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TITLE

Immune-Biomarkers In Advanced Non-Small Cell Lung Cancer

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Abstract

Background: Developing personalized immunotherapy-based approaches, through the identification of predictive immune-related biomarkers, is still a main topic for translational lung cancer research.
In the present study we investigated whether baseline tissue “immune-related gene signatures”, as well as plasma chemo-cytokines profiles, could predict sensitivity/resistance to immune-checkpoint inhibitors in pre-treated patients with advanced non-small cell lung cancer (NSCLC).

Methods: From September 2015 to September 2018, 150 patients with previously treated advanced NSCLC who received either anti-PD1 or anti-PD-L1 inhibitors in the second-third line setting were included within this translational study. All the patients underwent CT-scan every 6 cycles and responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST). Gene expression analysis was performed on 86 FFPE tissue samples by using the nCounter® PanCancer IO360™ Panel applied on NanoString platform, in order to analyze 770 genes involved in key immuno-oncology pathways. Peripheral blood samples were obtained from 57 patients at baseline and 37 patients at the 6^{th} week under IO-therapy. A panel of cytokines and chemokines (IL-6, IL-8, CXCL10, CX3CL1, CCL2, VEGF, and IFN- gamma) were quantified in plasma by Cytokine Bead Array and their association with OS and TTP was assessed by Adaptive Index Modeling multivariable analysis. NLR and PLR were also assessed for potential association with clinical outcomes.

Results: The gene expression levels of *IL12RB2*, *ESR1* and *CCL8* (*p-values* = 0.000308, 0.00162 and 0.0398, respectively) resulted significantly higher in responders versus non-responders patients. In patients with 0S $\geq$ 18 months, a significant upregulation of the *ESR1* (*p-value* = 0.00813) and *IL12RB2* (*p-value* = 2.48e-08) genes, as well as a higher score for lymphoid compartment (*p-value* = 0.0117), cytokine and chemokine signaling (*p-value* = 0.0268), costimulatory signaling (*p-value* = 0.0323), and cytotoxicity (*p-value* = 0.0412), was observed. As regards peripheral blood samples analysis, an Immune-suppressive blood index score (ISBIS) was identified clustering patients into 3 groups with progressively worsening TTP and OS. The score was composed by higher IL-8 and CCL-2 levels, higher NLR, and lower IFN-gamma level. The differences among both PFS and OS Kaplan Meyers curves across the different subgroups were statistically significant ($p < 0.0001$).

Conclusion: These data suggest that the systemic balance between neutrophil-related inflammation and lymphocyte anti-tumor immunity may condition response to immunotherapy in lung cancer, with clinical benefit primarily occurring in patients with such a preexisting, intra-tumoral T-cell adaptive immune response. Correlative tissue immune gene signatures as well as circulating cyto-chemokine emerged as potential indicators of a T cell–inflamed phenotype necessary for the clinical activity of PD-1/PD-L1 inhibitors.

Summary

Although the recent advent of immune-checkpoint inhibitors in the clinical setting produced a dramatic increase of long-term survival for advanced NSCLC patients, only a limited group of them achieved durable response in the real-word practice. Thus, the identification of immune predictive biomarkers driving patients' selection remains a crucial issue to optimize the clinical management of NSCLC patients.

In this study we performed a systematic collection of clinical data, tumor tissues and/or peripheral blood samples from two different cohorts of advanced NSCLC patients receiving ICIs as second-third line treatment, across different Italian Institutions, for the identification of immune-related biomarkers associated to clinical response/resistance to ICI therapies. Gene expression analysis was performed on FFPE tissue samples by using the nCounter® PanCancer IO360™ Panel, showing different levels of selected genes, key immunological pathways and cell type profiling between responders and non-responders patients. Conversely, baseline circulating immune cito-chemokines levels as well as peripheral blood count significantly correlated with survival outcomes of NSCLC patients under ICI-therapies. These data suggest that correlative tissue immune gene signatures as well as circulating cyto-chemokine profiles could be considered as potential indicators of a T cell–inflamed phenotype necessary for the clinical activity of PD-1/PD-L1 inhibitors in NSCLC patients.

CHAPTER 1

Background, Rationale and Objectives

1.1 Introduction

Lung cancer has been historically considered a poorly immunogenic disease because of the few evidence of immune responses in affected patients and the limited efficacy of immunomodulating strategies, including vaccines [1].

Nevertheless, the recent understanding of the molecular mechanisms leading to cancer immune evasion has allowed to develop a new class of drugs, known as immune checkpoint inhibitors (ICIs), able to reactivate host responses and to mediate outstanding clinical benefit in different tumor types, including non-small cell lung cancer (NSCLC) [2]. Particularly the stimulation of antitumor immune response through PD-1/PD-L1 blockade resulted in a significant and durable disease control in a relevant subgroup of patients with advanced NSCLC, with a remarkable impact on overall survival (OS) and notable improvement of patients' quality of life [3].

On the basis of these initial results, PD-1/PD-L1 immune-checkpoint inhibitors (ICIs) were initially recommended for the clinical treatment of pre-treated, both squamous and non-squamous NSCLC patients. Subsequently Pembrolizumab either as single agent or in combination with chemotherapy become the new upfront standard of care for non-oncogene addicted advanced NSCLC, worldwide. The extended follow-up of the main phase III randomized clinical trials, have recently demonstrated a dramatic increase of long-term survival for a subgroup of patients receiving ICIs both in second and in first-line setting, with respectively 15% and 30% of them alive at 5 years,

compared to just 5.5% in the chemotherapy era [3, 4]. These exciting data suggested for the first time we are near to “cure” a relevant fraction of lung cancer patients affected by metastatic disease, while the immune-oncology (IO) revolution is now moving to the early stages, thus progressively increasing the number of potential candidate to ICI-therapies. Although the recent advent of ICI-chemotherapy combinations in clinical practice allowed to extend the clinical benefit of immunotherapy also to patients with low or negative tumor PD-L1 expression, however only a limited group of them achieved durable response in the real-word practice [5]. Thus, the identification of immune predictive biomarkers driving patients’ selection remains a crucial issue to optimize the clinical management of NSCLC patients. Unlike target therapies, immunotherapy modulates a complex network of molecular and cellular pathways, making the discovery of predictive biomarkers a very hard process and a challenging task for translational research. In addition, the interaction between tumor and immune system observed in preclinical models may not reflect what occurs in cancer patients, thus it does not necessarily provide reliable information for human use. Hence, the process has to rely mostly on the clinical setting and on the availability of biological samples, collected in a systematic and standardized manner, from defined patients subsets, in order to provide reliable immune-biomarkers for clinical use.

1.2 Basis of cancer immunogenicity and immune-escape

The immune mechanisms leading to the induction of a response against the tumor cells are very complex, but they can be envisaged as the result of opposite forces regulating tumor/host interaction during the different disease phases. From one side, the immunogenicity of cancer cells, in other words, the expression of ‘altered’ proteins detected by the immune system as ‘antigens’, induces the priming of antigen-specific T-cells through the processing and presentation by dendritic cells (DC) of antigenic determinants in the context of the human leukocyte antigen (HLA) molecules. Antigenic determinants in NSCLC are provided by tumor proteins ectopically induced by neoplastic transformation (as for cancer/testis antigens) [6], epithelial molecules increased in expression density (EGFR, carcinoembryonic antigen (CEA), epithelial cell adhesion molecule

(EpCAM), mucin1 (MUC1)) [7] or mutated proteins deriving from cancer genetic instability that leads to the generation of new amino acid sequences able to be recognized by T-cells as 'neoantigens' [8]. The latter groups, globally quantified by measuring the tumor mutational burden, are considered to provide the most immunogenic epitopes and to associate with the presence of more efficient antitumor immune responses in course of immunotherapy [9]. Upon the engagement of their T-cell receptor (TCR) with the antigen/HLA complex expressed on tumor cells, lymphocytes mediate tumor cell lysis through the release of cytolytic granules (containing perforin, granzyme B and proapoptotic molecules such as FasL and TRAIL) and stimulatory cytokines such as IFN-γ, IL-2 and TNF-α. Sign of this reactivity is the presence of a T-cell infiltrate, most commonly composed by CD8+ T-cells that is often detected in solid cancers, including NSCLC, as linked to good prognosis [10], indicating that even in patients with clinically evident cancer, an active immune response can contribute in slowing-down disease progression. Nevertheless, when this immunity is unable to clear the tumor, it drives instead a sort of chronic 'evolutionary' process that causes the selection of immune-resistant tumor cell clones, according to a mechanism known as immunoediting, and including the onset of antigen- or HLA-loss tumor cell variants [11]. Concomitantly, multiple immune escape strategies take place within the tumor microenvironment (TME), mainly relying on two pathways: the upregulation on T-cells of immune checkpoints (PD-1, CTLA-4, TIM3 and LAG3) that, binding to cognate ligands also induced on tumor and stroma cells by IFN-γ, deliver inhibitory signals to T-cells causing their reversible paralysis (exhaustion) [12]; the accrual at tumor site of immune regulatory cells that exert suppressive activity on T lymphocytes and block their function in the attempt to maintain tissue homeostasis. To the latter belong regulatory T-cells (Tregs) and the large and heterogeneous family of myeloid-derived suppressor cells (MDSCs). Both cell subsets belong to the physiological process of immune homeostasis and represent the response of the host to chronic inflammation, aimed at contrasting persistent immune stimulation. Specifically, Treg (CD3+CD4+, FOXP3+, CD25 high) are cells devoted to control autoimmunity [13], and they result increased in number and activation state in blood, lymph nodes and tumor site of cancer patients, including NSCLC [14]. They exert immunosuppression on effector CD8+ T-cells through multiple pathways including blocking of IL-2 synthesis, release of T-

cell inhibitory cytokines such as IL-10 and TGF-β, and high expression of CTLA-4. Tregs restrain T-cell activation and their depletion is associated with more effective and durable response to immunotherapy, particularly to CTLA-4 blockade [15].

NSCLC lesions are also enriched in myeloid cells with respect to nontumor surrounding lung tissue, including cells of monocytic, granulocytic and macrophagic phenotype [14,16]. Aside from residential alveolar macrophages, most of these elements derive from the differentiation of immature myeloid cells coming from the blood and globally defined as MDSC [17]. It is indeed largely acknowledged that chronic immune stimulation, together with different cytokines and chemokines secreted by tumor cells, molds bone marrow myelopoiesis and promotes the mobilization of late precursors in the attempt to restrain inflammation and maintain tissue homeostasis. Pathological accrual of MDSC in blood, lymph nodes and TME of cancer patients, reproducibly occurring in association with poor prognosis [18] and limited response to immunotherapy, causes potent suppression of antitumor T-cells (via TGF-β, reactive oxygen species, nitric oxide synthase and indoleamine 2,3-dioxygenase [IDO]), stroma remodeling, VEGF-mediated angiogenesis and metastatic spread. These cells, defined as monocytic (CD14+HLA-DRneg) or granulocytic (CD15+HLA-DRneg) MDSC by cytofluorimetry, are measured as monocytes and granulocytes in routine blood test and this explains the negative prognostic impact that neutrophil or monocyte absolute counts, together with the relative ratio with lymphocytes, play in patients with cancer, including NSCLC [19,20] (Figure 1). This complex immune scenario featuring TME has been hard to modulate in a consistent and pleiotropic fashion until few years ago, when immune checkpoints and their role in favoring tumor immune escape were discovered. CTLA-4 and PD-1, along with the cognate ligand, PD-L1, are indeed the common denominators of many of the multiple immune cell subsets present in the TME, whose modulation, through antagonists MoAbs, has been recently shown to tilt the immune balance toward antitumor immune responses that can control disease progression in a substantial percentage of patients.

1.3 Mechanisms of action of PD-1 and PD-L1 inhibitors

CTLA-4 and PD-1 are two inhibitory receptors expressed on T-cells that, binding to their ligands (CD80/CD86 and PD-L1/PD-L2, respectively) expressed on several types of immune and tumor cells [21,22], play a central role in suppression of T-cell activity, thus regulating immune-response against tumor cells. CTLA-4 acts in the activation stage of T-cell priming, which occurs in regional secondary lymphoid organs and is necessary to generate a population of effector T-cells against the tumor. On the other hand, PD-1 acts in the secondary stage of T-cell activation, binding its ligands on tumor cells and other stromal cells of TME [23,24]. In the last 10 years, many clinical trials on antibodies against CTLA-4, PD-1 and PD-L1 have shown encouraging antitumor activity in various malignancies, including NSCLC [18,25–29]. The blockade of PD-1 receptor can restore the activity of chronically exhausted tumor-specific T-cells. After binding with its natural ligands – PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) – PD-1 receptor can negatively regulate the immune system, promoting self-tolerance and allowing tumor cells to escape from immune control [30]. PD-1 is a 288-aminoacid type-1 transmembrane protein of the immunoglobulin superfamily, encoded by the five exon Pdcd1 gene located on chromosome 1 in mice and chromosome 2 in humans and normally expressed on B cells, T-cells and monocytes [31–33]. After interaction with its ligands, the intracellular domain of PD-1 is phosphorylated, leading to the downmodulation of the TCR signaling [34,35] and to a limitation of T-cell activity in the TME [36]. Moreover, also CD80 or B7-1 complex has been shown to bind PD-1, inducing inhibitory signals in T-cells with a subsequent anergic state [37,38]. The centrality of PD-1 in the immune regulation after chronic tumor antigen stimulation was confirmed by the first demonstration of PD-1 high expression on lymphocytes infiltrating human tumors activated by melanoma specific antigens, as reported by Fourcade et al. in 2009, and by its high presence on Treg cells that are among the most important CD4+ T-cells with suppressive function [39–45]. PD-L1 is the principal ligand of PD-1 receptor and is expressed on activated hematopoietic and epithelial cells, while PD-L2 is more present on DC and macrophages [22]. Immunohistochemical (IHC) assays have revealed PD-L1 upregulation on different tumor cells types, including NSCLC [30]. Expression of PD-L1 on tumor cells is dependent on two main mechanisms: an intrinsic expression correlated to various aberrant oncogenic signal pathways, such as phosphoinositide 3-kinase/protein kinase B,

extracellular-signal regulated kinases/mitogen-activated protein kinase and Janus kinase (JAK2)/STAT, and an induced expression dependent on inflammatory signals such as IFN-γ release in the context of antitumor immune response [28,46–49]. The adaptive mechanism seems to be more frequently involved in PD-L1 expression in TME, suggesting that the presence of PD-L1 is directly correlated to a previous activation of T-cells specific for a tumor antigen [50]. High PD-L1 expression was demonstrated on melanoma, ovarian, lung and other types of cancer cells [51–58]. Moreover, high PD-L1 expression was associated with strong presence of tumor-infiltrating lymphocytes (TILs) and IFN-γ in melanoma TME, explaining how activation of PD-L1/PD-1 pathway is an immune-resistance mechanism used by cancer cells to escape from immune-related destruction [48]. Recent studies elucidated the molecular background underlying different PD-L1 expression NSCLC subsets, showing as mutations in KRAS, TP53, and MET are significantly associated with high PD-L1 expression, while EGFR/STK11 mutations as well as WNT pathway alterations are associated with PD-L1 negativity [59, 60]. These findings can explain the rationale to use PD-1/PD-L1 ICIs in the treatment of tumors expressing high PD-L1 levels, aiming to block tumor-induced immune suppression. Indeed MoAbs directed against PD-1 or PD-L1 can block the PD-1 pathway-mediated T-cell inhibition, restoring immune response; many preclinical studies demonstrated the antitumor activity of these antibodies in murine tumor models, leading to further investigation in cancer patients [45,61].

1.4 PD-1 and PD-L1 inhibitors in advanced NSCLC

On the basis of the encouraging activity observed in pre-clinical models and early-phase studies, different MoAbs targeting PD-1 and PD-L1 have been investigated in the clinical setting, showing a great clinical benefit in several tumor types, including NSCLC.

Pembrolizumab is a humanized monoclonal IgG4-κ isotype antibody targeting PD-1 receptor. In a Phase III randomized clinical trial including pretreated patients with advanced NSCLC with at least 1% of PD-L1-positive tumor cells [62], pembrolizumab, at a dose of 2 or 10 mg/kg of body weight (mg/kg) every 3 weeks or 10 mg/kg every 2 weeks, showed a significant superiority in terms of

survival outcomes and tolerability/quality of life, over docetaxel, leading to the regulatory approval as in PD-L1 positive patients who experienced disease progression after platinum chemotherapy. Subsequently the randomized KEYNOTE-024 study demonstrated a relevant improvement of survival and quality of life in favour of the anti-PD-1 agent pembrolizumab as compared to platinum-chemotherapy (CT) in the first-line treatment of non-oncogene addicted NSCLC with tumor PD-L1 expression higher than 50%, representing the current upfront standard for about 30% of patients with newly diagnosed metastatic disease [63]. More recently, the KEYNOTE 189 and 407 randomized clinical trials [64, 65], evaluated the addition of Pembrolizumab to platinum-based CT-regimens in advanced NSCLC patients with non-squamous and squamous subtype respectively, both proving that combination strategies are able to synergistically enhance individual immune response, leading to improved clinical outcomes as compared to CT alone, regardless of PD-L1 expression levels. According to this evidence pembrolizumab is currently approved in Italy as new standard first-line treatment either as single agent (PD-L1 expression in >50% of tumor cells, as assessed by the assay IHC 22C3 pharmDx), or in combination with platinum-CT (PD-L1 expression in <50% of tumor cells, as assessed by the assay IHC 22C3 pharmDx), for metastatic NSCLC patients.

Nivolumab (IgG4 anti-PD-1 MoAb) was the first ICI approved for the second-line treatment of advanced NSCLC patients who failed previous platinum-CT. The extended follow-up of the randomized CheckMate 017 and 057 studies, comparing nivolumab over docetaxel in pre-treated, advanced squamous and non-squamous NSCLC, respectively, has recently shown a pooled 5-year survival rate of 13.4% (nivolumab) versus 2.6% (docetaxel) in the overall included population, and of 8% versus 2% in the PD-L1 negative subgroup, confirming this is a valid therapeutic option for those patients who did not received pembrolizumab in first-line, regardless of tumor histology and PD-L1 expression [66]. More recently, the preliminary results of the CheckMate 9LA trial revealed that the association of nivolumab-ipilimumab combination with a short course (2 cycles) of CT significantly improved NSCLC patients' OS when directly compared to CT alone, regardless of PD-L1 expression levels, emerging as an additional potential upfront option requiring confirmation within longer follow-up [67].

Atezolizumab is the only humanized IgG1 MoAb anti-PD-L1 available for the clinical treatment of pre-treated advanced NSCLC, in Italy. The Phase III randomized OAK trial [68] showed a significant reduction of death risk in the overall tested population, regardless of PD-L1 status, histology, age, smoking status and presence of CNS metastases. The recent update of the trial revealed a 4-year survival rate of 16% (atezolizumab) versus 9% (docetaxel) in the overall included population, and of 14% versus 5% in the PD-L1 negative subgroup, confirming this as a valid therapeutic option for those patients who did not received pembrolizumab in first-line, regardless of tumor histology and PD-L1 expression [69]. The phase III randomized IMPower-150 study evaluated the addition of atezolizumab to the carboplatin-taxol-bevacizumab regimen in advanced non-squamous NSCLC, showing a significant increase of median survival in the overall, PD-L1 unselected, population, including a small subgroup of EGFR/ALK positive patients [70]. These data supported the regulatory approval of this combination as upfront standard option in non-oncogene addicted non-squamous NSCLC, as well as in EGFR-mutant and/or ALK-rearranged patients who failed prior TKIs, in the majority of European countries (not in Italy).

1.5 Rational and Objectives

In this exciting scenario, characterized by the continuous advent of innovative drugs and treatment combinations, tumour biological heterogeneity as well as innate/acquired resistance occurrence are well-known phenomena, which significantly affect ICIs' efficacy in individual patients [71]. Therefore, developing personalized IO-based approaches, through the elucidation of the determinant of response within the tumor microenvironment and the identification of predictive biomarkers for long-term survival, is a main topic for translational lung cancer research. Early clinical response under ICI treatment is currently considered as reliable surrogate of long-lasting clinical benefit in NSCLC patients [72]. However, also subgroups of patients who achieved stable disease under ICIs could experience long-term survival, thus limiting the role of imaging assessment as reliable clinical predictor. Tumor PD-L1 expression as well as tumor mutation burden (TMB), have been largely investigated within prospective clinical studies, showing to

partially overlap between responsive and non-responsive tumors, therefore suggesting that their stand-alone evaluation has significant shortcoming and could not be able to accurately predict tumor response to ICI therapies. More recently, a plethora of additional immune-biomarkers have been explored in the context of translational studies, including tumor lymphocyte infiltrate (TILs) [73, 74], tissue genomic alterations [75, 76], and immune-gene signatures [77], as well as molecular/immunological parameters detected in the peripheral blood [78,79], of lung cancer patients. The use of integrated multivariable models including cancer genomics and immune-related signatures is emerging as a reliable predictor of clinical ICIs' response in NSCLC patients [80, 81], revealing once again the complex interplay between tumor cells signaling pathways and immune microenvironment.

In this study we performed a systematic collection of clinical data, tumor tissues and/or peripheral blood samples from two different cohorts of advanced NSCLC patients receiving ICIs as second-third line treatment, across different Italian Institutions, for the identification of immune-related biomarkers associated to clinical response/resistance to ICI therapies.

In detail the main objectives of this research were:

- To investigate baseline tissue "immune-related gene signatures" in relation with clinical response/resistance to PD-1 blockade from advanced NSCLC patients
- To investigate baseline plasma cyto-chemokines levels and/or peripheral blood immune cells count in relation with clinical response/resistance to PD-1 blockade from advanced NSCLC patients

CHAPTER 2

Patients and Methods

2.1 Study Population

Approximately 150 eligible patients with previously treated advanced NSCLC who received either anti-PD1 or anti-PD-L1 inhibitors in the second-third line setting, were included in this retrospective study and grouped as follows:

- Cohort A: 86 patients with diagnostic tumor formalin-fixed paraffin embedded (FFPE) tissue specimens available for comprehensive tumor-immune microenvironment transcriptomic analysis
- Cohort B: 57 patients with pretreatment and/or on-treatment plasma samples available for cyto-chemokines and/or peripheral immune blood cells analysis

We retrospectively collected clinical and pathological data as well as routine blood tests from patients' charts and electronic medical records for eligible patients who have been treated at 7 different Italian institutions from September 2015 to September 2018. All included patients were followed until the end of data collection on December 2019.

Patients included in the analysis received ≥1 dose of either anti-PD1 or anti-PD-L1 inhibitors. The treatment was continued until disease progression or unacceptable toxicity, or the completion of permitted cycles (≤24 months). Radiological evaluation of treatment efficacy by CT-scan was performed at week 12 and every 12 weeks thereafter until disease progression and responses were evaluated by Response Evaluation Criteria in Solid Tumours (RECIST) v1.1. The study was

conducted in accordance with the International Conference on Harmonization Guidelines on Good Clinical Practice and the Declaration of Helsinki.

2.2 Samples collection and analysis

2.2.1 Tissue collection and analysis

Tissue collection

For patients included in the cohort A, either surgical or fine needle aspiration (FNA)/core biopsy tumoral tissue specimens were stored at seven different Italian Institutions and subsequently shipped to the Oncology Department of the University of Turin for centralized analysis. Formalin-fixed paraffin embedded (FFPE) blocks, were first triaged by a thoracic pathologist and a threshold of at least 50% of neoplastic cells was set, through analysis of hematoxylin-eosin (H/E) slides under light microscopy, in order to proceed with further analysis. Then, based on the specimens cellularity, different slides (4-µm-thick) were cut and placed in eppendorf tubes for RNA extractions.

Total RNA extraction and NanoString IO360 assay

Gene expression analysis was performed on 86 FFPE tissue samples. Total RNA was isolated from 2 to 6 FFPE tissue sections (5-µm-thick), collected in a sterile Eppendorf tube (the number of sections was increased in case of scant material). RNA isolation was performed using the FFPET RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocols. Total RNA concentration was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). 300 ng of total RNA from each sample were hybridized to the nCounter® PanCancer IO360™ Panel, according to the manufacturer's instructions (LBL-10504 Sept 2017 NanoString PanCancer IO360 Gene Expression Panel Best Practices Guide for FFPE Samples; JULY 2016 MAN-10023-11_nCounter_XT_Assay_User_Manual - NanoString Technologies, Seattle, WA, USA). This panel detects the expression of 750 immune related genes and 20 housekeeping genes. One lane of each cartridge was reserved to run the PanCancer IO 360™ Panel Standard, containing a pool of

synthetic DNA oligonucleotides corresponding to the target sequence of each of the 770 unique probe targets in the panel. Hybridized RNAs were processed on the NanoString nCounter preparation station using the high-sensitivity protocol. The cartridges were then scanned on the nCounter Digital Analyzer (NanoString Technologies, USA) using maximum scan resolution.

NanoString IO 360 data analyses

The analyses were set up according to the protocol provided by the manufacturer. Expression data were normalized and analyzed with the nSolver Analysis Software (version 4.0.62), as suggested by the manufacturer's protocol (LBL-10504 Sept 2017 NanoString PanCancer IO360 Gene Expression Panel Best Practices Guide for FFPE Samples - NanoString Technologies). The means of the supplied positive controls and the geometric mean of the housekeeping genes were used to normalize the measured expression values. Additionally, the Advanced Analysis module (version 2.0.115) was used to perform differential expression analyses between two selected conditions. Briefly, for each gene a single linear regression was fit using all selected covariates to predict expression. A volcano plot was generated to display each gene's -log10 (p-value) and log2 fold change with the selected covariate. Highly statistically significant genes fell at the top of the plot above the horizontal lines, and highly differentially expressed genes fell to either side. Horizontal lines indicated various p-value thresholds. In addition, data were uploaded to ROSALIND software v3.19.0.7 (OnRamp BioInformatics Inc., San Diego, CA) for pathway analyses and cell type profiling analyses. GraphPad Prism software version 7 (GraphPad Software, Inc., San Diego, CA) was used to generate heatmaps and to perform t-test analyses (unpaired, two-tailed; statistical significance *p-value* < 0.05).

RT-PCR validation

100 ng RNA of the same samples subjected to NanoString IO360 analysis were reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The RT-PCR was performed on 10 ng cDNA with the IQ SYBR Green Supermix (Bio-Rad Laboratories), using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories), and the following cycling conditions: 3 min at 95°C for denaturation and polymerase activation, followed by 42 cycles

of denaturation (15 s at 95°C) and annealing/extension (45 s at 60°C). Signals with threshold cycles (Ct) > 40 were considered non-specific. The primer sequences, deigned with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) were: *ESR1:* 5'-CAGGATCTCTAGCCAGGCAC-3' (f), 5'-ATGATCAACTGGGCGAAGAG-3' (r); *CD274/PD-L1*: 5'-CACGGTTCCCAAGGACCTAT-3' (f), 5'-GGCCCTCTGTCTGTAGCTAC-3' (r); *IL12BR2*: 5'-CACCTCCAAGAGCTCTCCAA-3' (r); 5'-TGAGGCTCCAGTTCTTCCAG-3' (r); *S14*: 5'-GGTGCAAGGAGCTGGGTAT3' (f), 5'-TCCAGGGGTCTTGGTCCTATTT-3' (r). The relative quantitation was performed by comparing each PCR product with the housekeeping gene *S14*, using the Bio-Rad Software Gene Expression Quantitation (Bio-Rad Laboratories). In all experiments, a negative control with RNAse-free water was included. The Cts of these samples were always > 42 cycles. ER+ breast cancer tissue, $CD3^+$T-lymphocytes from healthy donors and NSCLC cells obtained from the pleural effusion of a stage IV patient, were used as positive controls for *ESR11, CD274/PD-L1* and *IL12BR2*, respectively. In each of the positive controls the Ct was significantly lower ($p< 0.001$ for *ESR1,* $p< 0.01$ for *CD274/PD-L1* and IL12BR2) than the mean Ct of the samples from the analyzed NSCLC patients.

2.2.2 Plasma collection and analysis

Plasma collection

For patients included in the cohort B, pre-treatment and/or on treatment peripheral blood samples were collected at two different Italian Institutions. In detail pretreatment blood was drawn on the same day prior to the first administration of PD-1/PD-L1 inhibitor for 57 patients, while on treatment blood was drawn on the same day prior to the fourth administration (6^{th} week) of PD-1/PD-L1 inhibitor for 37 out of 57 enrolled patients.

Blood tests were obtained within one week prior to first and fourth (6^{th} week) administration of PD-1/PD-L1 inhibitor and included the white blood cell count with lymphocyte and neutrophil counts, from which the neutrophil to lymphocyte ratio (NLR) and the platelets to lymphocyte ratio (PLR) were deduced.

Plasma was derived from whole blood EDTA specimens. Samples were stored at room temperature until processing whereby plasma was separated by low-speed centrifugation for 10 to

15 min at room temperature, aliquoted, and then frozen at −80°C. Plasma samples from enrolled patients were subsequently shipped to the Unit of Immunotherapy of Human Tumors at the Milan National Cancer Institute for centralized analysis.

Cytokine Bead Array

BD™ Cytometric Bead Array (CBA) is a flow cytometry application that allows users to quantify multiple proteins simultaneously. The BD CBA system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes. Plasma samples (50 µl) were assayed for the presence of cyto-chemokines IL-6, IL-8, TNFα, CCL2, CXCL3CL1, Granzyme B, IFNgamma, CXCL10, CD62L, VEGF-A by cytokine bead array (CBA, Becton Dickinson) according to manufacturer's instructions. Samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed by the FCAP Array software (Becton Dickinson) software.

2.3 Statistical analysis

Standard descriptive statistics (absolute and relative frequencies for categorical variables, mean, medians, standard deviation and interquartile (IQ) ranges for continuos variables) were used to describe the sample pre-post and delta characteristics.

The Mann Whitney test was used for intergroup comparisons of two independent samples while X^2 or Fisher's exact test was used for categorical values, as appropriate.

Correlation analyses were done using non-parametric Spearman's rho coefficient to investigate pairwise associations among the biomarkers and clinical variables.

ORR was defined as the combined rates of complete response (CR) and partial response (PR). DCR was defined as the combined rates of CR, PR and stable disease (SD), as assessed by RECIST 1.1 criteria.

Overall survival and progression free survival were calculated as the intervals between the date of treatment start and the date of death for any cause/relapse, with censoring occurring at the date of

the last follow up visit for event-free patients. These endpoints were described by Kaplan-Meier curves and analyzed with univariable Cox regression models.

The machine learning method AIM (Adaptive Index Modeling) was used to dichotomize the variables and built the multivariate Cox regression models. Variables are sequentially added according to a forward selection procedure and the final number of variables retained in the model is chosen by means of cross-validation.

The output is an Index Score that, based on the AIM algorithm, selects a subgroup of variables exceeding the identified cutoffs at the individual patient level.

The conventional two-sided 5% level was chosen as the threshold of statistical significance.

All statistical analyses were performed with R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

CHAPTER 3

Results

3.1 Cohort A (Tissue analysis)

Patients' characteristics

Baseline demographic and clinicopathological characteristics of 86 patients included within the cohort A were summarized in Table 1. Overall patients' median age was 64 years, the majority were male (58%), ex- or current smokers at the time of diagnosis (87%). Forty-nine patients (57%) were diagnosed at advanced (stage IV) stage, with 83% bioptic specimens harvested at baseline during the first tumor characterization. At the histological evaluation the majority of patients' tumors had adenocarcinoma subtype, with a small fraction (22%) harboring other histologies. ICIs were administered preferentially at second line treatment and in most cases patients received nivolumab. At the time of treatment initiation only a slight fraction of patients reported a poor performance status (17%) while the majority of them presented good clinical conditions. About half of the patients (53%) experienced objective response/stable disease as best response, at the time of data collection, and were therefore assigned to the "responders" group, while non-responders included patients who experienced disease progression at the first radiological evaluation. Patients' median follow-up was 25.5 months (range 4-119) at the end of data collection (December 2019).

Gene expression analyses using NanoString IO360 assay

The gene expression profile of our cohort was characterized through transcriptome analysis based on the NanoString nCounter platform. A high-level exploratory view of the data is reported in Figure

2; the heatmap of the normalized data, scaled to give all genes equal variance, was generated via unsupervised clustering through the nSolver Advanced Analysis module. Interestingly, the NSCLC tissue samples analyzed in our study were clustered into 7 different subgroups indicating a significant immune transcriptional heterogeneity within the study population.

A differential gene expression analysis was observed between male and female subjects, as shown in Figure 3A: *ESR1* and *IFI6* were up-regulated (*p-value*s = 0.013 and 0.0352, respectively) while *IL12RB2* resulted down-regulated (*p-value* = 0.0343) in females, as compared to the males samples. *IL12RB2* was up-regulated (*p-value* = 0.00306), along with *CD209* and *MET* (*p-values* = 0.0082 and 0.0187, respectively) in metastatic/biopsy as compared to the surgical samples. *ESR1*, on the contrary, resulted down-regulated in the same group (*p-value* = 0.0187) (Figure 3B). Stratifying cases according to treatment response, *C5* gene was up-regulated (*p-value* = 0.0321) in non-responder subjects, while *IL12RB2*, *ESR1* and *CCL8* (*p-values* = 0.000308, 0.00162 and 0.0398, respectively) genes expression levels resulted significantly higher in responder patients (Figure 3C). Patients were also stratified according to long-term survival (cut-off: OS $\geq$ 18 months). In patients with 0S $\geq$ 18 months, a significant upregulation of the *ESR1* (*p-value* = 0.00813) and *IL12RB2* (*p-value* = 2.48e-08) genes, was observed (Figure 3D).

Pathway score analyses using NanoString IO360

The Pathway Score analysis was interrogated to summarize data coming from pathways' genes into a single score, in order to understand if our cohort was affected by immunological pathways changes in relation to treatment response and OS.

Figure 4A shows the pathway scores mapped against response: several signatures resulted down-regulated in non-responder group. In particular, samples from patients who experienced clinical benefit showed higher scores for the following pathways: angiogenesis (*p-value* = 0.0098), antigen presentation (*p-value* = 0.0298), cytokine and chemokine signaling (*p-value* = 0.0102), cytotoxicity (*p-value* = 0.0269), immune cell adhesion and migration (*p-value* = 0.0261), interferon signaling (*p-value* = 0.0321), lymphoid compartment (*p-value* = 0.0250), matrix remodeling (*p-value* = 0.0241), myeloid compartment (*p-value* = 0.0366) and PI3K-Akt (*p-value* = 0.0369). The boxplots reported in Figure 5 summarize the single scores. The pathway scores were also mapped against OS:

patients with 0S>18 months presented a higher score for lymphoid compartment (*p-value* = 0.0117), cytokine and chemokine signaling (*p-value* = 0.0268), costimulatory signaling (*p-value* = 0.0323), and cytotoxicity (*p-value* = 0.0412) (Figure 4B). The boxplots reported in Figure 6 summarize the single scores.

Cell type profiling analyses using NanoString IO360

Gene expression profiling illustrates the fluctuation of different immunological cell types within the tumor microenvironment of analyzed NSCLC tissue samples. Through the nSolver Advanced Analysis module and ROSALIND software, the abundance of these cell populations' was assessed.

The cell type scores were statistically different between responders and non-responders *(p-value* = 0.0103). A higher expression level related to the following cell populations was identified in responder patients: macrophages (*p-value* = 0.0405), dendritic cells (*p-value* = 0.005), T-cells (*p-value* = 0.0425), CD8 T-cells (*p-value* = 0.0472) and cytotoxic cells (*p-value* = 0.0122) (Figure 7A). Statistically significant differences were also identified between patients with OS< 18 months versus 0S>18 months (*p-value* < 0.0001). In patients with 0S>18 months higher levels of the following cell type scores were detected: dendritic cells (*p-value* = 0.0172), T-cells (*p-value* = 0.0246), CD8 T-cells (*p-value* = 0.0175), Th1 cells (*p-value* = 0.0353), Treg (*p-value* = 0.0336) and cytotoxic cells (*p-value* = 0.0337)(Figure 7B). Boxplots of single cell type profiling related to clinical response and OS are illustrated in Figure 8 and 9.

RT-PCR validation

We then set up RT-PCR of ESR1, IL12RB2 and *CD274* in each sample evaluated by Nanostring analysis, with the aim of identifying the optimal conditions that make RT-PCR data comparable to Nanostring analysis in terms of sensitivity. We detected a striking direct correlation between the relative expression level of *ESR1*, *IL12RB2* and *CD274/PD-L1* as RNA molecules number detected by Nanostring and the relative expression measured in RT-PCR (Figure 10 A-B-C). A similar correlation was observed after the normalization of Nanostring data with the nSolver Analysis Software and plotting them against the relative expression obtained with RT-PCR (Figure

10 D-E-F). This data suggested that routinely performed RT-PCR on primary bioptic samples, including relative small amount of RNA (10 ng), well mirrored the Nanostring analysis.

3.2 Cohort B (Plasma analysis)

Patients' characteristics

Clinical characteristics of the 57 patients included in the cohort B were summarized in Table 2. Median age was 66 years (range 51-80); the majority of patients were males (71.1%), current or former smokers (84.4%) and exhibited an ECOG PS <2 (82.2%). The bone was the most common metastatic site (44.4%) followed by liver (37.8%), adrenal gland (28.9%) and central nervous system (CNS) (15.6%). The majority of patients (55.5%) had adenocarcinoma subtype. ICIs were preferentially administered as second-line treatment and in most of the case patients received nivolumab. Median PFS and OS in the overall population were 5 months and 12 months, respectively. Patients' median follow-up was 9.1 months (range: 0.1–29.7).

Blood immune-biomarker analysis

To identify potential baseline biomarkers associated with ICIs' effectiveness, a panel of cytokines and chemokines (IL-6, IL-8, CXCL10, CX3CL1, CCL2, VEGF, and IFN-gamma) were quantified in pre-treatment plasma samples of 57 NSCLC patients by Cytokine Bead Array and their association with both PFS and OS was assessed. Pre-treatment IL6, IL8, and CXCL10 levels were significantly higher in patients with median PFS < 5 months (Figure 11A) and OS < 12 months (Figure 11B) while increased IFN-gamma levels were found in patients with median PFS > 5 months and OS > 12 months (p=0.04) (Figure 11C). In addition an increased absolute number of neutrophil and NLR (Figure 12A), as well as of platelets and PLR (Figure 12B) were observed in patients with median OS < 12 months and PFS < 5 months (Figure 12C), while the absolute lymphocyte count was higher in the subgroup of patients with median PFS > 5 months and OS > 12 months (Figure 13).

Among 37 out of 57 patients evaluable for cyto-chemokines and/or NLR/PLR analysis after 4 cycles of ICI therapy, no major changes in the levels of immune soluble cyto-chemokines and peripheral blood cells count have been observed as compared to the baseline estimations (Figure

14). Specifically looking to the small cohort of patients experiencing hyper-progression under ICI-therapies, we observed a significant increase between on-treatments versus pre-treatments plasma levels of IL-6, IL-8, CXCL10, PD-1, NLR and PLR, as compared to the remaining population (Figure 15).

Immune-suppressive blood index score

An Immune-suppressive blood index score (ISBIS) was identified clustering patients into 3 groups with progressively worsening TTP and OS. The score was composed by higher IL-8 and CCL-2 levels (above the cut-offs of 8.58 pg/ml and 9.77 pg/ml, respectively) higher NLR (above the cut-off of 8.11) and lower IFN-gamma level (below cut-off of 11 pg/ml). Patients with score 0-1 (i.e. with none or 1 altered parameter; n= 15) displayed a median PFS of 20 months (95% CIs= 12-NA) and median OS of 23 months (95% CIs= 12-NA); in contrast patients with score 2 (2 altered parameters; n=19) showed a median PFS of 5 months (95% CIs= 3-NA) and median OS of 12 months (95% IC= 2-8); finally patients with score 3-4 (3-4 altered parameters; n=23) showed a median PFS of 2 months (95% CIs= 2-3) and median OS of 5 months (95% IC= 2-6). The differences among both PFS and OS Kaplan Meyers curves across the different subgroups were statistically significant ($p < 0.0001$) (Figures 16, 17).

CHAPTER 4

Discussion

This retrospective study aimed to identify both tissue and plasma immune-related biomarkers which may serve as reliable predictors of clinical response/resistance to ICIs-therapies in advanced NSCLC patients.

We assessed pretreatment tumor biopsy specimens, capturing immune-related gene signatures, as robust indicators of a T cell–inflamed phenotype necessary for the clinical activity of PD-1/PD-L1 inhibitors. Inspection of the gene expression profiling from analyzed NSCLC tissue samples illustrates the fluctuation of specific immunological cell types/signaling pathways within the tumor microenvironment of NSCLC patients experiencing long-term survival under ICIs therapies, including IFN-γ, cytokine and chemokine signaling, cytolytic activity, antigen presentation, lymphoid compartment, as well as inhibitory mechanisms, which modulate T-cell homeostasis. The data suggest that the response to anti–PD-1 blockade occurs primarily in patients with such a preexisting, intra-tumoral T cell adaptive immune response and these correlative gene signatures may represent a novel method for capturing the complexity of the dynamic immune response at single patient level by distinguishing between tumors with preexisting inflammatory components and noninflamed ones, a classification that is likely to be of high clinical relevance. The significant association of selected genes, such as *IL12RB2 and ESR1,* with long-term survival under ICI therapies, confirmed the pivotal contribution of IFN-γ-signaling to the definition of a T cell–inflamed microenvironment [82], but suggested also that additional aspects of T cell biology might be involved in the modulation of ICIs response at single patients' level. Indeed, the EREs (Estrogen

Response Elements) sequences linked by ESR1 are located within the promoter sequences of several immune-related genes, therefore the estrogens might directly modulate the activity of the immune system, including the PD-1/PD-L1 axis [83]. Relevant differences of immune system function in men and women are well known, relying on complex interactions among genetic, hormonal, and behavioral features, as well as commensal microbiome composition [84-86].

Preclinical studies suggest that sex hormones regulate the expression and function of PD-1 and that the hormone-mediated effects on PD-1 pathway is important for mediating autoimmunity [87].

Sex-related differences in anticancer immune response has been described in the amount and composition of intra-tumoral immune infiltrates as well as in tumor PD-L1 expression levels across a large spectrum of tumors, including NSCLC [88, 89].

The clinical question of potential sex-based ICIs' efficacy differences in cancer patients is still matter of debate, with scientific evidences rather controversial [90].

The results of recent meta-analysis, evaluating the effectiveness of anti-PD1/PD-L1 as well as anti-CTLA-4 monoclonal antibodies in patients with different solid tumors stratified by gender suggested a lower benefit of ICIs in women [91]. Conversely, female patients with advanced NSCLC seems to gain greater benefit from anti-PD1/PD-L1 agents and chemotherapy combinations [92]. Overall, the high heterogeneity of the included studies, showing inconsistent benefit from each trial, suggest the difficulty to address this debate by meta-analysis alone. Looking at the large sample size and multi-omics data from TCGA [93] and investigating sex-associated molecular differences in immune components for 22 cancer types with ≥20 samples in both female and male groups it's interesting to note that female patients with lung squamous cell carcinoma (LUSC) had significantly higher levels of immune-biomarkers, including relative abundance of activated CD4+ T cells and activated CD8+ T cells, as well as immune checkpoints and TCR richness, suggesting a sort of female-bias regarding immune features in lung cancer.

In this research context, the results of our study showed that the expression of the ESR1 gene is associated to a greater benefit of single agent PD1/PD-L1 inhibitors in pre-treated patients with advanced NSCLC. Interestingly, our analysis showed that ESR1 expression was significantly higher in the female population included in the study, revealing an additional potential

pathophysiological mechanism underlying the greater activity of PD-1/PD-L1 inhibitors in female NSCLC patients, which requires further investigation in dedicated studies.

As demonstrated in the tumor microenvironment, the analysis of circulating immune-biomarkers confirmed that a prevalence of immunosuppressive soluble factors (IL-6, IL-8) and cells is associated with worse survival outcomes, while an enrichment of activated lymphocytes and IFN-gamma signaling exert a protective effect in NSCLC patients undergoing ICIs therapies. These data suggest that the systemic balance between neutrophil-related inflammation and lymphocyte anti-tumor immunity may condition response to immunotherapy in lung cancer.

Interest in circulating biomarkers associated to immunotherapy efficacy is rapidly growing, with many potential candidates, such as soluble PD-L1 [94], blood-based TMB [95], serum chemokines/cytokines [96], ctDNA [79], and circulating immune-cell subsets [78], currently under investigation in clinical studies. In this scenario the NLR, a reliable index of systemic inflammation, represents an easy and accessible biomarker with potential application in the clinical context. Although its prognostic role in lung cancer is well established, however the clinical ability of NLR in predicting ICIs efficacy remains far from clear. The majority of available evidences suggested that high pre-treatment NLR is associated to poor response and survival in advanced NSCLC patients treated with ICIs [97-100]. Conversely, other trials did not find any significant correlation between pre-treatment NLR and clinical response to nivolumab, revealing that only NLR at 6th week was significantly associated to patients' survival [101]. More recently pre-treatment NLR has been evaluated along with other clinical-pathological features in the context of more complex prognostic scores aiming at identifying patients unlikely to benefit from immunotherapy [102]. Higher levels of interleukin 6 (IL-6) have been described as independent risk factors for poor response to PD-1 inhibition in melanoma and triple-negative breast cancer [103, 104], while a decrease in IL-6 levels under ICI therapies was associated with improved outcomes in lung cancer patients [105]. IL-6 has immunosuppressive functions and may drive a myeloid compartment that contributes to innate treatment resistance, such as the accumulation of T-cell suppressing neutrophils as was observed in mouse models of IL-17 and KRAS-driven NSCLC sensitive to IL-6 depletion [105].

Soluble factors are strictly linked to circulating blood immune cells, whose absolute number or reciprocal ratios could be implemented to potentiate the already known predictive/prognostic value. In this regards the use of machine-learning multivariate approaches is effective to build multifactorial scores in which the contribution of the single immune variables implements the predictive value. In particular, in our cohorts the ISBIS, including baseline plasma levels of IL-8, CCL2, IFN-gamma and NLR was able to predict resistance to nivolumab in pre-treated patients with advanced NSCLC, suggesting a potential role in the real-time monitoring of immunotherapy resistance. Validation of these results in independent cohorts is crucial and is currently proceeding with other retrospective sample sets.

Conversely, dynamic (on-treatment) immune-biomarkers seem more difficult to capture reliable predictive/prognostic information in our cohort, likely because the need to follow kinetics of immune response in a tighter fashion. Further research with larger patient cohorts sampled at uniform early on-treatment timepoints is needed to understand the predictive accuracy of plasma cytokines and NLR decreases for radiological evaluation.

This study has some limitations, including the retrospective design, the low number of patients, and the heterogeneity of clinical-pathological characteristics, which may have produced selection bias impairing survival outcomes. Nevertheless, we performed a thorough evaluation of a real-world series providing preliminary evidence on the potential role of both tissue and circulating immune-biomarkers, which require further investigation in prospective studies.

In conclusion, considering that the systemic balance between neutrophil-related inflammation and lymphocyte-mediated anti-tumor immunity may influence clinical response to immunotherapy, the systematic assessment of an individual patient's immune repertoire in tumor microenvironment as well as in the peripheral blood may provide useful insights to the personalization of immunotherapeutic approaches in NSCLC patients.

CHAPTER 5

Tables and Figures

Table 1. Cohort A: Baseline Patients' Characteristics

Characteristics	%	All patients (n=86) n.
Age		
30-49	7	6
50-69	66	57
70-89	27	23
Gender		
Female	42	36
Male	58	50
Smoking		
Never	13	11
Ex/Current	87	75
TNM stage*		
IB-IIIC	43	37
IV	57	49
Tissue timing		
Baseline	83	71
Re-biopsy	17	15
Histology		
Adenocarcinoma	78	67
Non-adenocarcinoma	22	19

ECOG-Performance Status		
0	26	22
1	57	49
2	17	15
Treatment line		
2nd line	59	51
3rd line	41	35
ICIs		
Nivolumab	76	65
Pembrolizumab	24	21
Best response		
CR+PR+SD	53	46
PD	47	40
OS at 18 months		
> 18 months	34	29
< 18 months	66	57

Table 2. Cohort B: Baseline Patients' Characteristics

Characteristics	All patients (n= 57) n.	%
Median age (years - range)	66 (51 - 80)	
Gender		
Male	40	71.1
Female	17	28.9
Histology		
Adenocarcinoma	31	55.5
Non-adenocarcinoma	26	44.5
Smoking history		
Never	9	15.6
Current/Former	48	84.4
ECOG-Performance status		
<2	47	82.2
≥2	10	17.8
Stage IV subgroup		
IVA	14	24.4
IVB	43	75.5
Metastatic sites		
Bone	25	44.4
Liver	21	37.8
Adrenal	16	28.9
Brain	9	15.6
Prior line of therapy		
<2	28	48.9
≥2	29	51.1
ICIs		
Nivolumab	41	71.6

Pembrolizumab	12	22.2
Atezolizumab	4	6.2
Median survival **PFS: 5 months (95% CI: 3-7)** **OS: 12 months (95% CI: 6-24)**		

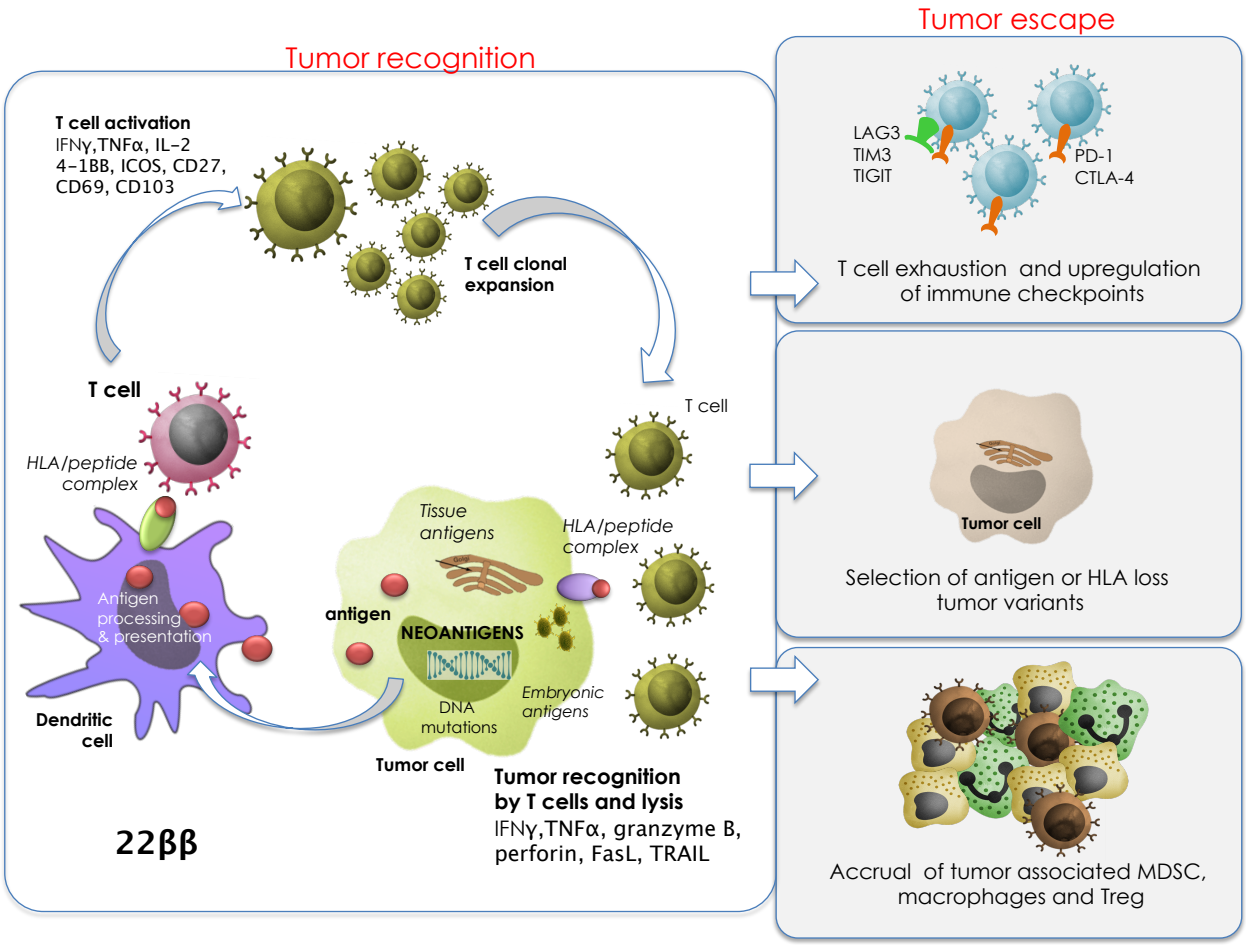

Figure 1. Basis of cancer immunogenicity and immune-escape

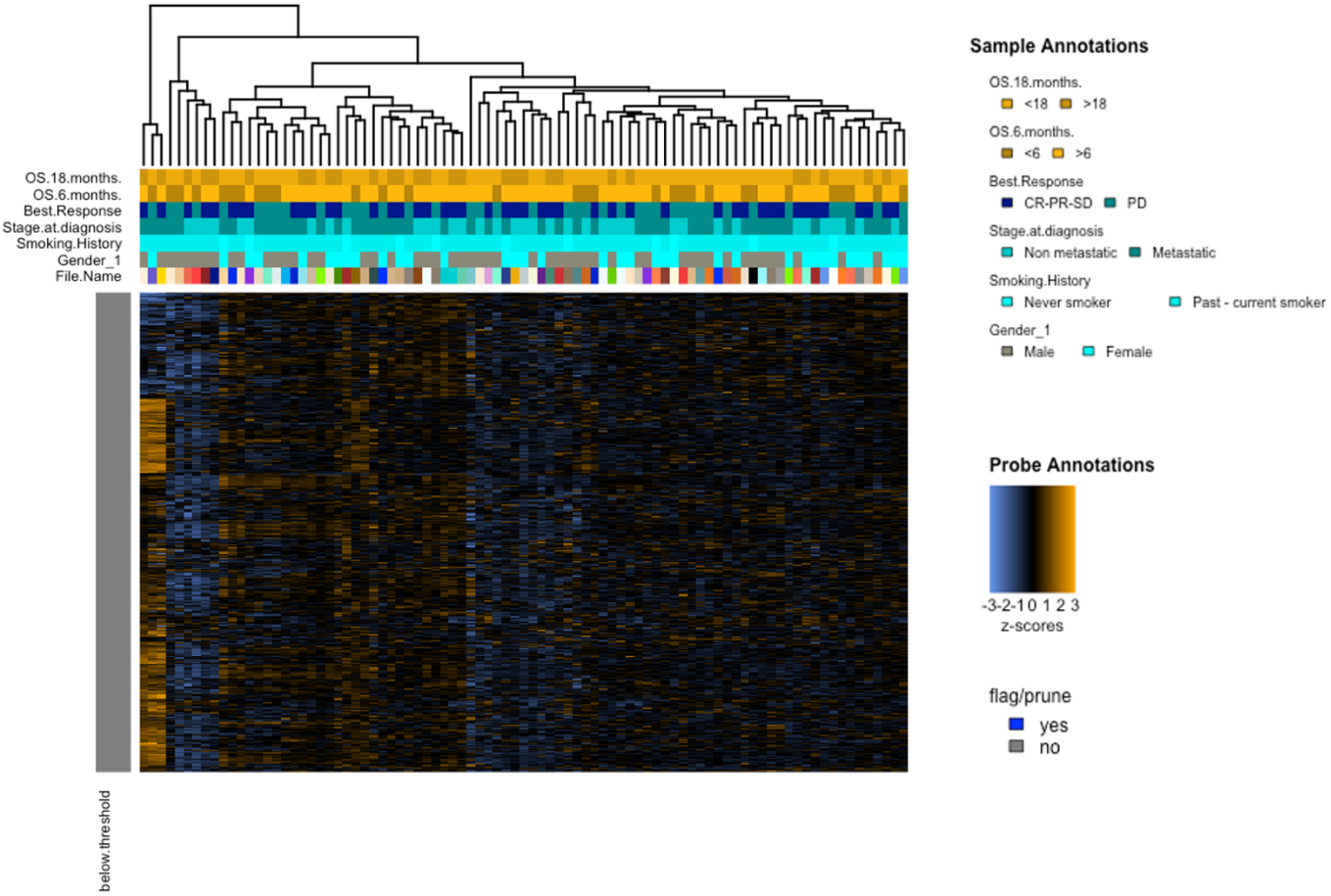

Figure 2. Gene-expression profile of NSCLC samples by NanoString IO360 assay

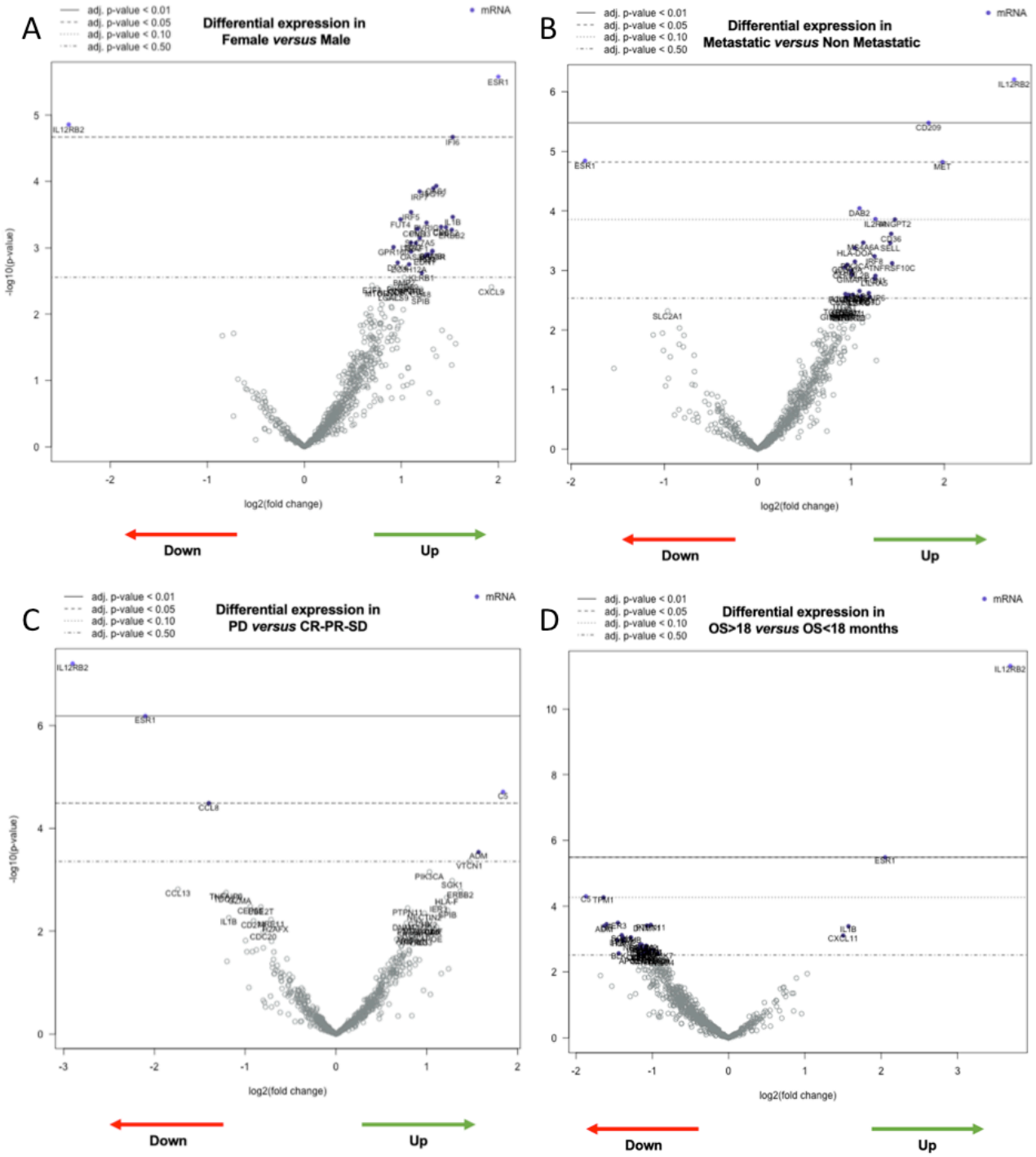

Figure 3. Gene-expression profile of NSCLC samples by NanoString IO360 assay comparing: A. Female versus Male; B. Metastatic versus non-metastatic disease; C. Progression versus non-progression disease; D. OS > 18 months versus OS < 18 months

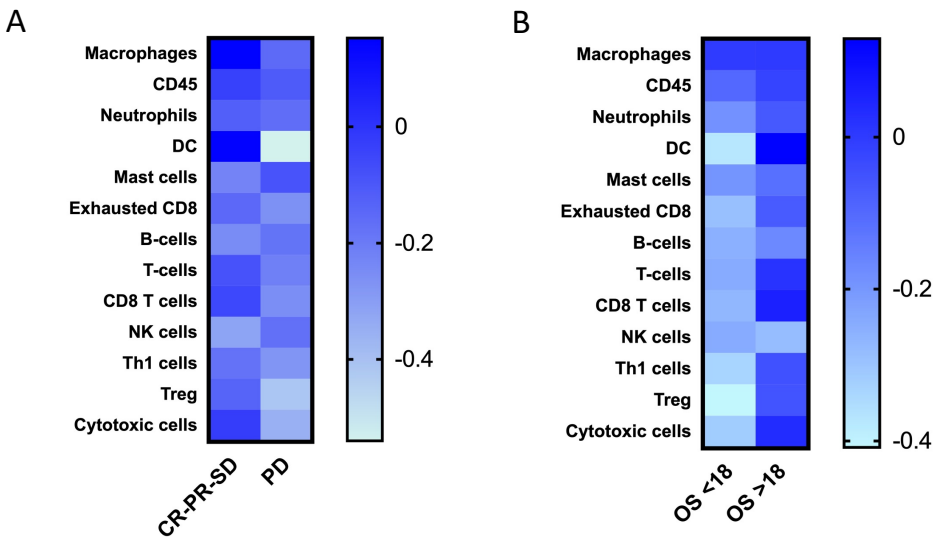

Figure 4. Pathway scores of NSCLC samples by NanoString IO360 assay comparing: A. Progression versus non-progression disease; B. OS > 18 months versus OS < 18 months

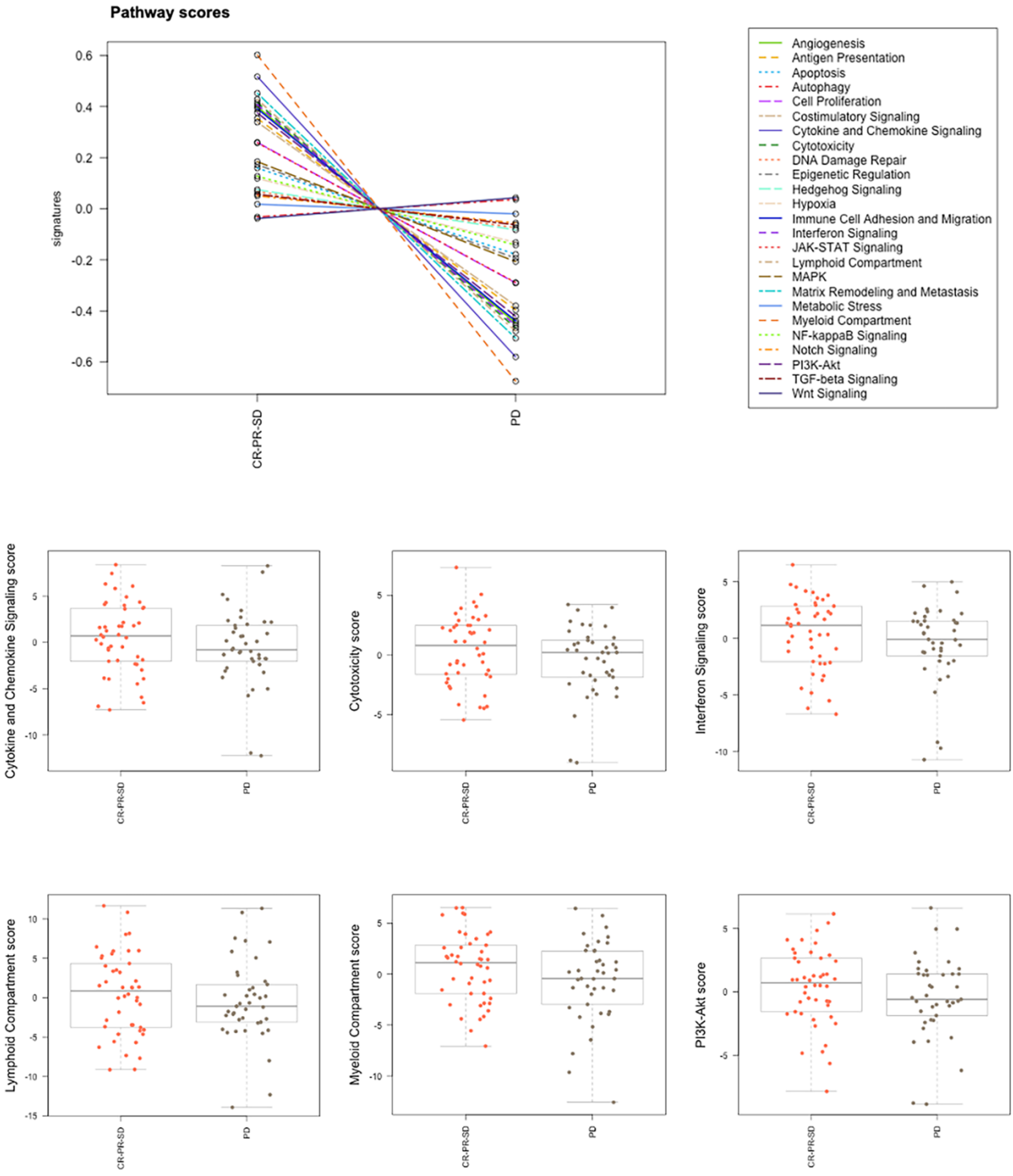

Figure 5. Single Pathway scores of NSCLC samples by NanoString IO360 assay comparing Progression versus non-progression disease

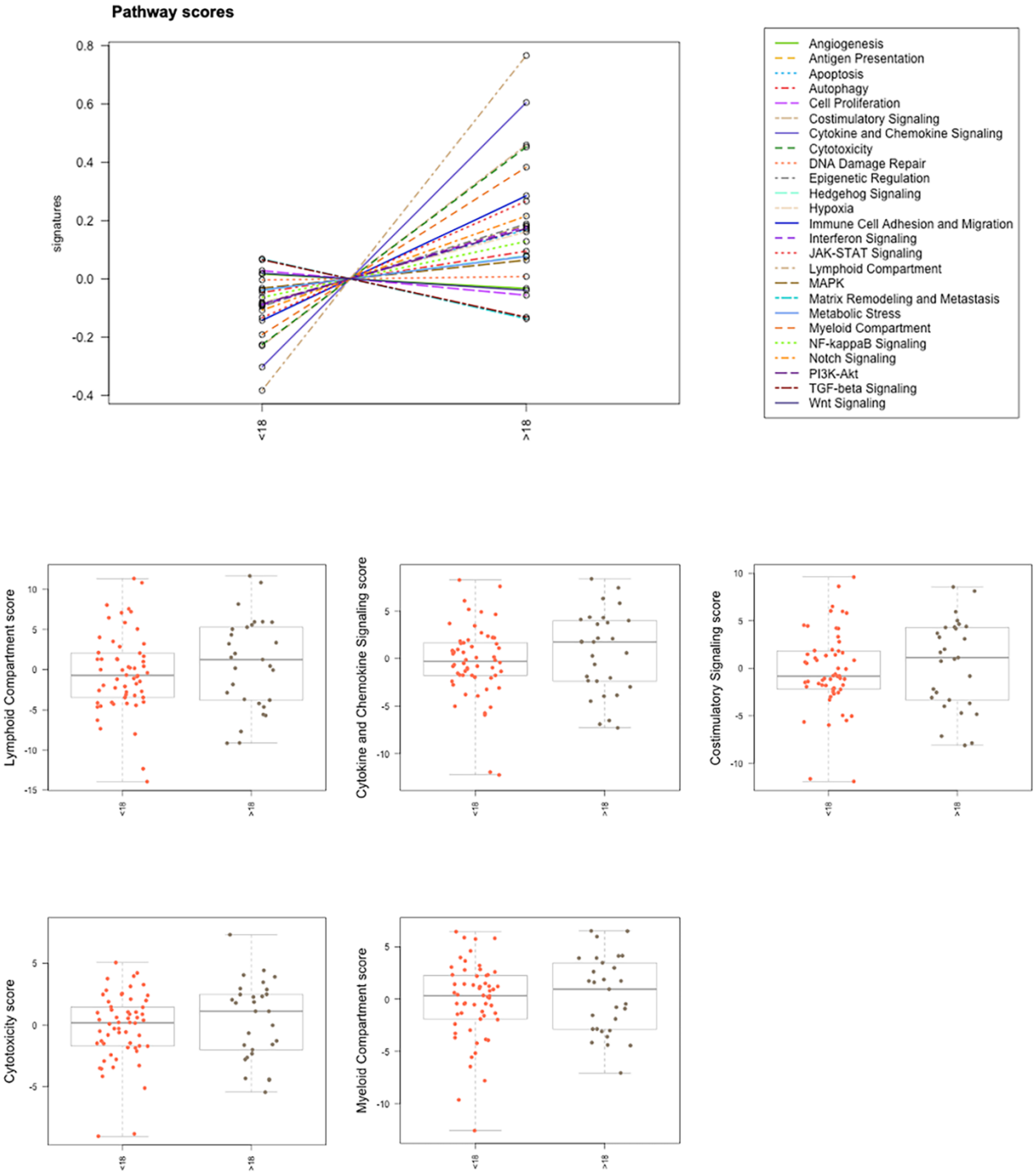

Figure 6. Single Pathway scores of NSCLC samples by NanoString IO360 assay comparing OS > 18 months versus OS < 18 months

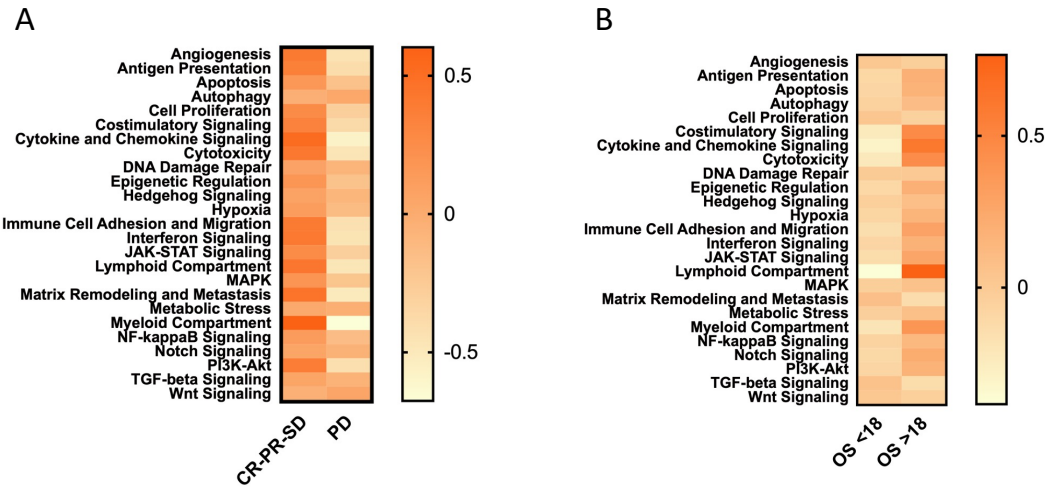

Figure 7. Cell type scores of NSCLC samples by NanoString IO360 assay comparing: A. Progression versus non-progression disease; B. OS > 18 months versus OS < 18 months

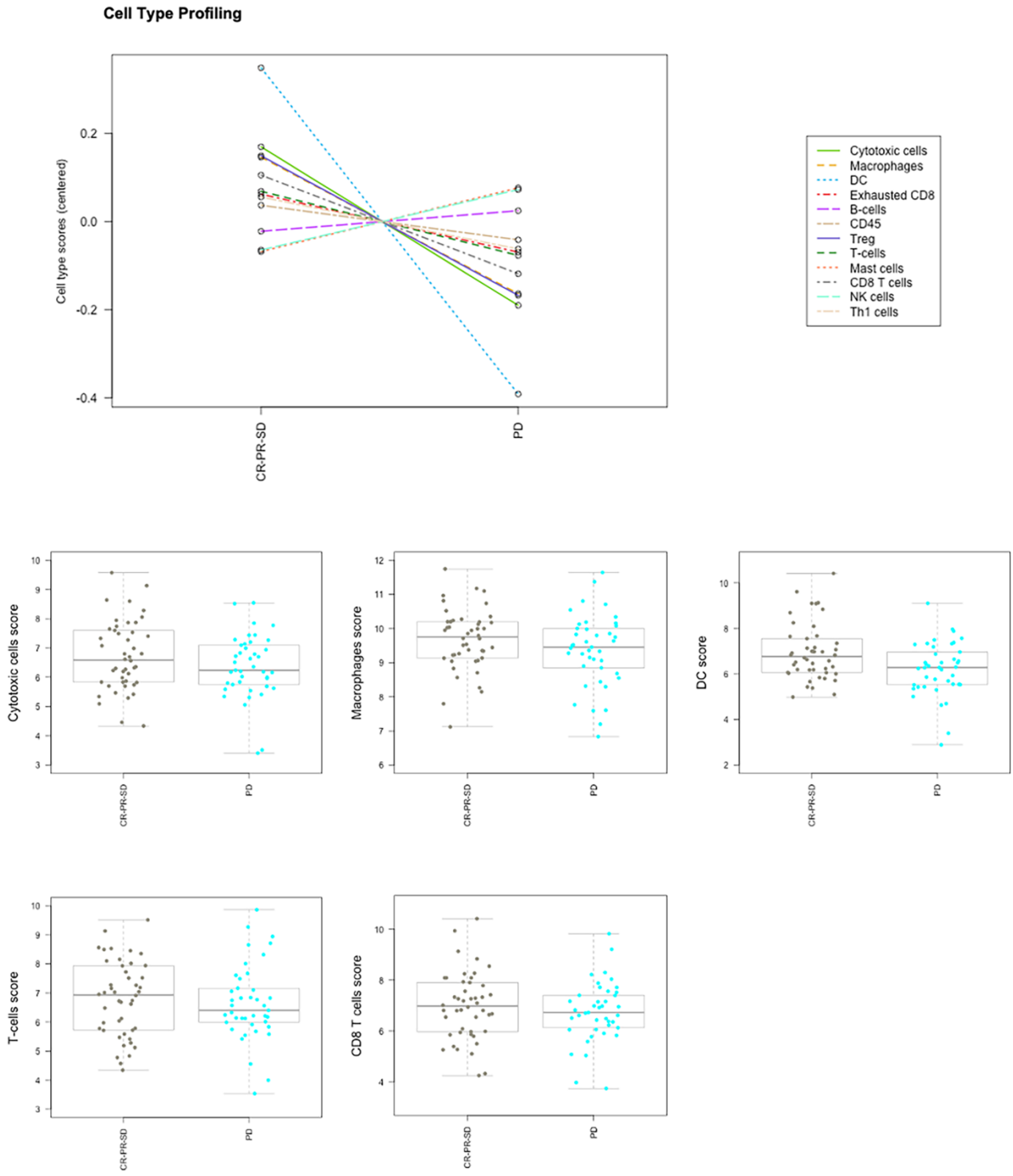

Figure 8. Single cell type scores of NSCLC samples by NanoString IO360 assay comparing Progression versus non-progression disease

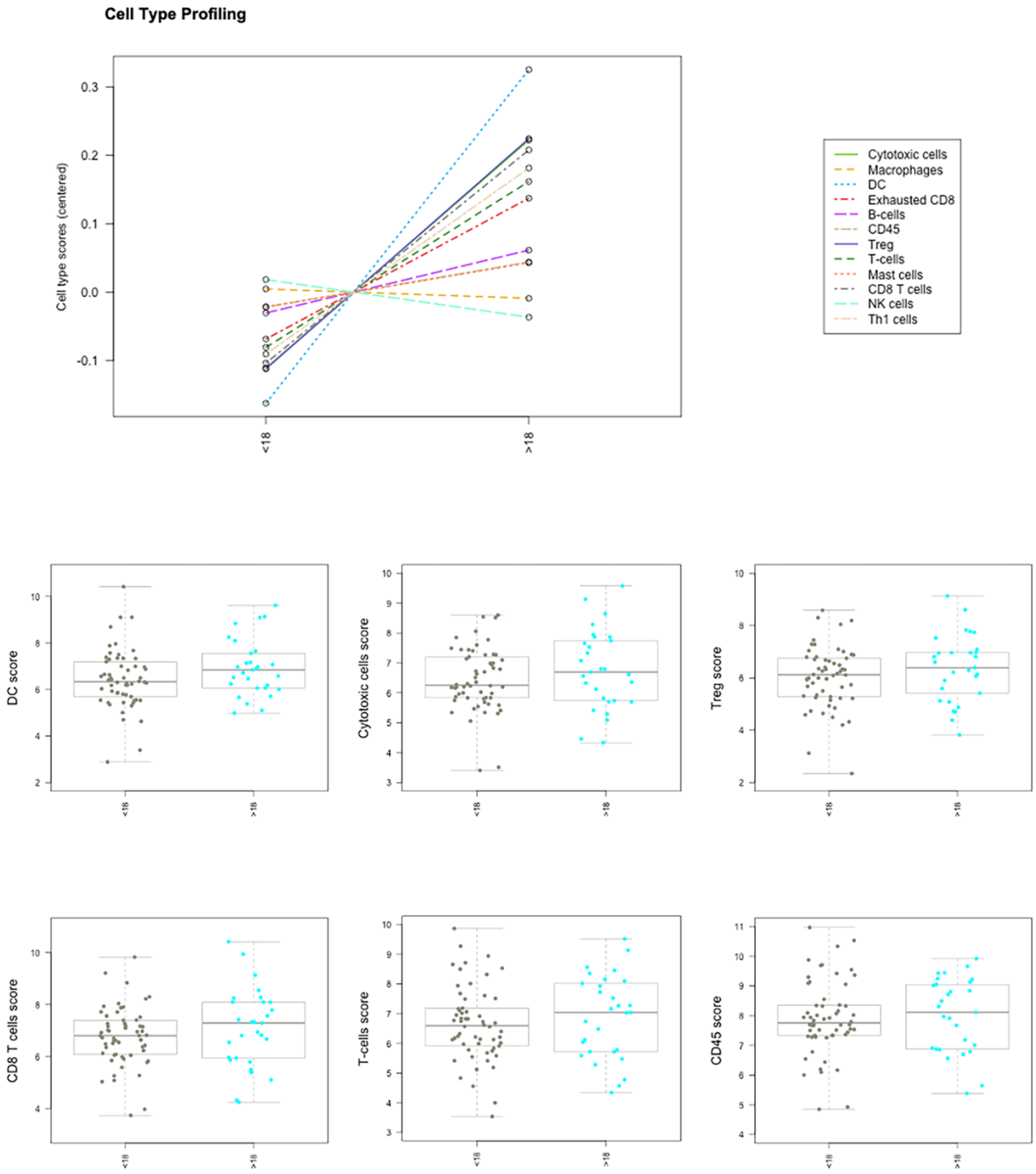

Figure 9. Single Cell type scores of NSCLC samples by NanoString IO360 assay comparing OS > 18 months versus OS < 18 months

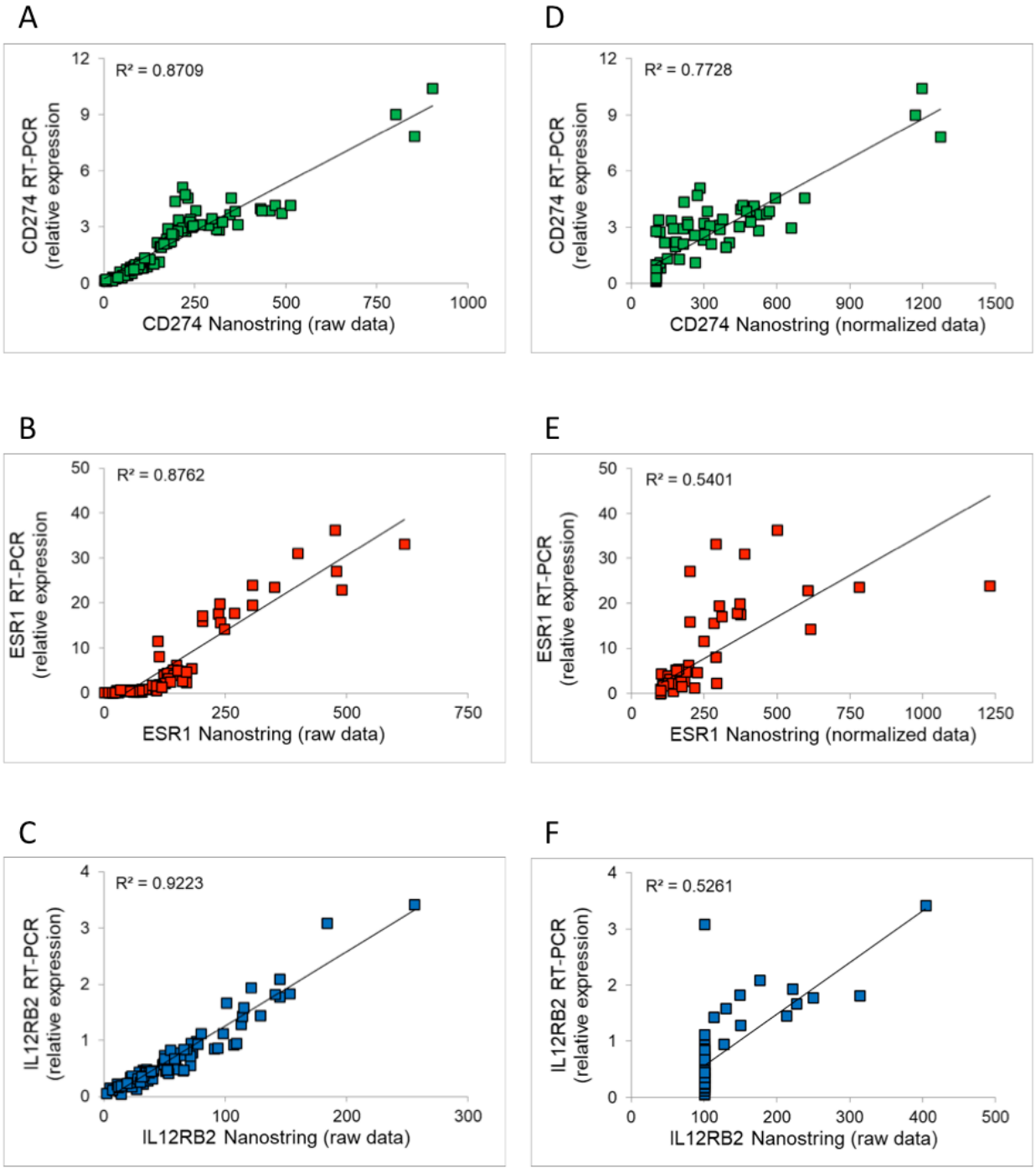

Figure 10. Correlation of the relative expression of selected genes in NSCLC samples as assessed by NanoString IO360 assay versus RT-PCR

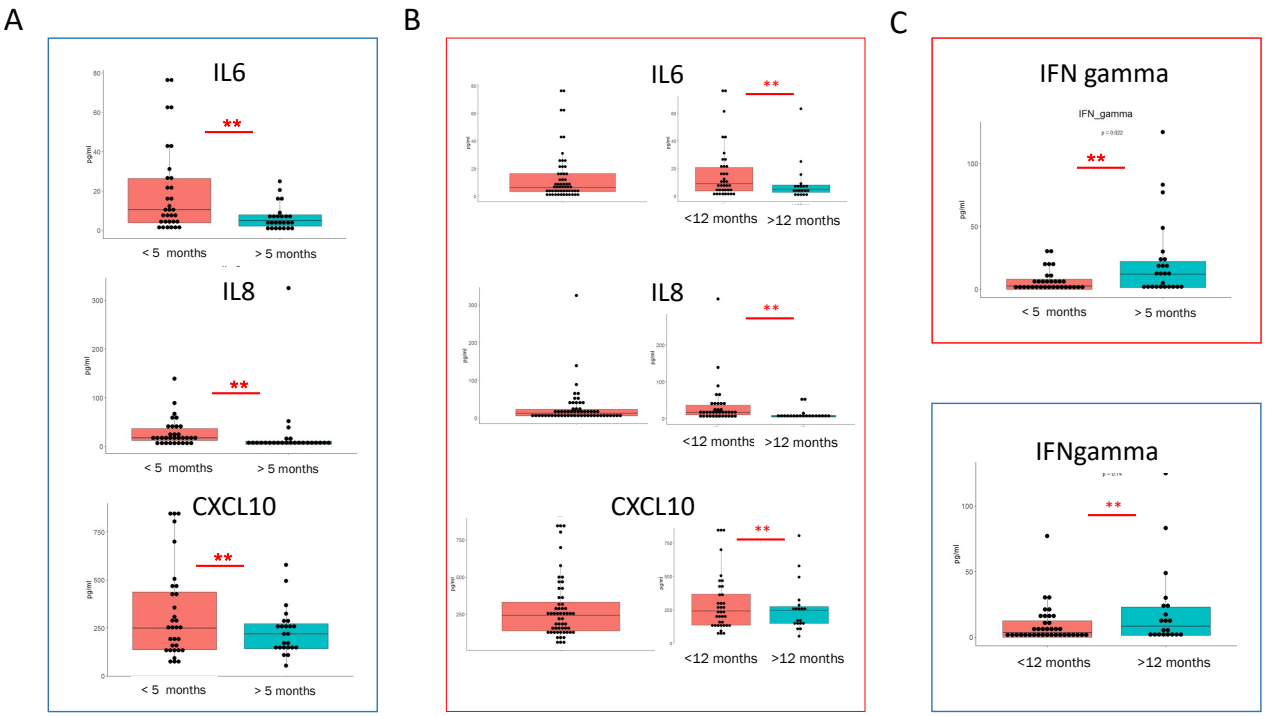

Figure 11. Blood cytokines levels according to survival outcomes in advanced NSCLC patients: A. IL6, IL8, CXCL10 levels in patients with PFS < 5 months versus > 5 months; B. IL6, IL8, CXCL10 levels in patients OS < 12 months versus > 12 months; C. IFN gamma levels in patients with PFS < 5 months versus > 5 months and OS < 12 months versus > 12 months

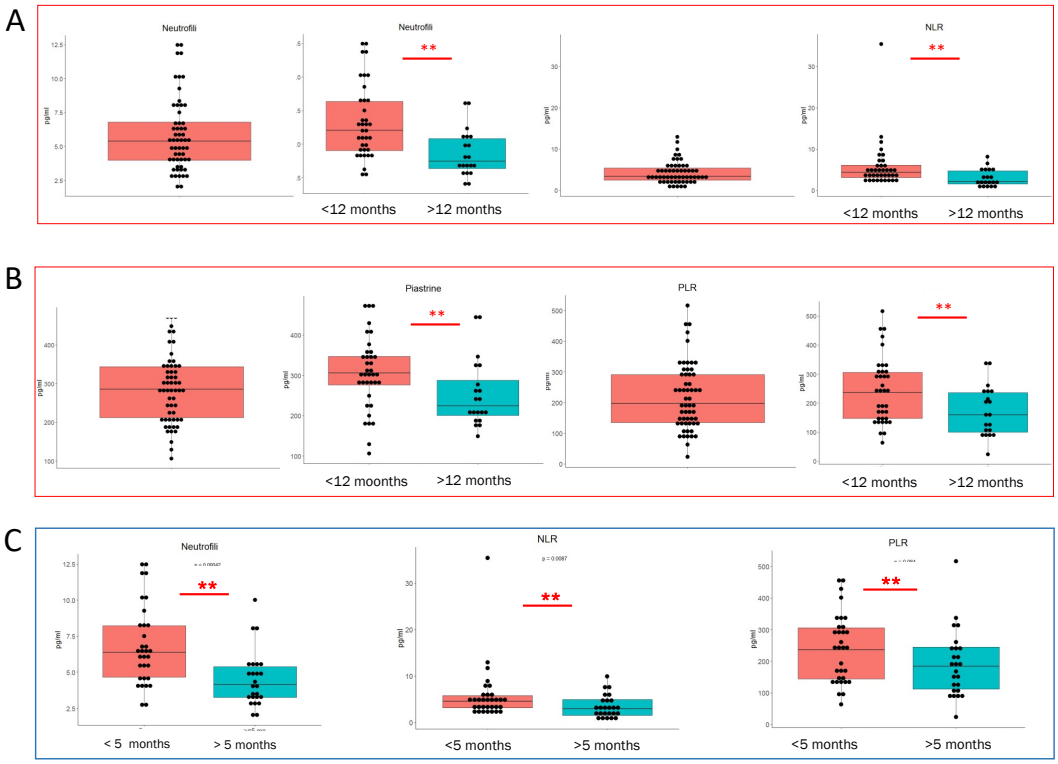

Figure 12. Peripheral blood cells according to survival outcomes in advanced NSCLC patients: A. Absolute neutrophil count and NLR levels in patients with OS < 12 months versus > 12 months; B. Absolute platelets count and PLR levels in patients OS < 12 months versus > 12 months; C. Absolute neutrophil count and NLR/PLR levels in patients with PFS < 5 months versus > 5 months.

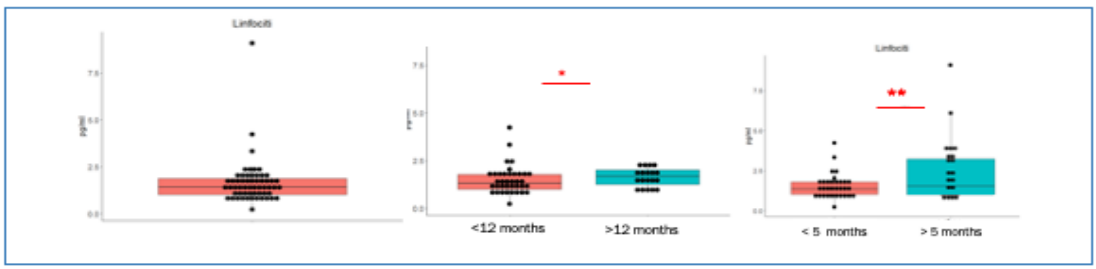

Figure 13. Peripheral blood lymphocyte count according to survival outcomes in advanced NSCLC patients

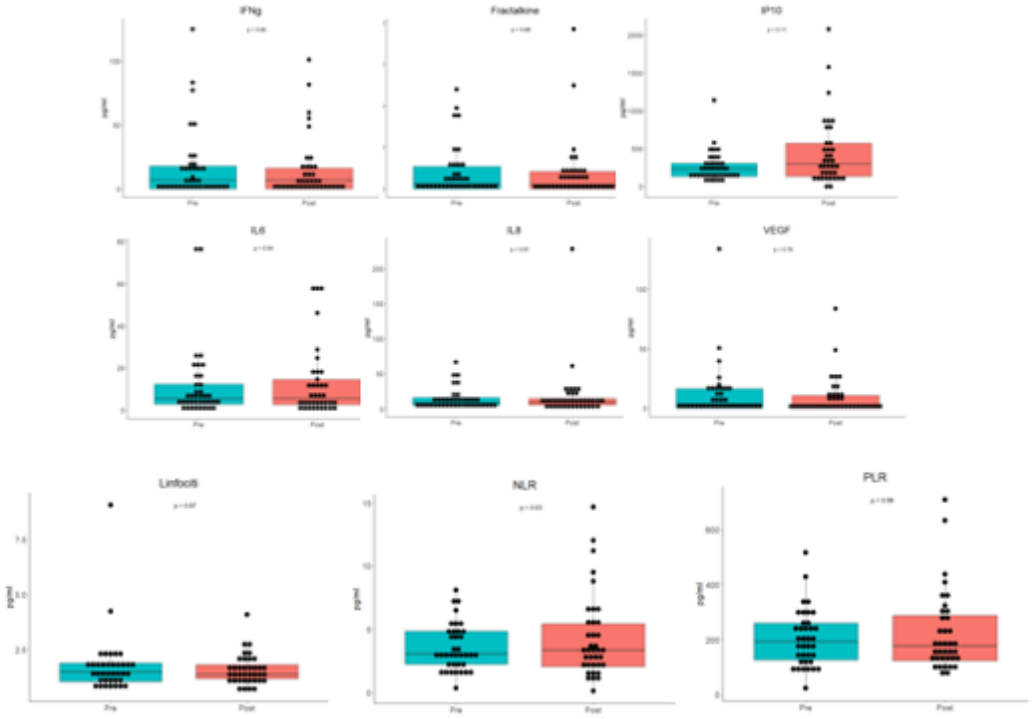

Figure 14. Blood cytokines levels and peripheral blood cells count dynamic variations in advanced NSCLC patients after 4 cycles of ICI therapy

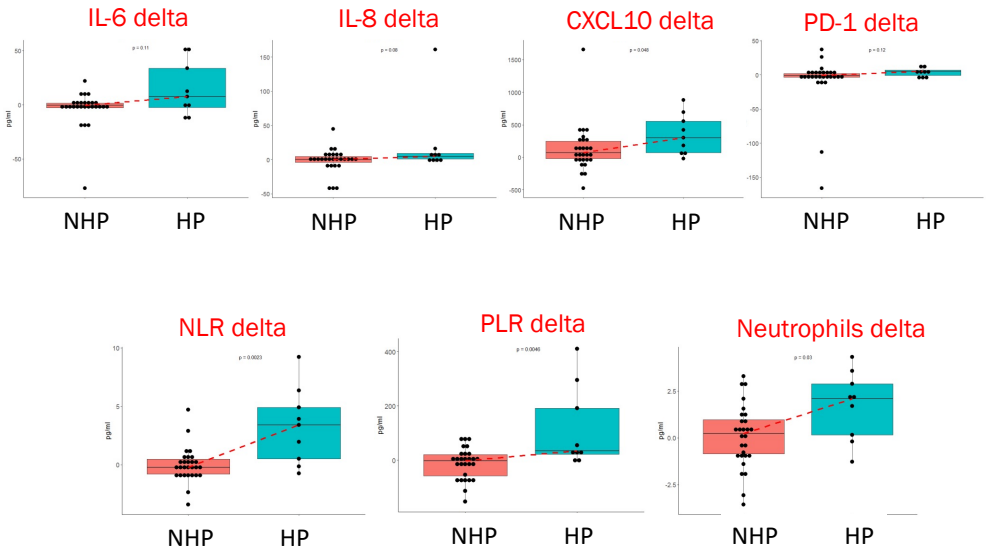

Figure 15. Blood cytokines levels and peripheral blood cells count dynamic variations in advanced NSCLC patients experiencing hyper progression after 4 cycles of ICI therap

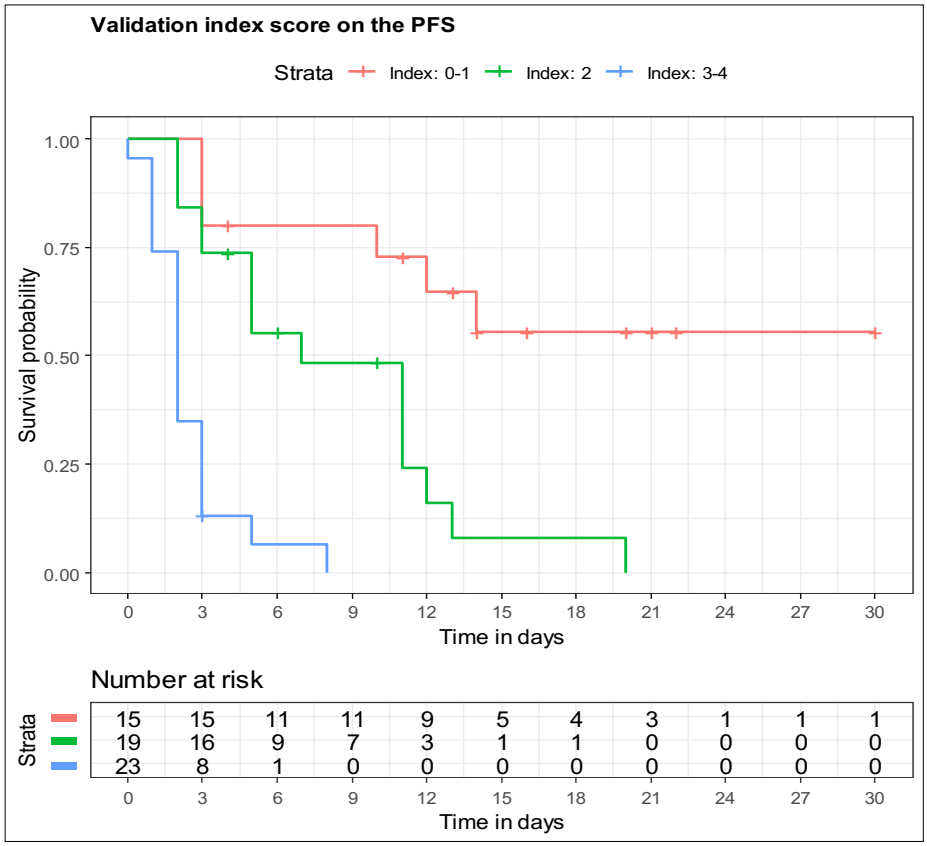

Figure 16. Progression free survival according to the Immune-suppressive blood index score (ISBS) in advanced NSCLC patients receiving ICI therapy

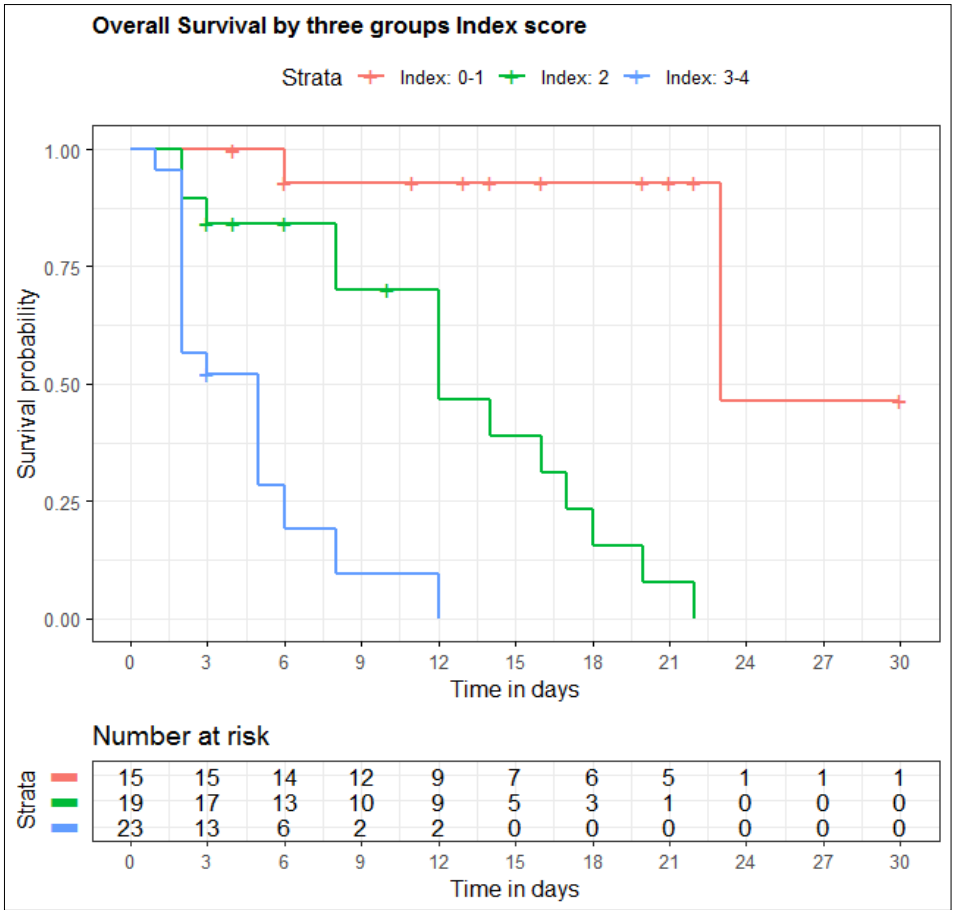

Figure 17. Overall survival according to the Immune-suppressive blood index score (ISBS) in advanced NSCLC patients receiving ICI therapy

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Scientific Products:

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