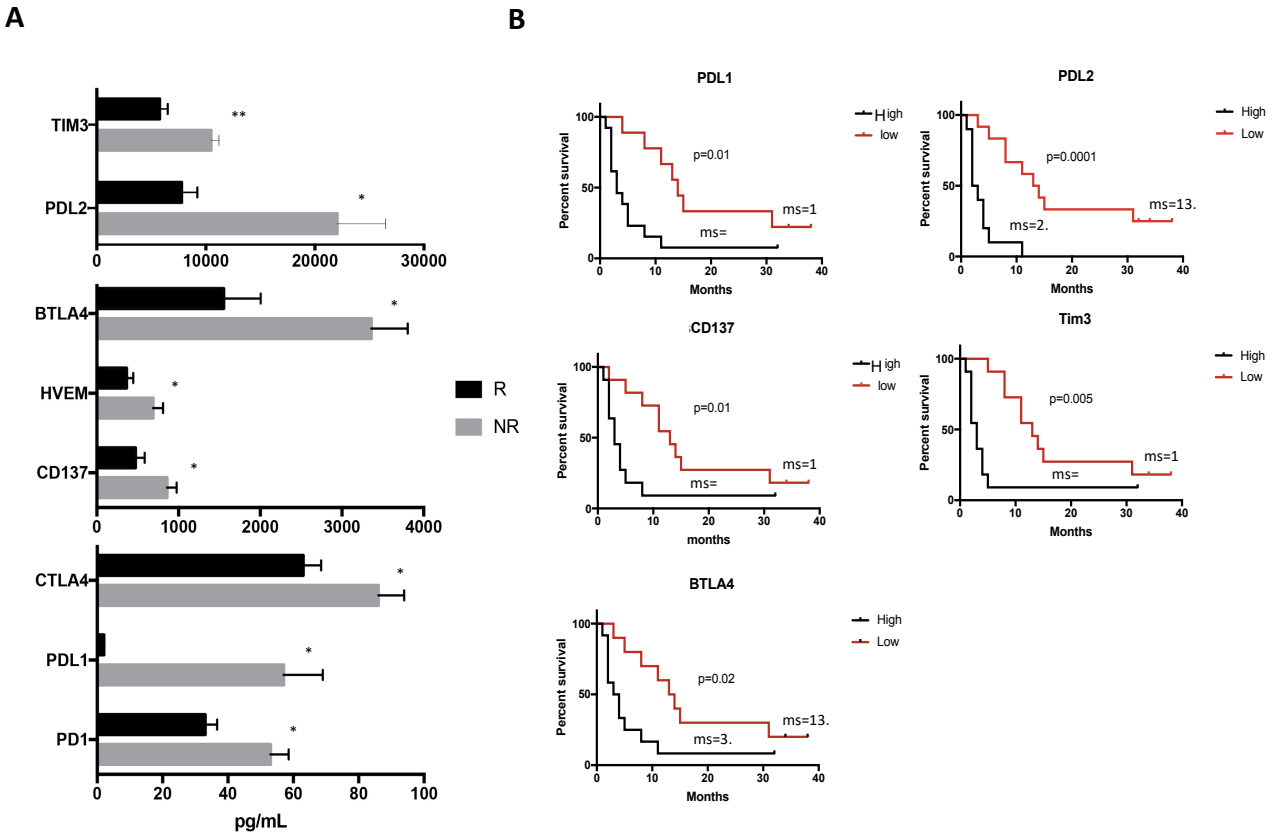

Figure 17: Levels of sICs evaluated in R and NR patients at first clinical evaluation (>T0) and their correlation with duration of response. (**A**) Soluble immunecheckpoint-related molecules were evaluated in the serum of R (Black histogram) and NR (grey histograms) patients after three months of the beginning of Nivolumab treatment (>T0) by Luminex multiple assays. Histograms represent the concentration mean values ± SEM of PD1 (R 31±4 pg/mL *vs* NR 53±6 pg/mL), PDL1 (R 1,7±0,06 pg/mL *vs* NR 57±12 pg/mL), PDL2 (R 7,3±1,4 ng/mL *vs* NR 22±4,4 ng/mL), BTLA4 (R 1,4±0,4 ng/mL *vs* NR 3,3± 0,4 ng/mL), HVEM (R 332±82 pg/mL *vs* NR 687±124 pg/mL), Tim3 (R 5,4±0,8 ng/mL *vs* NR 10±0,7 ng/mL), CTLA4 (R 61±5 pg/mL *vs* NR 86±8 pg/mL) and CD137 (R 429±113 pg/mL *vs* NR 859±117 pg/mL). **(B)** Survival analysis carried out at >T0 in NSCLC patients with high and low levels of sICs. The median values used to define patients with high and low levels of sICs are the following: PDL1 (20pg/mL), PDL2 (7.7ng/mL), CD137 (624pg/mL), Tim3 (8.1ng/mL) and BTLA4(2.2 ng/mL). *p* values ≤ 0.05 were considered significant. * p≤0.05, **p≤0.01. ms=median survival.

sICs Are Differently Modulated According to ECOG PS Scale

Moreover, for a more complete picture the immune profile was related to clinical parameters like performance status. This represents a measurement of clinical condition of cancer patients and their cancer progression in terms of their ability to take care of themselves.

Thus, sICs were analyzed and correlated with the Eastern Cooperative Oncology Group (ECOG) PS scale. According to this, NSCLC patients were stratified into 2 groups: PS = 0 and PS = 1,2, in particular 9 were classified as PS = 0 and 13 as PS = 1,2 (11 patients with PS = 1; 2 patients with PS = 2). Monitoring the variation of sICs during treatment in these groups we observed that in patients scored as PS = 0 there was a significant decrease of sPD1 during Nivolumab administration (PD1: T0 59 ± 14 pg/mL vs. >T0 32 ± 4 pg/mL, $p = 0.04$. Instead in PS=1,2 patients sPD1 levels remained similar (PD1: T0 71 ± 14 pg/mL vs. >T0 69 ± 13 pg/mL, $p = 0.8$). Moreover, the analysis displayed a trend of association between low levels of sICs and PS = 0 (Figure 18). This group of patients compared to PS=1,2 group showed low levels of the inhibitory molecules sPDL2 and sGITR and seems to have a better immune fitness before the beginning of therapy. Moreover, at first clinical evaluation (>T0), concentration of sPD1, sPDL1, sCTLA4 and sHVEM were less abundant in PS = 0 patients compared to PS = 1,2 group. These data suggest that Nivolumab treatment appears to be more efficient in patients with PS = 0, decreasing sPD1 and maintaining low levels of immune-evasion molecules.

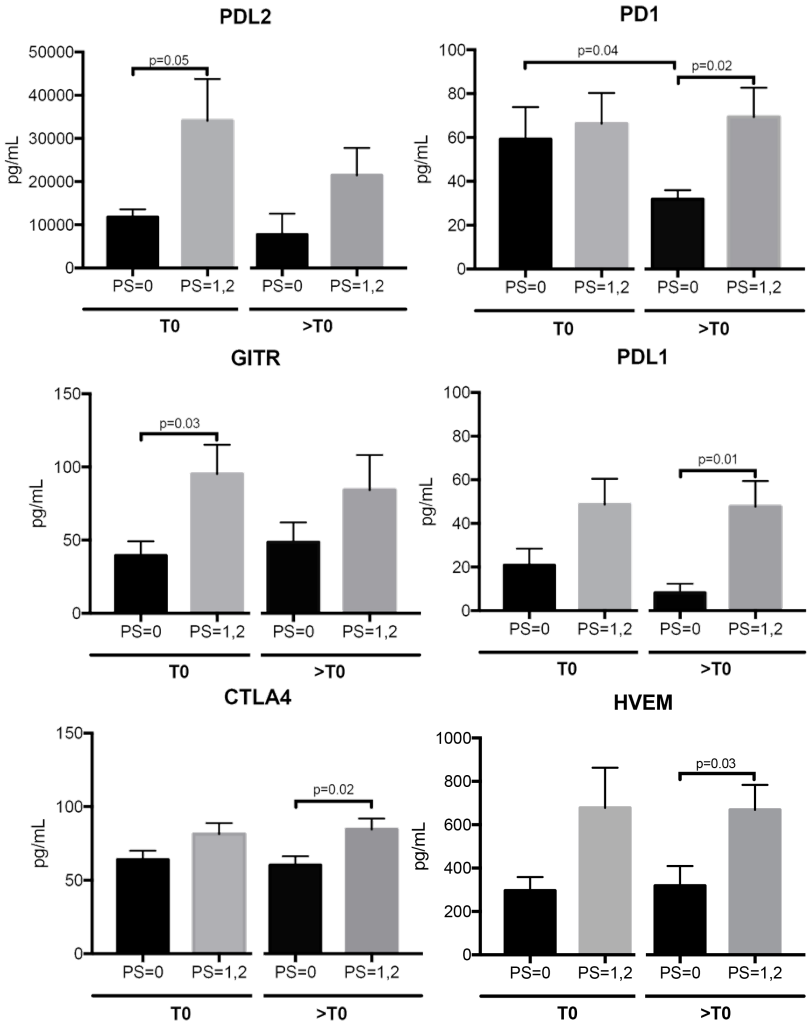

Figure 18: Levels of sICIs evaluated in NSCLC patients scored as performance status (PS)=0 and PS=1,2 before the beginning of Nivolumab treatment (T0) and at first clinical evaluation (>T0). Histograms represent the concentration mean values±SEM of sICs detected in patients with PS=0 (black histograms) and PS=1,2 (grey histograms) at T0 and >T0. The median values ± SEM of sICs at T0 are the follow: PDL2: PS=0 *vs* PS1,2, 12± *vs* 36± ng/mL; GITR: PS=0 *vs* PS1,2, 39± *vs* 95± pg/mL; CTLA4: PS=0 *vs* PS1,2, 64± *vs* 81± pg/mL; PD1 PS=0 *vs* PS1,2, 59± *vs* 71± pg/mL; PDL1: PS=0 *vs* PS1,2, 21± *vs* 49± pg/mL; HVEM: PS=0 *vs* PS1,2, 296± *vs* 677± pg/mL. At >T0 the mean values are: PDL2: PS=0 *vs* PS1,2, 8± *vs* 21± ng/mL; GITR: PS=0 *vs* PS1,2, 48± *vs* 84± pg/mL; CTLA4: PS=0 *vs* PS1,2, 60± *vs* 84± pg/mL; PD1 PS=0 *vs* PS1,2, 32± *vs* 69± pg/mL; PDL1: PS=0 *vs* PS1,2, 8± *vs* 48± pg/mL; HVEM: PS=0 *vs* PS1,2, 319± *vs* 668± pg/mL. *p* values ≤ 0.05 were considered significant.

Responder Patients Have a High Proportion of Eubiosis-Associated Gut Metabolites

An important district that could impact the immunological fitness and influence patients response is represented by gut microbiota and its metabolomic profile [68]. Several data demonstrated that specific gut microbiota and metabolome profiles have been associated with eubiosis or dysbiosis status [69]. To analyze the gut microbiota and the proportion of eubiosis/dysbiosis-associated gut metabolites in R and NR patients, stool samples derived from 11 NSCLC patients (6 R and 5 NR) were collected at T0 and 9 out of 11 were analyzed.

The analysis of the gut metabolome profile showed a set of 114 metabolites, 67 (59%) volatile organic compounds (VOCs) and 47 (41%) non-volatile. Among these compounds, 42 were related to eubiosis or dysbiosis (14 and 28, respectively).

In particular, VOCs showed the eubiosis related metabolites belonging to a chemical class of short chain fatty acids (SCFAs) (i.e., butyric, proprionic, acetic and pentanoic) and terpenes; on the contrary, the metabolites probably associated with dysbiosis were aldehydes (i.e., butanal 3-Methyl, benzeneacetaldehyde), alcohols (i.e., ethanol, 2-Octanol) and phenols. Evaluating the concentration average values of these 42 metabolites in R and NR patients, 31 of them showed a difference of at least two-fold in their concentration between the two groups and were further analyzed (Figure 19). In R patients, a total of 14 compounds were found, 9 (64%) potentially related to eubiosis and 5 (36%) to dysbiosis, while NR patients showed dysbiosis, while NR patients showed 17 metabolites, 2 (12%) might be associated with eubiosis and 15 (88%) with dysbiosis.

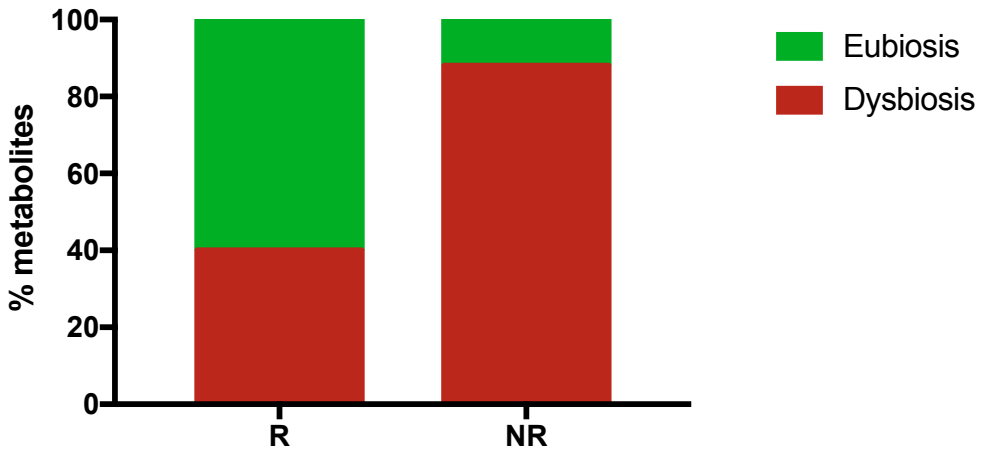

Figure 19: Percentage of eubiosis/dysbiosis gut metabolites in R and NR patients evaluated before the beginning of nivolumab treatment (T0). Gut metabolites were evaluated in the fecal samples of 9 NSCLC patients. Volatile organic compounds were analysed by gas chromatography-mass spectrometry, while non-volatile metabolites were analysed by proton nuclear magnetic resonance spectroscopy. In R patients 14 metabolites were found, 9 related to eubiosis and 5 to dysbiosis. The histograms represent the percentage of eubiosis (in green) and dysbiosis (in red) associated gut metabolites evaluated, considering the 14 and 17 metabolites found in R and NR patients as 100%, respectively.

CD137$^+$ subset as an immune biomarker to define the wellness status of immune system

To better define immune profile, it's fundamental taking into account the cellular component in terms of circulating T lymphocytes. The analysis of the immune profiling revealed that the expression of CD137 molecule on T cells was associated to clinical response in several cancer setting treated with TKIs or ICIs.

CD137 is a co-stimulatory receptor expressed on activated antigen-specific T cells. The engagement with its ligand, CD137L, enhances T cells proliferation and effector function[70]. It is considered a marker of antigen-specific cells.

Specifically, figure 20A, showed that in mRCC setting treated with TKIs, responder patients had a significantly higher percentage of CD3$^+$CD137$^+$ T cells (2.7% ± 0.92%) compared to non-responder (0.9% ± 0.87%) both at baseline (T0) that during TKIs therapy (>T0) (R: 2.6% ± 0.78%; NR 0.67% ± 0.4%) (respectively p = 0.003 and p = 0.0001). Then, evaluating CD3$^+$CD137$^+$ subpopulations, it's interestingly to note that this significance was ascribable only to CD8. Indeed, at T0, the expression of CD137 on CD8$^+$ T cells was significantly higher in R patients (2.02% ± 0.7%) compared to NR patients (0.6% ± 0.5%) (p = 0.001). This data became even more significant also during therapy (>T0), observing a percentage of CD8$^+$CD137$^+$ subpopulation equal to 1.91% ± 0.75% in responder patients vs. 0.43% ± 0.25% in NR (p = 0.0008). Instead, no significant difference was obtained for CD4$^+$ T-cell subpopulation (%CD4$^+$CD137$^+$at T0: 0.6% ± 0.2% in responder patients vs. 0.27% ± 0.18% in NR, p = 0.28; at >T0: 0.87% ± 0.28% in R vs. 0.23% ± 0.08% in NR, p = 0.18).

These results showed that CD137$^+$ T cells could represent a possible biomarker able to identify patients that could clinically benefit from TKI treatment.

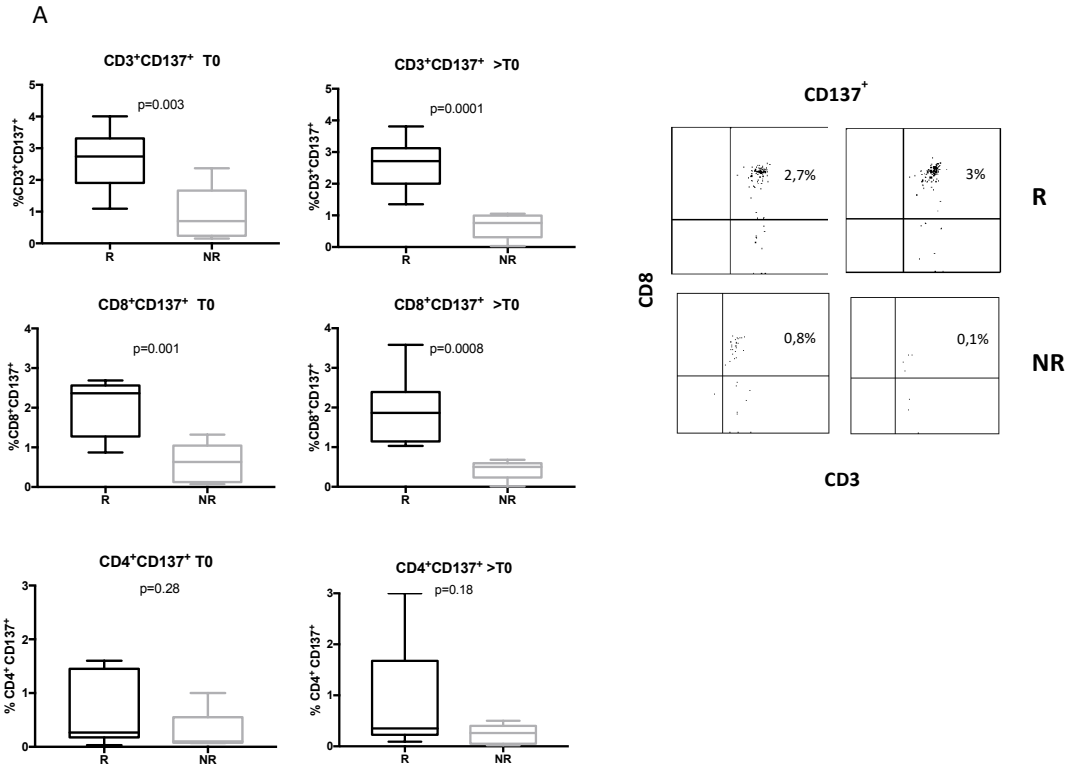

Figure 20: CD137⁺ expression on T lymphocytes in mRCC patient treated with TKIs

Immune cells subpopulations were evaluated using flow cytometry and analyzed by FACSDiva Software. To analyze the CD137⁺ T cells, lymphocytes were first gated on FSC-A and SSC-A and then the CD3⁺ T cells sub-population was selected from lymphocytes. CD3⁺CD137⁺ T cells were selected and then analyzed for the expression of CD4 and CD8. The results are shown as percentage of CD3⁺CD137⁺, CD8⁺CD137⁺ and CD4⁺CD137⁺ T cells in R and NR patients at baseline and during TKIs therapy. The dot plot analysis of CD3⁺ CD8⁺CD137⁺ T lymphocytes is shown in the right side of panel A. The results are representative of one R patient and one NR mRCC patient.

Subsequently, CD137 circulating levels were examined in 66 cancer patients with different tumor diagnosis, all treated with anti-PD1 and then compared to 20 healthy donors (HD). Flow cytometry analysis showed that HD expressed a significantly higher percentage of CD3⁺CD137⁺ T cells (3.2% ± 1.2%) than tumor patients (% 1.8 ± 1.6) ($p<0.0007$) (Figure 21A), suggesting a role as possible parameters to monitor a wellness status of immune system for CD3⁺CD137⁺.

Later, correlating this cellular subset to clinical outcome it was observed that R patients had a percentage of CD3⁺CD137⁺ T cells significantly higher (2.2% ± 1.8%) than NR patients (1.3% ± 1.1%) ($p<0.03$) (Figure 21B) This data was mainly ascribable to CD8⁺CD137⁺ T cells ($p=0.0001$). No association was found between the levels of CD4⁺CD137⁺ T cell subset and the clinical response (Figure 21C) ($p=0.2$)

Further analysis examined the frequency of $CD137^+$ related to several clinical parameters, i.e. PS, number of metastasis (n° met), toxicity and previous therapies. Results obtained are shown in figure 21D.

Patients with PS=0-1 and with n° met < 2 expressed a more elevated levels of $CD3^+CD137^+$ T cells (respectively p=0.02 and p=0.006) suggesting that a better clinical status is correlated with high levels of $CD137^+$ T cells.

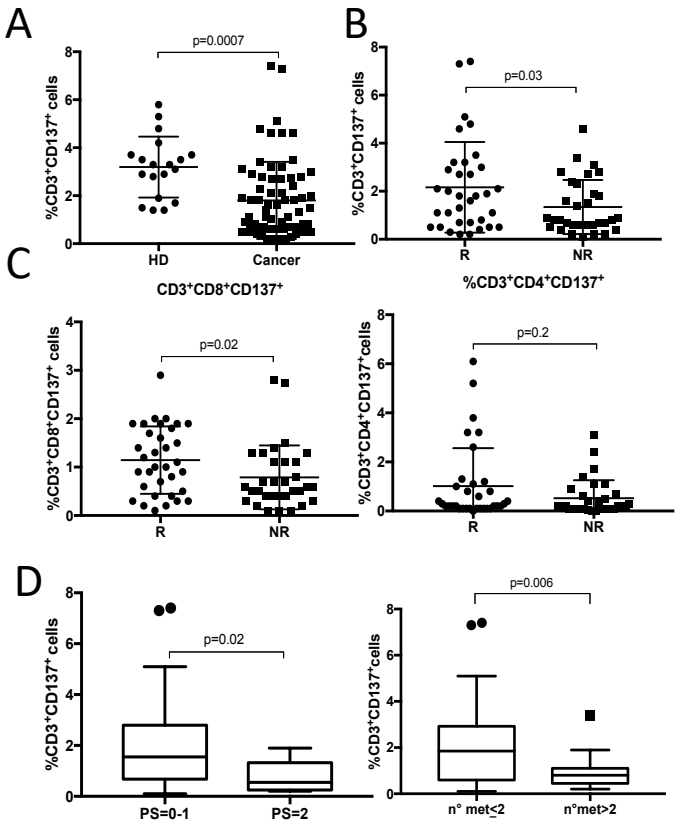

Figure 21: The high frequency of CD137+T cells correlates with the response to treatments and with clinical parameters. (A) The scattered dot plot represents the percentage values of circulating $CD3^+CD137^+$ cells evaluated by cytofluorimetry in 20 healthy donors (HD) and 66 cancer patients (Cancer) ± standard deviation (SD). The scattered plots show the percentage of circulating $CD3^+CD137^+$ cells **(B)**, $CD3^+$ $CD8^+CD137^+$ cells and $CD3^+$ $CD4^+CD137^+$ **(C)** in responder (R) and non-responder (NR) patients to anti-PD1 treatment ± SD.

(D) Tukey's box plots represent the median distribution of $CD3^+CD137^+$ cells according to performance status (PS) and number of metastasis (n°met) + the lowest and the largest data point excluding any outlier. Unpaired Student't test was used to compare the different groups. *p* values<0.05 were considered significant.

CD137⁺ T cells as a predictive and prognostic biomarker for progression-free survival and overall survival in cancer patients treated with immunotherapeutic agents

Once identified CD137⁺ T cells as a biomarker of immune wellness, we evaluated its predictive and prognostic role by the correlation with survival either in mRCC patients treated with TKIs or cancer patients treated with anti-PD1.

Kaplan-Meier curves for mRCC patients treated with TKIs, with high and low concentrations of CD8⁺CD137⁺ T cells are represented in Figure 22 showing a longer survival in patients with high percentage of this population. While at baseline survival curves showed a trend for this data, during treatment survival curves showed an undefined survival for patient with high percentage of CD8⁺CD137⁺ and a median survival equal to 12 months for low CD8⁺CD137⁺ group (p = 0.04, log-rank test), suggesting that the maintenance of CD8⁺CD137⁺ T cells in circulation is associated with the duration of the response to TKIs.

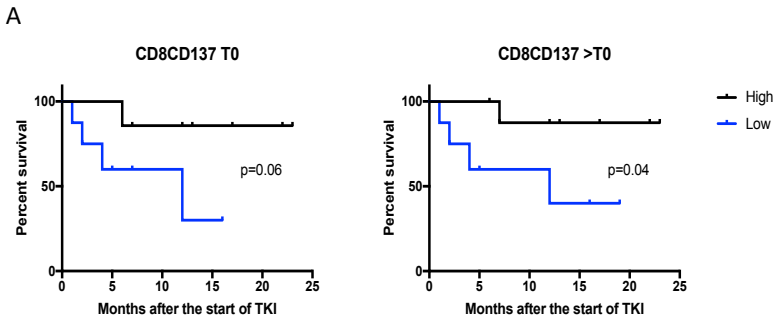

Figure 22: Kaplan-Meyer curves of PFS based on Survival analysis of mRCC patients at baseline and during TKI treatment. According to median value of CD3⁺ CD8⁺CD137⁺ T cells (T0: 1,4 % ; >T0 1,3%) patients were divided in those with high or low percentage of CD3⁺ CD8⁺CD137⁺ T cells. Log-rank test compared the survival between two groups.

The prognostic role of CD137 T cells was observed at baseline in the cohort of 66 patients belonging to different cancer setting. According to median value of CD3⁺CD137⁺ and CD8⁺CD137⁺ (respectively equal to 1.2% and 0.8%) these patients were stratified in those with high and low percentage of CD137⁺ T cells. Figure 23 showed that high CD3⁺CD137⁺ and high CD8⁺CD137⁺ group of patients had a significantly longer PFS (figure 23A) and OS (Figure 23B). Instead, high levels of CD3⁺CD4⁺CD137⁺ were associated with PFS (p=0.02), but not with OS (p=0.05).

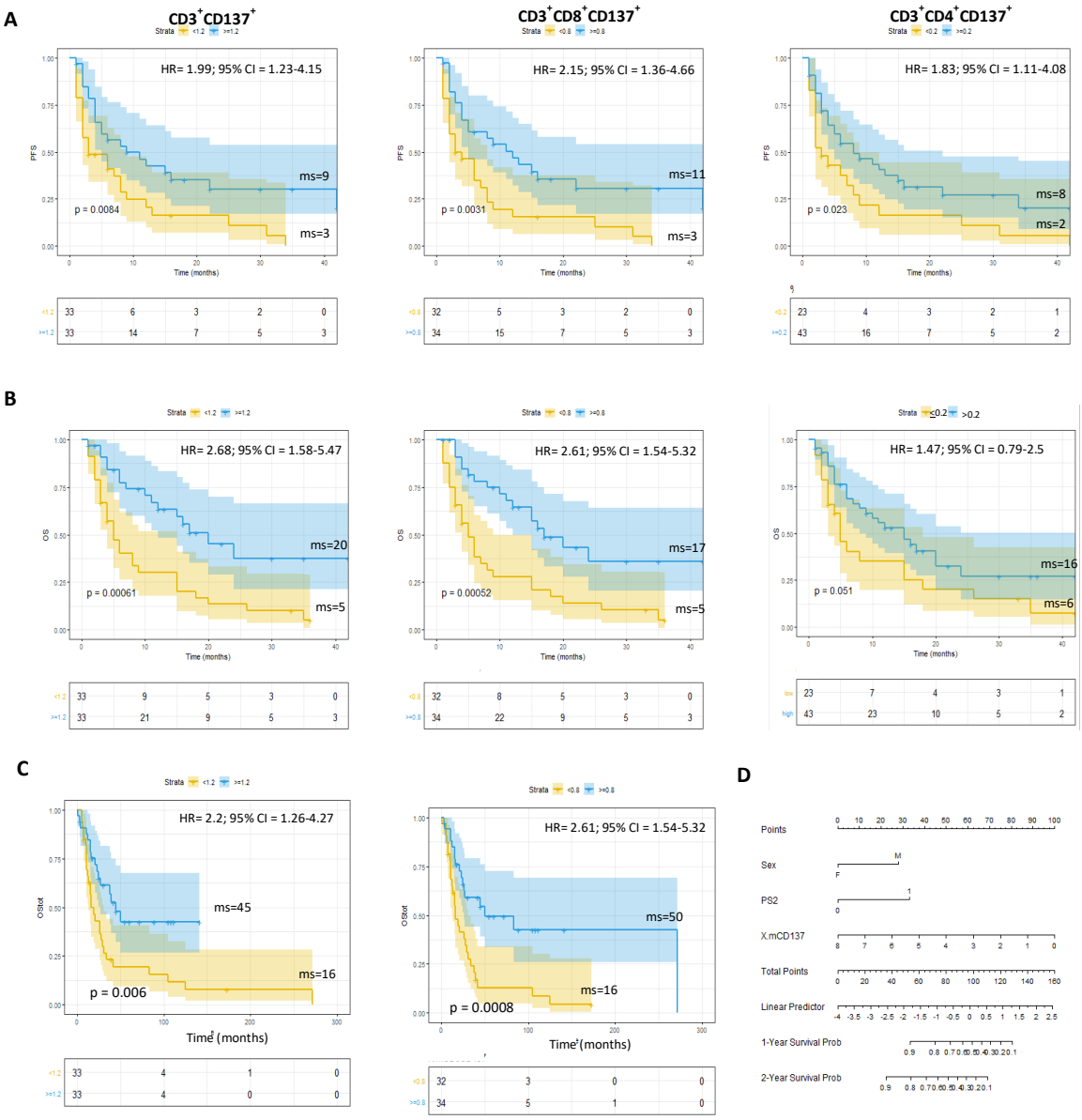

Figure 23: Kaplan Mayer curves of PFS after anti-PD1 treatment **(A)** and OS evaluated at the beginning of anti-PD1 treatment (OS) **(B)** and at diagnosis (OStot) **(C)** analyzed considering the median values of the percentage of circulating $CD3^{+}CD137^{+}$ (1.2%), $CD3^{+}CD8^{+}$ $CD137^{+}$ (0.8%), and $CD3^{+}CD4^{+}CD137^{+}$ (0.2%), cells. Log-rank test was used to compare survival between the two groups. ms=months; **(D)** Prognostic nomogram of OS probability at 1 and 2 years in metastatic cancer patients treated with anti-PD1. $p<0.05$ were considered significant.

Moreover, correlation with clinical parameters showed that PS resulted associated with an increased PFS (Table 4) and OS (Table 5). In particular, patients with PS=0-1 had a longer PFS (PS=0-1 vs. PS=2: median survival 8 months vs. 1,5 months) and OS (PS=0-1 vs. PS=2: median survival 15 months vs. 3 months) than patients scored as PS=2.

Table 4: predictive and prognostic factors for PFS

	Univariate Analysis		Multivariate Analysis	
Variables	**HR (95% CI)**	***p value***	**HR (95% CI)**	***p value***
Age (<64y vs ≥ 64y)	0.99 (0.55 to 1.79)	*p = 0.99*		
Sex (Female vs Male)	1.64 (0.96 to 3.17)	*p = 0.06*		
Performance Status (0,1 vs 2)	0.17 (0.03 to 0.82)	***p < 0.0001***		
n° Metastasis:				
1 vs ≥ 2	0.9 (0.44 to 1.82)	*p = 0.76*		
2 vs ≥ 3	0.69 (0.32 to 1.26)	*p = 0.19*		
Toxicities (Yes vs No)	0.96 (0.53 to 1.72)	*p = 0.88*		
Previous Lines (Yes vs No)	0.73 (0.37 to 1.42)	*p = 0.35*		
% $CD3^+CD137^+$ ≥ 1.2	1.99 (1.23 to 4.15)	***p = 0.008***		
% $CD3^+CD8^+CD137^+$ ≥ 0.8	2.15 (1.36 to 4.66)	***p = 0.003***	0.44 (0.25 to 0.79)	**0.0062**
% $CD3^+CD4^+CD137^+$ ≥ 0.2	1.83 (1.11 to 4.08)	***p = 0.02***		

CI = confidence interval; HR = Hazard Ratio

Table 5: predictive and prognostic factors for OS evaluated from the beginning of immunotherapy

	Univariate Analysis		Multivariate Analysis	
Variables	**HR (95% CI)**	***p value***	**HR (95% CI)**	***p value***
Age (<64y vs ≥ 64y)	0.93 (0.5 to 1.7)	*p = 0.81*		
Sex (Female vs Male)	2.16 (1.2 to 4.16)	***p = 0.01***	0.37 (0.18 to 0.73)	**0.004**
Performance Status (0,1 vs 2)	0.19 (0.04 to 0.86)	***p < 0.0001***	3.41 (21.29 to 8.96)	**0.01**
n° Metastasis:				
1 vs ≥ 2	0.78 (0.37 to 1.63)	*p = 0.52*		
2 vs ≥ 3	0.48 (0.19 to 0.81)	*p = 0.083*		
Toxicities (Yes vs No)	0.88 (0.47 to 1.62)	*p = 0.68*		
Previous Lines (Yes vs No)	0.67 (0.34 to 1.35)	*p = 0.28*		
% $CD3^+CD137^+$ ≥ 1.2	2.68 (1.58 to 5.47)	***p = 0.0006***	0.37 (0.19 to 0.70)	**0.002**
% $CD3^+CD8^+CD137^+$ ≥ 0.8	2.61 (1.54 to 5.32)	***p = 0.0005***		
% $CD3^+CD4^+CD137^+$ ≥ 0.2	1.75 (0.99 to 3.76)	*p =0.05*		

CI = confidence interval; HR = Hazard Ratio

Another clinical characteristic that showed a correlation with the OS was the patients' gender (females vs. males) (Table 5). Females appeared to have longer survival compared to males (females vs. males: median survival 26 months vs. 7 months) after the beginning of the anti-PD1 treatment.

On the other hand, multivariate analysis (MVA) revealed that a high level of $CD3^+CD8^+CD137^+$ cells was an independent prognostic factor of PFS (Table 4) and that the female gender, PS=0-1 and high levels of $CD3^+CD137^+$ cells were significantly associated with longer OS evaluated after the beginning of immunotherapy (Table 5).

These three factors were integrated as part of a dynamic prognostic nomogram that evaluated the survival probability at 1 and 2 years of cancer patients treated with immunotherapy (Figure 23D). In the nomogram a final score was obtained by summing the point value of each variable. Finally, from the total points, a vertical line needed to be drawn to get the value of 1- or 2-year OS probability. The female sex corresponded to 0 points, the PS=2 corresponded to 36 points, while the levels of %$CD137^+$ T cells of 5 corresponded to 37.5 points. The total point of 73.5 corresponded to a 1- and 2-year OS of about 0.8 (80%) and 0.6 (60%), respectively. It is interesting to note that a poor OS was observed when patients showed lower values of $CD137^+$ cells.

At the end, considering the OS of cancer patients from tumor diagnosis, UVA confirmed the results described above, i.e. PS=0-1, high levels of $CD3^+CD137^+$ and $CD3^+CD8^+CD137^+$ cells characterized patients with longer survival (Figure 24C). Moreover, at the multivariate analysis, the high levels of $CD3^+CD137^+$ cells resulted as an independent predictive factor for patients' survival.

The prognostic role of $CD137^+$ T cells was validated in an indipendent cohort of 43 metastatic cancer patients (NSCLC and HNSCC) treated with anti-PD1. Univariate analysis confirmed the association of $CD137^+$ T cells cut- off=1.2) with the OS (HR 8.26, 95% CI: 1.34-12.8; p=0.001) (Figure 24A), and with the OStot (HR 6.87, 95% CI: 1.13-11.24; p=0.02) (Figure 24B) Data exhibited a favorable survival outcome for those patients with a percentage of $CD3^+CD137^+$ >1.2 before the beginning of therapy (T0). Moreover, also the frequency of $CD8^+CD137^+$ T cells (cut-off=0.8%) found in the identification cohort was confirmed as prognostic factor of PFS (HR 5.2, 95% CI: 1.02-10.59; p=0.04) (Figure 24C).

To definetely validate our results, the nomogram obtained in the identification cohort was used to evaluate the survival probability of cancer patients of the validation group. The point assignment was the same used in the identification cohort (female/male: 0/28 points; PS=0,1/PS=2: 0/36 point; the levels of %$CD137^+$ T cells of 5 corresponded to 37.5 points). The nomogram score was assigned to each patient and plotted as a Kaplan-Mayer curve (Figure 24D). Patients with a score lower than the median value showed a poor survival (p=0.01), confirming the analysis carried out in the identification cohort. The factors were the same used in the identification cohort.

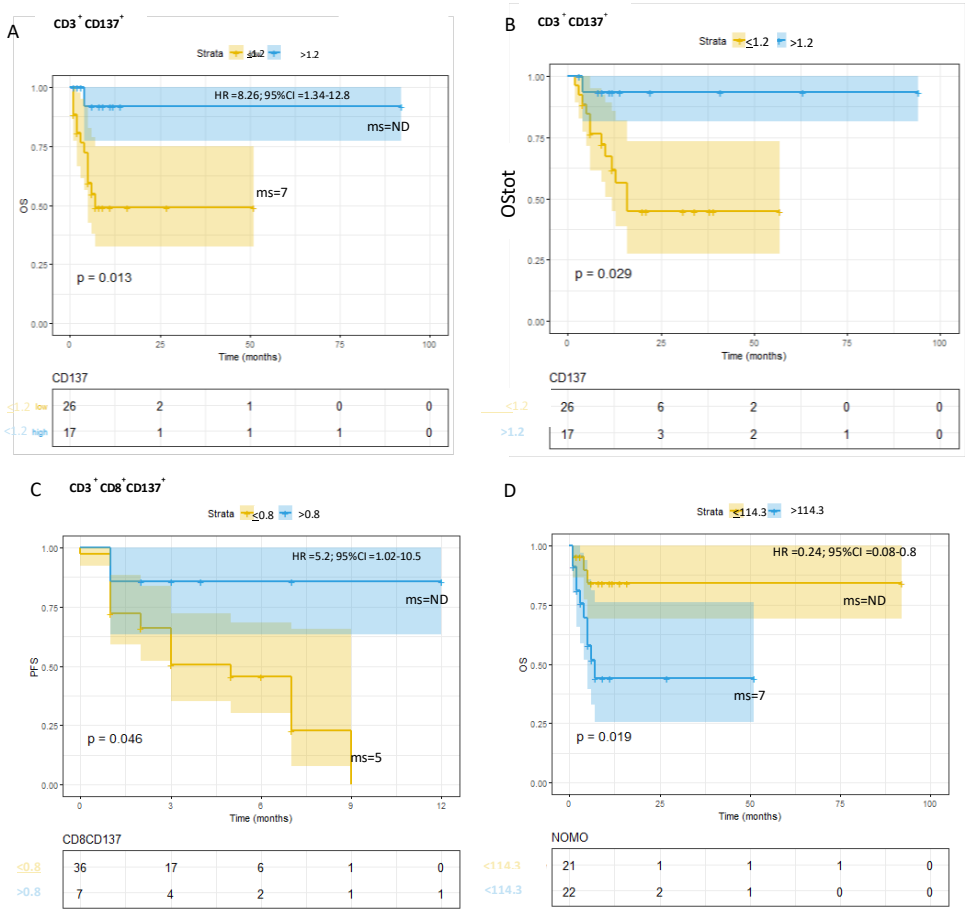

Figure 24: CD137 was validated as prognostic factor to survival.

(A) Kaplan Mayer curve of OS after anti-PD1 treatment calculated in the validation cohort considering the median percentage of CD137 (1.2%). **(B)** Kaplan Mayer curve of OS evaluated at diagnosis, analysed considering the median values of the percentage of $CD137^+$ T cells (1,2%) in the validation cohort **(C)** Kaplan Mayer curve of PFS after anti-PD1 treatment calculated in the validation cohort considering the median percentage of CD8+CD137+ T cells (0.8%). **(D)** Kaplan Mayer curve of OS evaluated in the validation cohort using the median score of 114.3 obtained assigning to each patient belonging to the validation group the nomogram score used in the identification cohort. Log-rank tests were used to compare survival between two groups. p values<0.05 were considered significant. ms=months; ND= not determined.

Cancer patients with high levels of $CD137^+PD1^+$T cells showed a longer OS

PD1 molecules is a marker of activated T cells, but in the last years has been demonstrated that its expression identify also exhausted lymphocytes [73]. Recently, it has been demonstrated that the expression of CD137 molecule plays a critical role in the discrimination of the activated and exhausted T cells identifying the $CD137^+PD1^+$ subset as the most functionally active T cells population [74]. Therefore, the double positive expression of T lymphocytes to CD137 and PD1 was analyzed in our cohort of cancer patients and in healthy donors and then correlated with clinical parameters previously described. Figure 25A showed that the frequency of $CD137^+PD1^+$T cells in HD was significantly higher (7.7% ± 3.3%) compared to cancer patients (2.7% ± 2.3%). Then, associating this population with clinical parameter, we observed that the percentage of $CD137^+PD1^+$ cells was inversely correlated with n* of metastasis (Figure 25B). However, when this population was analysed in regard to clinical response to anti-PD1 treatment, no significant differences between responders and non-responders were observed (Figure 25C).

Moreover, we assessed the frequency of $CD3^+CD137^+PD1^+$ in relation to PFS and OS observing that, before starting treatment, high levels of $CD3^+CD137^+PD1^+$ (>1.85%), were associated with a longer OS evaluated at diagnosis (figure 25D), but not with a PFS. Instead, regarding OS calculated from the start of immunotherapy we observed a difference between patients with $CD3^+CD137^+PD1^+$ percentage < 1.85% and >1.85%, even if it was only a trend and not a statistically significant difference (p=0.055) (Figure 25E).

To understand the abundance of CD137 expression in the different $PD1^+$ T cell subsets and their correlation with clinical parameters, the frequency of $CD8^+CD137^+PD1^+$ and $CD4^+CD137^+PD1^+$T cells was analyzed. The levels of $CD3^+CD8^+CD137^+PD1^+$ (0.43% ± 0.3%) and $CD3^+CD4^+CD137^+PD1^+$ (0.49% ± 0.9%) cells represent respectively the 44% and 63% of the total $CD3^+CD8^+CD137^+$ and $CD3^+CD4^+CD137^+$. Analyzing this cellular subset according to clinical outcome, we observed that responder patients showed increased levels of $CD3^+CD8^+CD137^+PD1^+$ (0.59%± 0.4%) compared to NR (0.34± 0.28) (p=0.01), while no significant difference was obtained for $CD3^+CD4^+CD137^+PD1^+$ T cell subpopulation (R vs. NR: %0.68±1 vs. % 0.34±0.73%; p=0.09) (Figure 25F). These two populations were also analyzed in regard to PFS and OS, however, no significant correlation with the survival was obtained ($CD3^+CD8^+CD137^+PD1^+$ cells, PFS: HR:1.52, 95% CI:0.87-3.1, p=0.12; OS:

HR:1.32, 95% CI:0.72-2.56, p=0.3; $CD3^+CD4^+CD137^+PD1^+$ T cells, PFS: HR: 1.3, 95% CI: 0.69-2.56, p=0.3; OS: HR:1.2, 95% CI: 0.68-2.38, p=0.6).

Finally, to evaluate the activation status of $CD8^+CD137^+PD1^+$ respect to $CD8^+CD137^-PD1^+$ this cellular subset was analyzed using the proliferation marker Ki67. As reported in figure 25G, the expression of Ki67 was significantly higher $CD8^+CD137^+PD1^+$ T cells population compared to $CD8^+CD137^-PD1^+$(49.65%±16.86 vs. 38%±19.08 respectively; p=0.004), demonstrating that the CD137 marker mainly identify those lymphocytes with the higher proliferation capacity.

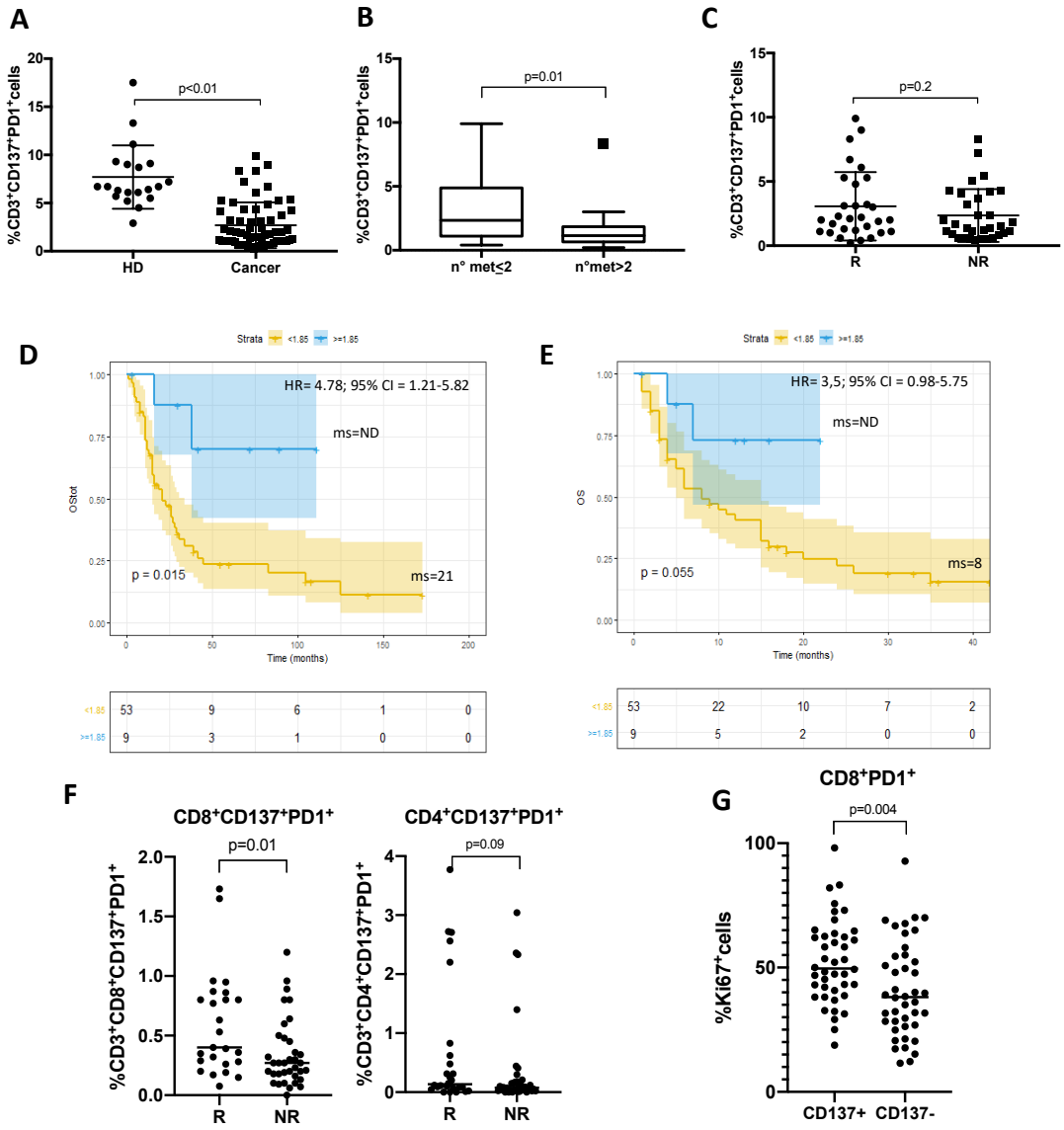

Figure 25: High levels of CD137⁺PD1⁺ T cells correlate with a better clinical status and survival

(A) The scattered dot plot represents the percentage values of circulating CD137⁺PD1⁺ T cells evaluated in 20 HD and 66 cancer patients (Cancer) ± SD by flow cytometry. The values of CD137⁺PD1⁺ T cells were calculated gating on CD3⁺PD1⁺ cells. **(B)** The tukey's box plots represent the median distribution of CD137⁺PD1⁺ T cells according to the number of metastasis (n°met) + the lowest and the largest data point excluding any outliers. **(C)** The scattered dot plot shows the percentage of CD137⁺PD1⁺ T cells in R and NR patients. Unpaired Student't test was used to compare the different groups **(D)** Kaplan Mayer curves of OS evaluated at diagnosis (OStot) considering the median value of the percentage of CD137⁺PD1⁺T cells (1.85%). **(E)** Kaplan Mayer curves of OS evaluated from the beginning of immunotherapy (OS). **(F)** The scattered plots show the percentage of circulating CD3⁺CD8⁺CD137⁺PD1⁺and CD3⁺CD4⁺CD137⁺PD1⁺ cells in R and NR patients to anti-PD1 treatment. **(G)** Percentage of Ki67 expression on CD8⁺CD137⁻PD1⁺ T cells and CD8⁺CD137⁺PD1⁺ T cells. p values <0.05 were considered significant. ms= months; ND=not yet defined.

High levels of sCD137 in serum are associated with a poor survival

Several evidence demonstrated the existence of a soluble form of CD137 and its negative role in immune response [67]. Thus, levels of this molecule were measured at baseline in serum of 66 cancer patients and then associated with survival. As expected, the sCD137 didn't correlate with CD137 expressed on the plasma membrane of T cells. Indeed, no correlation of sCD137 with $CD137^+$ (r = -0.26, 95% CI: -0.53-0.05, p=0.09), $CD8^+CD137^+$ (r = -0.2, 95% CI: -0.47-0.41, p=0.09) and also with overall $CD8^+$ (r = 0.1, 95% CI: -0.19- 0.41, p=0.4) T cells was found.

Patients with a serum concentration of sCD137 >158 pg/mL showed a shorter PFS and OS calculated both from the beginning of anti-PD1 therapy (OS) and from tumor diagnosis (OStot) (Figure 26A, B, C, respectively), confirming the negative impact of this molecule on the clinical outcome.

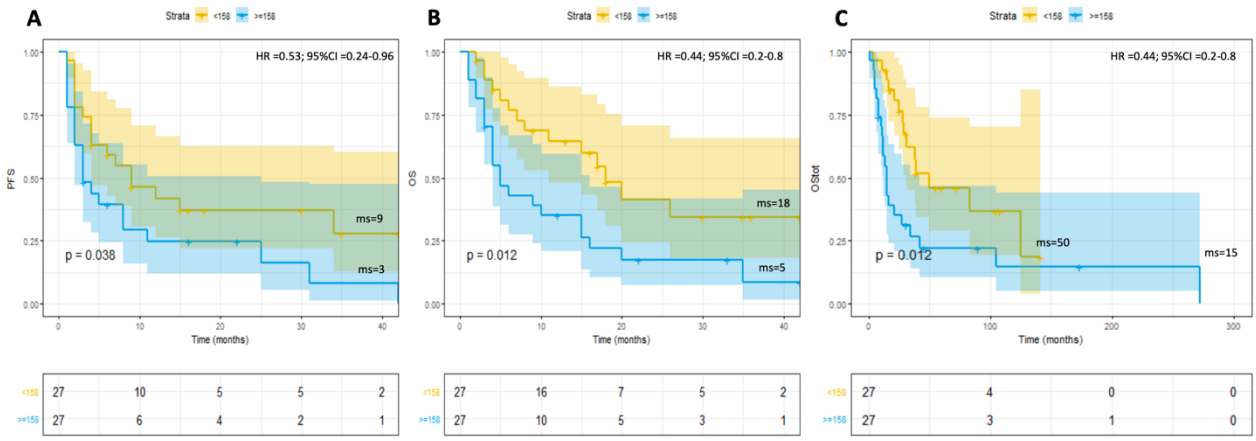

Figure 26: Low concentration of sCD137 is correlated with a better survival.

Kaplan Mayer curves of PFS after anti-PD1 treatment **(A)** and Overall Survival evaluated at the beginning of anti-PD1 treatment (OS) **(B)** and at diagnosis (OStot) **(C)** calculated considering the concentration median values of sCD137 (158 pg/mL). Log-rank tests were used to compare survival between two groups. p values<0.05 were considered significant. ms=months.

The presence of CD137$^+$ T cells in the tumor microenvironment appears to be associated with a complete pathological response to immunotherapy

At the end, to understand whether circulating cells correspond to lymphocytes in the tumor nests, the expression pattern of CD137$^+$T cells in the tumor microenvironment was analyzed in tumor samples from three oligometastatic NSCLC patients who underwent radical surgery to achieve the complete local control after immunotherapy treatment. As pictured in figure 22, a detailed histological examination of tertiary lymphoid structure (TLS) showed the distribution of CD137$^+$, CD8$^+$ and CD4$^+$ cells in these representative TLSs. Results demonstrated that the distribution of CD137$^+$ T cells in the tumor microenvironment appeared to differ among patients in relation to the response to immunological treatment. Indeed, Patient 1 showed a complete pathological response with a high tumor regression grade. The tumor bed was characterized by proliferative fibrosis, neovascularization, and high numbers of TILs and TLSs.

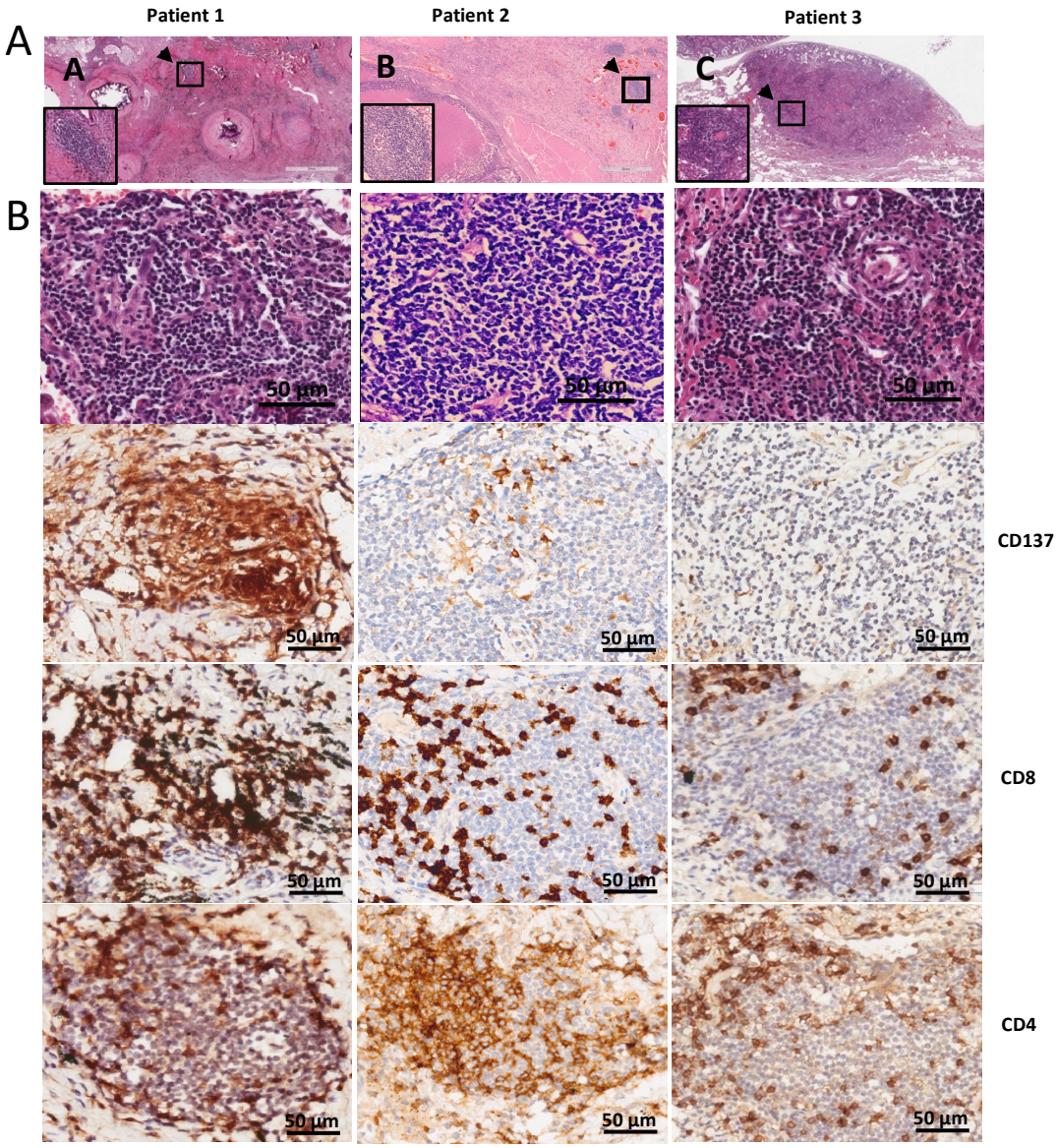

Figure 22: Paraffine tumor slides derived from three NSCLC patients with different responses to anti-PD1 treatment. (A) Patient 1 (first column): Pathologic complete response with diffuse proliferative fibrosis, calcifications, cholesterol clefts and intense inflammatory infiltrates with several tertiary lymphoid follicles (TLF) (arrows, insert); Patient 2 (second column): pathologic partial response with a small nest of residual cancer (left) associated with proliferative fibrosis, necrosis, inflammatory infiltrates with foamy macrophages and occasional TLF (arrows, insert); Patient 3: Absence of pathologic response: scanty interstitial inflammatory infiltrates and rare TLF within neoplasia (arrows, insert). Original magnification 1x**. (B)** Patient 1: TLF from a lung cancer sample with pathological complete response show a high number of lymphocytes positive for both CD137 and CD8. A small number of $CD4^+$ T lymphocytes is also present; Patients 2: TLF from a lung cancer sample with pathological partial response show a lower number of lymphocytes positive for both CD137 and CD8 as compared to case 1 while the amount of $CD4^+$ T lymphocytes is higher; Patients 3: tertiary lymphoid follicles from a lung cancer sample with absence

The representative TLS found around the tumor showed a high number of $CD137^+$ and $CD8^+$ cells suggesting the involvement of cytotoxic $CD137^+$ cells in the elimination of tumor cells. Patient 2 showed a pathologic partial response with a small nest of residual cancer associated with proliferative fibrosis, necrosis, inflammatory infiltrates with foamy macrophages and occasional tertiary lymphoid follicles. TLS had a low infiltration of $CD137^+$ and $CD8^+$ T cells compared to patients 1. Patient 3 classified as non-major pathological response (non-MPR), showed a very limited pathological response, with extensive residual neoplasia. The residual tumor was characterized by the presence of sparse inflammatory infiltrates with rare TLSs. The number of both $CD137^+$ and $CD8^+$ cells in the TLS was scarce. The amount of $CD4^+$ T lymphocytes was similar in the three patients. Interestingly, the number of lymphocytes positive for both CD137 and CD8 within the inflammatory infiltrates was inversely related to the extent of pathological response, while $CD4^+$ T cells seemed to not correlate with the response. Similar results were obtained analyzing the TILs derived from two patients who suffered from cutaneous melanoma and HNSCC (Figure 23) treated with immunotherapy and chemotherapy, respectively, before surgery. The melanoma patient showed a complete response and exhibit high levels of infiltrating $CD137^+$ and $CD8^+$ T cells in the TME. The HNSCC patient, with a pathological incomplete response (non-MPR), showed a low level of $CD137^+$ and $CD8^+$ T cells in the tumor bed. These data further suggested that the presence of $CD137^+$ cells is strongly associated with tumor regression and with response to therapy independently by the type of treatment.

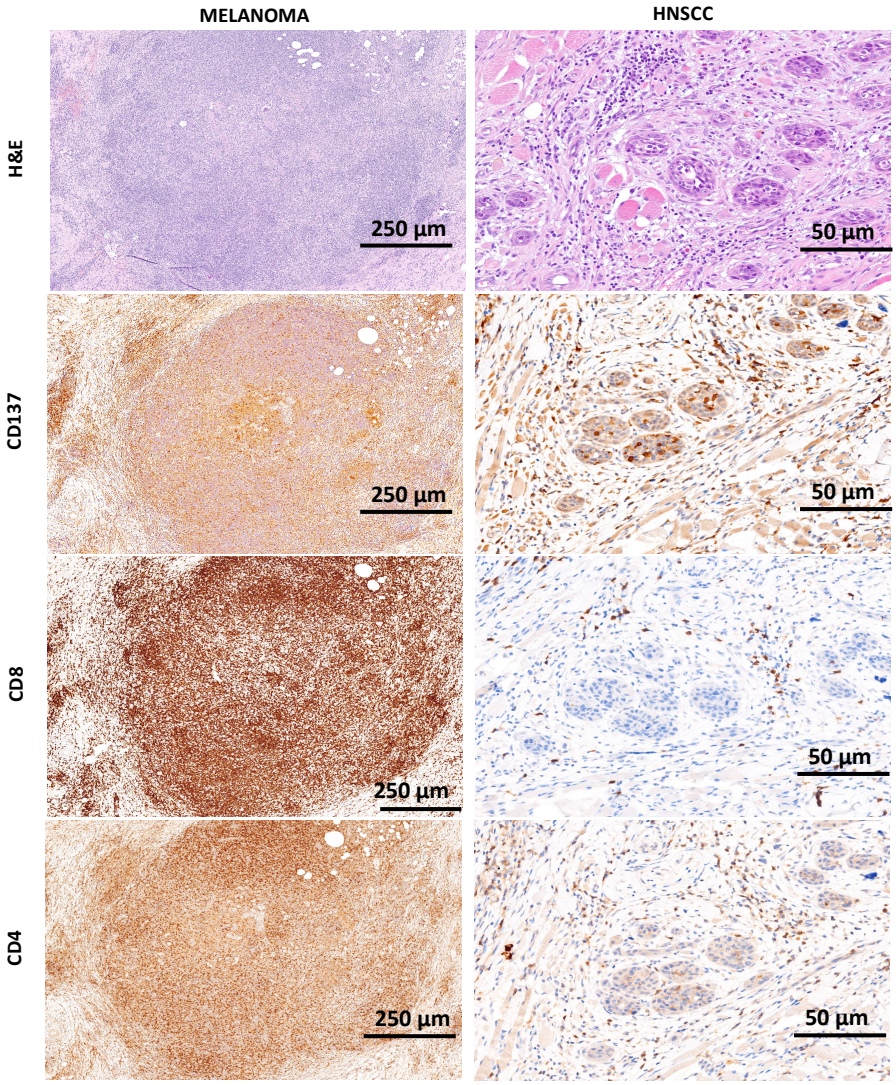

Figure 23: TILs analysis in melanoma and HNSCC tumor slide.

Surgical sample of melanoma (first column) with pathological complete response to immunotherapy showing a marked inflammatory infiltrate with abundant TLFs. A high number of lymphocytes positive for both CD137 and CD8 are shown. A small amount of $CD4^+$ T lymphocytes is also present. Original magnification 4X; Surgical sample of squamous cell carcinoma of the oral cavity (second column) with pathological incomplete response. Inflammatory infiltrates are scanty with low number of lymphocytes positive for both $CD137^+$ and $CD8^+$ cells; a small amount of $CD4^+$ T lymphocytes is present. Original magnification 20X.

Discussion

The immune profile of each individual is the result of their own immune history such as lifestyle, the encounter with pathogens, age, gender, microbiota composition and several other factors including predisposing and heritable conditions. This complexity further increases in cancer patients. We believe that the wellness of immune system represents a crucial point to define the success of anti-cancer immunological treatment.

Despite the exciting immunological treatment that, targeting the immune system, contributes to modify the activation state of the immune cells, a high percentage of patient doesn't benefit from this type of therapy and develops toxicities or adverse events. In this scenario the identification of biomarkers predictive and prognostic could be a useful tool to maximize response to treatment. Beyond the most known and validated biomarkers, it's fundamental identify new biomarkers with a simple and not invasive method, thus peripheral circulating biomarkers come into play.

In this project we investigated, across different tumor types, the role of peripheral immune cells and circulating immune molecules in patients treated with TKIs and immunotherapy, evaluating patient's immune profile at baseline and during treatment, to figure out whether eventual changes could impact on clinical outcome.

Our results suggest that TKIs and ICIs modulate the release of several sICs respectively in mRCC and in NSCLC patients. In mRCC patients, several sICs decreased during treatment (i.e. sPDL2, sHVEM, sPD1, and sGITR), but sPDL2 resulted the unique biomarker downregulated in responder patients during TKIs therapy. This data is supported by a previous work that identified sPDL2 as significant predictive biomarker of recurrence risk in ccRCC [66].

Instead, in NSCLC patients Nivolumab affected the decrease of sPD1 and sPDL1 during therapy but only sPD1 resulted reduced in responder patients during treatment. The role of sPD1 is the most discussed and it is also controversial. Several studies suggested a positive role in anti-tumor immunity to PD1, hypothesizing that the binding between soluble PD1 and the membrane-bound PDL1/PDL2 might prevent T cell inhibition [72] and that high levels of this soluble protein are associated with longer PFS and OS in NSCLC patients after two cycles of Nivolumab[73]. On the contrary, other studies describe soluble PD1 as a negative regulator: low levels of PD1 favor the activation of the immune system inducing the maturation of dendritic cells and decreasing the threshold of T cell activation[74].

The importance of biomarker's identification is highlighted not only at baseline, but also during treatment. In fact, we observed low levels of soluble immune-related proteins in mRCC and NSCLC responder patients during treatment. We demonstrated that IFNγ, sPDL1 and sCTLA4 play important roles in regulating the response to TKI treatment in mRCC patients. Interestingly, we observed that low levels of IFNγ correlated with the response to TKI therapy, both at baseline and after 3–4 months from starting treatment and that at baseline they seem to be associated with a better PFS. IFNγ has a dual and opposite role both as anti- and pro-tumor cytokine[75]. It induces various genes, such as PDL1, PDL2, CTLA4, and IDO, involved in cancer cells immune evasion. [76] Our results showed that the presence of sPDL1 and sCTLA4 is associated with a poor response to TKI confirming their role for poor prognosis and failure to response during TKI therapy.

Likewise, our data in NSLCS group showed low levels of several sICs in responder patients during treatment, that are also associated with duration of response to Nivolumab (PFS).

Moreover, the efficiency of immune system could be related to clinical condition of cancer patients like performance status that represents a clinical measure to establish cancer progression.

Most studies do not enroll patients with poor PS (PS ≥ 2) because this is a negative prognostic factor for response to treatment and survival and a predictive factor of adverse events [77]. Patients belonging to PS ≥ 2 group had moderate or severe comorbidities, frequently required the use of antibiotics or corticosteroid which influence the activation of the immune response and the response to ICI. In our study, sICs seem to be modulated by Nivolumab treatment according to patients' PS. NSCLC patients with a PS = 0 were linked to a better immunological fitness at the beginning of therapy. Nivolumab seems to perform its immunoregulatory function more effectively in this setting of patients reducing PD1 and maintaining low levels of several sICs with immunosuppressive functions.

In addition, an important district that could influence the immune systems response is represented by gut microbiota and its metabolomic profile.

In NSCLC setting we investigated the metabolomic profile of R and NR patients observing differences in microbial composition associated respectively to eubiosis and dysbiosis. R patients showed higher levels of SFCA, whose production is essential for gut integrity by the regulation of the luminal pH, action on mucosal immune function and mucus production. NR patients showed a prevalence of dysbiosis-associated metabolites like alcohols, the principal mediators to the

development of non-alcoholic steatohepatitis (NASH), and aldehydes that could promote mutagenesis.

Due to the strict correlation between bacterial metabolism and immune fitness, it is conceivable to believe that the presence of eubiosis-related compounds in responding patients contributes to maintaining optimal the wellness of the immune fitness, thus resulting in a better and durable response to Nivolumab treatment. Eubiosis, together with sICs and performance status could be used as possible biomarkers of response in NSCLC patients.

The other important parameters evaluated in this project are the circulating immune cells and their role in cancer patients treated with immunological therapies.

In particular, we identified the $CD137^+$ T cells, as a predictive biomarker of response to therapy. We showed that mRCC patients who benefited from TKI treatment had high percentage of $CD3^+CD137^+$ T cells and $CD8^+CD137^+$ T cells both at baseline and during therapy, observing that the maintenance of $CD8^+CD137^+$ T in circulation is associated with a better PFS. Numerous studies have demonstrated that although both activated $CD4^+$ and $CD8^+$ T cells express CD137, signals through CD137 are more biased toward CD8+ T cells, both in vitro and in vivo.

In line with the analysis in mRCC patients, we proposed the $CD137^+$ T cell subset as a driver of successful anti-tumor therapy in a larger cohort of patients treated with immunotherapy demonstrating that it could define the "quality" of the immune activation thus predicting the patients' clinical outcome independently from tumor histotype, previous therapies, as well as toxicity. Indeed, we showed that the frequency of $CD3^+CD137^+$ is higher in healthy donors and in those patients with a better clinical status, who presumably have a fully or less dysregulated active immune system. The $CD137^+$ T and in particular $CD8^+CD137^+$T cells subset seem to have a crucial role in response to anti-PD1 therapy, in fact high levels of these cells correlated with survival and have been identified as independent prognostic factors. The $CD4^+CD137^+$ cells influence the patients' survival but resulted not associated with response. As reported above, several studies underlined the main involvement of $CD8^+CD137^+$ population compared the $CD4^+CD137^+$ subset. These evidences are supported by anti-CD137 agonistic antibodies that promote the expansion of $CD8^+$ T cells in several diseases including cancer [78]. These therapies not only are positively correlated with clinical outcome, by increasing the frequency of anti-tumor specific memory T cells

that provides a long-lasting anti-tumor immune response, but they also enhance the recruitment of specific lymphocytes in TME that act by reducing Treg and MDSC[79,80].

Interestingly combining the levels of $CD137^+$ T cells with PS and patients' gender in a nomogram analysis, could identify the profile of the patients who will benefit from immunotherapy in terms of survival. Indeed, a poor OS is observed in patients that had low expression of CD137 on T cells. All these studies confirm the hypothesis that the frequency of CD137 represents a key point to obtain an efficacious anti-tumor immune response.

Later, we analyzed the frequency of these cells also in TME observing the distribution of $CD137^+$ T cells in the Tertiary Lymphoid Structures that surround the tumor of three NSCLC patients with a different clinical outcome. We identified high levels of $CD137^+$ and $CD8^+$ T cells only in TLS of patients with a complete tumor response, confirming the hypothesis that the intensity of the anti-tumor immune response is strictly correlated with the amount of $CD137^+$ T cells in the tumor bed.

Our data were supported by previous studies that analyzed the distribution of CD137 in milieu identifying this molecule as a biomarker to detect and isolate the full repertoire of tumor-specific $CD8^+$ T cells distributed in the tumor site.

$CD137^+$ T cells have been also analyzed for the expression of PD1 molecule. The $CD137^+PD1^+$ and $CD8^+CD137^+PD1^+$ subsets were associated with the response to anti- PD1 therapy, and with a longer OS. The co-expression of these two molecules identifies those lymphocytes that exhibit a higher tumor reactivity. For instance, in the hepatocarcinoma, $CD137^+PD1^{high}$ T cells show a transcriptomic profile correlated with a T cell activation and a high proliferative capacity[81]. Our data showed that $CD8^+CD137^+PD1^+$ had a higher proliferation capacity when compared to $CD8^+CD137^-PD1^+$ cells (evaluated by the expression of Ki67 marker), demonstrating the existence of several T cell populations with different levels of activation based on the expression of CD137 and PD1, and suggesting the critical role of CD137 markers in determining the activation state of T cells.

CD137 could be released as soluble form, an immunoinhibitory molecule produced by overactivated immune cells, including Treg that allow a faster tumor progression in vivo[82]. In hypoxic conditions, several cancer cells release high levels of this molecule independently by the expression of the membrane-bound CD137, suggesting its beneficial effect for cancer survival.

In this study we evaluated the concentration of sCD137 in our cohort of cancer patients, observing that high levels of this molecule are associated with a shorter PFS and OS, but no correlation was found between sCD137 and several $CD137^+$ T cell subsets.

All these results identified and validated the role of $CD137^+$ T cells as a biomarker of immune wellness able to predict the success of anti-cancer immunotherapy.

Conclusion

The results obtained in this project highlighted the importance of the immune profiling, in order to identify predictive biomarkers of patients' clinical outcome as well as to elucidate why some patients fail to respond to immunological treatment. Moreover, we observed that these therapies have a strong impact on immunological components, and they contribute to changes patients' immune system in the course of treatment, underlying the importance of a comprehensive and longitudinal immune monitoring as a dynamic process.

Due to the urgent need to increase the number of responding patients treated with immunological therapies we believe that these prognostic and predictive biomarkers could help clinicians in the decision-making. Moreover, these findings translated into large-scale studies, using a network-based approach that includes clinical and experimental data, could be the basis for a personalized medicine.

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