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IMMUNE-BLOOD

Peripheral Immune Response Biomarkers Study On Blood And Tissue Samples Collected Over Time

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RESUMEN

Introducción: Las inmunoterapias (ICI) han revolucionado los protocolos terapéuticos de la mayoría de cánceres pero muchos pacientes continúan sin beneficiarse de ellas. Es obligatorio mejorar la selección de candidatos a ICI.

Métodos: las muestras de sangre fueron recogidas en el ciclo 1 (C1D1), en el ciclo 2 (C2D1) y en cada evaluación de respuesta tumoral hasta observar una progresión tumoral (PD). La historia médica electrónica fue revisada para recoger todos la información clínica relevante y los resultados de las analíticas realizadas por práctica habitual. Los scores pronósticos conocidos fueron calculados previos al inicio del tratamiento (LNR: lymphocyte neutrophil ratio. dNLR: derive neutrophil lymphocyte ratio. LIPI: lung immune prognosis index. RMH: Royal Marsden Hospital score. PMH: Princess Margaret Hospital score. GRIm: Gustave Roussy Immune Score. PIPO: Phase I Prognostic Online score). Una vez que habíamos selecionado el major score, analizamos su asociación al C1D1, C2D1 y sus dinámicas entre ambos con la supervivencia global (OS) y la progresión libre de enfermedad (PFS). El estado de PDL1 se recogió de la historia clínica. Las muestras de archivo previas a recibir la ICI fueron recogidas para analizar los linfocitos infiltrantes de tumor (TILs) y los niveles de PD1 mRNA. Las evaluaciones tumorales se revisaron centralmente por criterios RECIST 1.1 / RANO. Las asociaciones con las tasas de respuesta global (ORR), el beneficio de control de enfermedad (DCB), el beneficio clínico duradero, la PFS y la OS fueron estudiadas mediante regresiones logísticas o de Cox univariables/multivariables cuando procedía. Kapplan Meier curves, ANOVA, comparaciones de pares, regresiones logisticas o de Cox, hazard ratios (HR), odd ratios (OR), y áreas bajo la curva (AUC), fueron realizadas cuando aplicaba.

Objetivos: El objetivo primario fue el análisis de la relación de las subpoblaciones linfocitarias de forma seriada como biomarcador de respuesta o resistencia a la ICI.

Los objetivos secundarios incluyen el estudio de 1) la correlación de los factores clinicopatológicos basales con las respuestas y la supervivencia en una población multitumor tratada con ICI, 2) el papel de los niveles de PD1 mRNA, PD-L1 y TILs como predictor de respuesta o resistencia a ICI y 3) la correlación de los scores pronósticos publicados con los resultados del tratamiento con ICI.

Resultados: se reclutaron 173 adultos diagnosticados de tumores sólidos que eran candidatos a tratamiento con ICI. Tras una media de seguimiento de 38,3 meses (95%CI: 36.1-58.6), PFS media de 2.5 months (95%CI: 2.0-3.7), OS media de 13.3 meses (95%CI: 9.9-17.4), con progresiones o muertes rápidas (≤4 meses desde el inicio

del ICI) en el 60.7% (95%CI: 53.0%-68.0%) de los casos y una ORR de 16.8% (95%CI: 11.5%-23.2%). Los niveles de la subpoblación linfocitaria CD3+CD4+PD1+ se asoció con menores ORR (C1D1: OR 0.93, 95%CI: 0.88-0.99, p=0.01; C2D1: OR 0.94, 95% CI: 0.89-1.00, p=0.04), DCB (C1D1: OR 0.93, 95%CI: 0.88-0.99, p=0.01; C2D1: OR 0.94, 95% CI: 0.89-1.00, p=0.04) y PFS (C1D1: HR= 1.03, 1.01 – 1.05, p=0.01; C2D1: HR= 1.03, 1.00 – 1.06, p=0.02). Los niveles de la subpoblación linfocitaria "CD3+CD4+ Highly Differentiated" se asoció con una menor OS tanto al medirla en C1D1 (HR= 1.01, 1.00 – 1.01, p=0.03) como en C2D1 (HR=1.01, 1.00 –1.01, p=0.03). El estado inmuno naive se asoció independientemente del tipo tumoral con mejor PFS (p = 0.005). Se detectó un punto de corte de PD-L1 del 10% entre los buenos y malos respondedores en términos de ORR (univariado p = 0.011, multivariado p = 0.028) y DCB (univariado p = 0.043). Los niveles de PD1 mRNA se asociaron a las respuestas completes (p = 0.021). LIPI en C1D1 fue el mejor predictor de progresiones rápidas, PFS y OS independientemente del tipo tumoral en comparación con otros scores. El LIPI basal fue capaz de diferencia 3 categorías de pacientes con diferencias significativas en OS (p < 0.001) y PFS (p = 0.013). Lo mismo se observó en C2D1 para OS y PFS (ambos p = 0.020). Retener la categoría de LIPI bueno o experimentar un cambio hacia una categoría mejor se asoció a una mejor OS (p = 0.009) y PFS (p = 0.006).

Conclusión: la selección óptima de pacientes todavía es una cuestión no resuelta pero identificamos varios factores que pueden ayudarnos a mejorar la selección de nuestros candidatos. El estado inmuno-naive, la categoría de LIPI bueno basal así como mantenerla en C2D1, los niveles elevados de PD1 mRNA y de PDL1 parecen asociarse a beneficio a ICI mientras que los niveles elevados de linfocitos CD3+CD4+PD1+ parecen relacionarse con peores resultados.

ABSTRACT

Background: Immune-checkpoint inhibitors (ICI) have revolutionized the therapeutic landscape of cancer but many patients remain not benefiting from them. Improve patient selection is mandatory.

Methods: Blood samples were collected at cycle 1 (C1D1) and 2 (C2D1) and at the time of every tumoral response assessment until the occurrence of progressive disease (PD). Electronic patient charts were reviewed to collect relevant clinical information and blood test results. Baseline prognostic scores were calculated (LNR: lymphocyte neutrophil ratio. dNLR: derive neutrophil lymphocyte ratio. LIPI: lung immune prognosis index. RMH: Royal Marsden Hospital score. PMH: Princess Margaret Hospital score. GRIm: Gustave Roussy Immune Score. PIPO: Phase I Prognostic Online score). Once we had selected the best score, we analyzed its association at C1D1, C2D1 and its dynamics with overall survival (OS) and progression free survival (PFS). PDL1 status was collected from patient charts. Pre-ICI archived tissues were retrieved to evaluate tumor-infiltrating lymphocytes (TILs) and PD1 mRNA levels. Tumor assessments were centrally reviewed by RECIST 1.1 / RANO criteria. Associations with overall response rates (ORR), disease control benefit (DCB), durable clinical benefit, PFS and OS were performed with univariable/multivariable logistic and Cox regressions, where appropriate. Mean and standard deviations of each subpopulation/timepoint were calculated. Kapplan Meier curves, ANOVA, pairwise comparison, logistic regressions, hazard ratios (HR), odd ratios (OR), Cox regressions and area under curve (AUC), were performed where appropriate.

Objectives: Primary objective was the serial assessment of lymphocytes subpopulation levels over time as biomarker of response or resistance to immunotherapies.

Secondary objectives included the study of 1) the correlation of baseline clinicopathological factors to response and survival in ICI a multitumor ICI treated population, 2) the role of PD1 mRNA levels, PD-L1 and TILs levels as predictor of response or resistance to immunotherapies and 3) the correlation of published prognosis scores and early dynamics with ICI outcomes.

Results: 173 adult patients with metastatic solid tumors candidates to ICI were prospectively recruited. At a median follow up of 38,3 months (95%CI: 36.1-58.6), median PFS was 2.5 months (95%CI: 2.0-3.7), median OS was 13.3 months (95%CI: 9.9-17.4), rapid progressions. At a median follow-up of 38.3 months (95%CI: 36.1-58.6), median PFS was 2.5 months (95%CI: 2.0-3.7) and median OS was 13.3 months (95%CI: 9.9-17.4), with rapid progression or death ≤4 months from ICI initiation observed in

60.7% (95%CI: 53.0%-68.0%) cases and ORR 16.8% (95%CI: 11.5%-23.2%). Levels of CD3+CD4+PD1+ lymphocytes subpopulation showed an association with shorter ORR (C1D1: OR 0.93, 95%CI: 0.88-0.99, p=0.01; C2D1: OR 0.94, 95% CI: 0.89-1.00, p=0.04), DCB (C1D1: OR 0.93, 95%CI: 0.88-0.99, p=0.01; C2D1: OR 0.94, 95% CI: 0.89-1.00, p=0.04) and PFS (C1D1: HR= 1.03, 1.01 – 1.05, p=0.01; C2D1: HR= 1.03, 1.00 – 1.06, p=0.02). CD3+CD4+ Highly Differentiated subpopulation demonstrated an association with shorter OS when measured at C1D1 (HR= 1.01, 1.00 – 1.01, p=0.03) and C2D1 (HR=1.01, 1.00 –1.01, p=0.03). Immunotherapy-naïve status was independently associated with better PFS (p = 0.005). A PD-L1 10% cut-off detected worse/best responders in terms of ORR (univariate p = 0.011, multivariate p = 0.028) and DCB (univariate p = 0.043). PD1 mRNA levels were strikingly associated to complete responses (p = 0.021). C1D1 LIPI was the best predictor of rapid PD, OS and PFS, regardless of cancer type, compared to other scores. Baseline LIPI detected three categories of patients with significantly different OS (p < 0.001) and PFS (p = 0.013). The same was observed at C2D1 for OS and PFS (both p = 0.020). Retaining a good LIPI or experiencing a shift towards a better prognostic class was associated to improved OS (p = 0.009) and PFS (p = 0.006).

Conclusion: optimal patient selection is still an unmet need but we identified several factors that may help us improve the selection of our candidates. Immune naïve status, good LIPI baseline score and retaining it prior to the second infusion as well as higher levels of PD1 mRNA and PDL1 seem to correlated with ICI benefit while higher baseline levels of peripheral CD3+CD4+PD1+ lymphocytes subpopulations might correlate with worse outcomes.

Table of Contents

Chapter 1. Introduction

In the last decade, immunotherapy with immune-checkpoint inhibitors (ICI) has revolutionized the therapeutic landscape of many solid tumors. ICI-based therapeutic approach is based on the disruption of the activity of several immune system inhibitory mechanisms, so to unleash a potent immune response directed towards the tumor (1,2). Nevertheless, there are plenty of patients treated with ICIs who are exposed to their potential adverse events but do not benefit from them. Therefore, it is critical to find a way to improve patient selection, treating only those with higher probabilities of ICI benefit and offering to the rest other alternative options that may give them higher chances of benefit.

Immune System Overview

The innate immunity is the first defense action of our immune system which is antigen independent and does not create immune memory. Several cell types are involved in this innate response as macrophages, neutrophils, dendritic cells, mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid cells. Dendritic cells, within others, express in their surface proteins called “Major Histocompatibility Complex” (MHC) class I (also known as Human Leukocyte Antigen; [HLA] A, B or C) or class II, that allow them to act as Antigen Presenting Cells (APC) and trigger the adaptive immunity(3).

The adaptive immunity is the second barrier against foreign agents in which lymphocytes are key players. Stem cells from the bone marrow derive to naïve T cells (CD3+CCR7+CD45RA+) which migrate to the thymus for maturation. During this process, T cells that would attack to antigens usually found in our body are eliminated while the others survive. These cells express in their surface specific proteins able to bind unique antigens called T cell receptor (TCR). Once APCs capture foreign antigens, expose them linked to its MHC in an MHC-antigen complex, activates the TCR and the T cell secretes cytokines that spread the immune response. This antigen presentation process stimulates their differentiation into either cytotoxic/effector T cells (CD3+CD8+ cells) or T helper (Th) cells (CD3+CD4+ cells)(3).

APCs present antigens by MHC I molecules, activating effector T cells (CD3+ CD8+) which are responsible of the destruction of foreign agents by releasing substances that induce apoptosis of target cells. Once the foreign agent is cleared, most of these effector T cells die and just a few of them become memory cells (central memory lymphocytes CD3+CCR7+CD45RO+ / effector memory lymphocytes CD3+CCR7-CD45RO+) that could differentiate into effector T cells in the future if exposed to the same antigen(3).

T helper cells (CD3+CD4+) are not able to kill or phagocyte foreign agents but play a main role in the immune response regulation. Th cells are activated through TCR recognition of antigen bound to class II MHC molecules and once activated, they release cytokines that influence many other immune cells including their activating APC. Regulatory T cells are a subset of CD4+ T cells (CD3+CD4+CD25+FoxP3+) that play an important role in the immune response by limiting and suppressing immune responses, avoiding abnormal responses against self-antigens and the development of autoimmune diseases(3).

Apart from antigen recognition by TCR, T cell activation is modulated by costimulatory and inhibitory signals. Receptors such as CD28 and ICOS transduce signals necessary to fully activate T cells while receptors like CTLA-4 and PD-1 transduce inhibitory signals against lymphocyte activation. The balance between positive and negative inputs determines immune responses or tolerance(4).

CD28 is a protein expressed in T cell surface that is essential for T cell activation. It binds to either CD80 (B7-1) o CD86 (B7-2) which are costimulatory molecules or ligands family, found on APCs surface. CTLA4, which belongs to the CD28 subfamily, is an inhibitory protein stored intracellularly in T cell naïve cells until their activation, when it is transferred to the cell surface. Once they are in the surface, both CTLA4 and CD28 are able to bind CD80 and CD86 ligands on APCs but CTLA4 has higher affinity, blocking CD28 co-stimulation and preventing T cell response (4–6).

Human T cells undergo a natural differentiation process following the initial antigen recognition, characterized by the progressive loss of CD27 and CD28 surface expression, and acquisition of memory and effector functions so they can be classified as poorly differentiated (CD27+/CD28+), intermediately differentiated (CD27neg/CD28+) and highly differentiated (CD27neg/CD28 neg). HD T cells include memory, effector and senescent T cells (7).

PD1 is another transmembrane protein that belong to the CD28 subfamily, that is expressed on T cells after TCR stimulation, with coinhibitory effect. When PD1 binds PDL1 (also known as B7-H1) o PDL2 (also known as B7-DC) which are present on APCs, it changes its conformation, phosphorylates its intracellular tail, recruits SHP-2 in the proximity of TCR attenuating its activity and affects downstream signaling pathways like PTEN-PI3K-AKT and RAS-MEK-ERK. The final outcomes are multiple, including inhibition of T cell proliferation, activation, survival, cytokine production, alteration of T cell killer functions and occasional death of activated T cells (6). PD1/PDL1 axis is crucial for controlling the continued activation and proliferation of differentiated effectors, being

able to activate apoptosis or induce a state of T cell dysfunction called T cell exhaustion (4,5,8–12). Unfortunately, tumor cells can take advantage of this mechanism by upregulating PD1/PDL1 axis to induce T cell exhaustion and facilitate cancer cells survival (12). In fact, the expression of PD1 on tumor infiltrating T cells and PD1 ligands on tumors may be poor prognostic factors in several cancers (13,14).

Hematopoietic stem cells from bone marrow also generate B cells. Following their maturation they are released expressing a unique antigen-binding receptor on their membrane. B cells can recognize antigens directly, without APCs, through specific antibodies expressed on their surface. Their principal function is the secretion of antibodies against foreign antigens which requires their further differentiation but they also interact with T cells which are key for antitumor T cell mediated cytotoxicity (15). When activated by foreign antigens to which they have an appropriate antigen specific receptor, B cells proliferate and differentiate into antibody-secreting plasma cells or memory B cells. Therefore, B cells play a major role in humoral o antibody-mediated humoral immune response (3)

During the oncogenesis, neoantigens are released and captured by dendritic cells that act as APCs. Their surface MHC class I or II will display fragments of those antigens to T cells, resulting in the activation of effector T cells against cancer specific antigens as long as they are considered foreign (3). Activated T cells, travel by the blood stream and finally infiltrate the tumor environment, recognize and bind to the specific cancer cell through the interaction between its TCR and the MHC-antigen. Finally, it kills their target cancer cell and release more tumor antigens, increasing the immune response. Nevertheless, this immune cycle may fail in oncology patients. Tumor antigens may not be detected o considered self-antigens instead of foreign, T cells may not infiltrate tumors, o tumor microenvironment factors may inhibit T cells (16).

Clinicopathological factors

As of today, anti-PD1 (e.g. pembrolizumab, nivolumab), anti-PD-L1 (e.g. atezolizumab, durvalumab) and antiCTLA4 monoclonal antibodies have become some of the most widely prescribed anticancer therapies and are recommended, in monotherapy or combination with other ICI or chemotherapy, in a broad spectrum of cancer types (1,17). However, the degree of benefit is different according to the cancer type and within each tumor type, and only a limited proportion of patients seem to benefit. Furthermore, ICIs may lead to harmful and potentially lethal immune-mediated side effects. Therefore, the identification of proper biomarkers of response is crucial to improve therapeutic outcomes, avoid unnecessary toxicities and optimize resources, since ICIs are

considered an expensive treatment that can yearly cost more than $100,000 per patient. (18–23). Unfortunately, few biomarkers have proved to be effective for a proper patient selection and with several cancer-specific and/or technical-related limitations (24–27).

Baseline clinical/hematological characteristics and previous medical history may influence the chances of benefit of our candidates. The optimal metastatic therapeutic setting (earlier or further lines), the efficacy in immune-pretreated patients, the effects of exposure to immediately previous or concurrent radiotherapy (RT), and the optimal duration of treatment remain questions unanswered. To note, the impact of systemic corticosteroids and exposure to antibiotic (ATB) therapy on response to ICI are another major concern, with only few and/or conflicting data being published so far (28–37).

Prognostic Scores in Oncology

Several prognostic scores have been identified for different settings based on analytical and clinical factors so they are easy-to-detect with a relatively low cost. Our inflammatory status is balanced by several immune cells within which we can find lymphocytes and neutrophil. These populations are used to calculate the Neutrophil Lymphocyte Ratio (NLR = absolute counts of neutrophils / lymphocytes), a score which elevation has been correlated with worse overall survival and progression free survival in multiple tumor types. Optimal cut-off has been controversial within different studies although equal or above five has been the most widely used to define the high NLR category (38–48).

Derived Neutrophil to Lymphocyte Ratio (dNLR) score, calculated by neutrophils/[leucocytes-Neutrophils] has mainly been explored in non small cell lung cancer (NSCLC), where dNLR high (≥3) was associated with worse overall survival (OS) and progression free survival (PFS) than the dNLR low group (<3). Although dNLR has been explored in some specific histologies beyond NSCLC, no multitumor studies have been performed to analyze its relationship across tumor types (49–53)

Interestingly, the dynamic changes in dNLR with ICI over time (baseline and prior to second cycle [C2]) have been also explored in a large cohort of patients with NSCLC. In the low dNLR group, those that changed to high dNLR at C2 presented poorer outcomes than those that remained low. Similarly, in the high dNLR group those that decreased to low dNLR had better outcomes than those that remained high (50).

Lung Immune Prognostic Index (LIPI), based on dNLR and LDH was used to stratify patients in 3 categories: good (dNLR ≤3 and LDH ≤ upper limit of normal [ULN]), intermediate (dNLR >3 or LDH > ULN) and poor (dNLR >3 and LDH > ULN). Initial studies explored the performance of baseline LIPI in lung cancer patients showing the correlation of good score with better PFS/OS. Following these results several studies

were conducted to validate the results across tumor types showing initial correlation between good score and better outcomes. Nevertheless, further studies are needed to validate its potential as biomarker in a tumor agnostic population (54–59).

Gustave Roussy's Early Drug Development Department analyzed their ICI treated population in order to identify a potential score to identify prognostic factors to potential benefit with ICIs. Based on the analysis of 155 patients treated with ICI in their unit they generated a new score (Gustave Roussy Immune Score; GRIm score) using albumin serum level (<35 g/l = 1), LDH (>ULN = 1) and NLR (>6 = 1). Patients were classified in low risk score (0 o 1) or high risk score (2 o 3). Further 113 patients were analyzed confirming that high risk score patients presented worse OS than low risk score population (60).

Other research sites have developed score to improve patient selection for experimental therapies. These scores may identify patients with better prognosis or probability to benefit from a new therapy. Therefore, it would be worth exploring their potential with ICIs.

The Royal Marsden Hospital performed a retrospective analysis of patients recruited in early phase clinical trials. The analysis of 212 consecutive patients showed statistical correlation of outcomes with LDH, albumin and number of metastatic sites. A risk score (Royal Marsden Hospital score; RMH score) based on the results of the outcome of the multivariate analysis (LDH normal = 0 vs LDH > UNL = 1, albumin ≥ 35 g/l= 1 Vs albumin < 35 g/l = 0, site of metastasis ≤ 2 = 0 vs >2=1) demonstrated that patients with a good prognosis score (0 o 1) had a significantly longer OS than the poor prognosis group (2 o 3) (61).

The Princess Margaret Hospital analyzed their population to validate RMH score and identify predictors of mortality at 90 days (90DM) after treatment initiation. In multivariate analysis, albumin < 35 g/L, > 2 metastatic sites and ECOG > 0 were significantly associated with 90DM. In order to construct their score (Princess Margaret Hospital Index; PMHI), one point was assigned to each variable, classifying patients in low risk (0-1 points) and high risk (2-3 points). Low risk score was associated with lower 90D mortality rate than high risk group(62).

Vall dHebron Institute of Oncology (VHIO) also explored potential prognostics factors of survival. They collected data from 799 consecutive patients recruited in clinical trials and identified in the multivariable analysis, six prognostic variables were independently associated with their survival (ECOG, number of metastatic sites, presence of liver metastases, dNLR, albumin and LDH levels) that were used to create a prognostic

survival calculator online (Phase 1 prognostic online (PIPO): https://pipo.vhio.net/). Ordinal and laboratory variables were dichotomized and patients classified into prognostic groups (good =0-2 points, intermediate = 3-4 points, poor = 5-6 points). Within other populations, they analyzed patients who had received ICI showing statistical differences in OS between groups (63).

In conclusion, several scores based on baseline characteristics and laboratory test have been developed to predict survival o potential benefit to therapies. Nevertheless, most of the studies were retrospective, focused in specific histologies or not ICI specific and did not provide comparison between scores. Therefore, further prospective analysis in a pan-cancer setting treated with ICI of the performance of these scores and their direct comparison is needed.

ICI Tumoral Tissue Biomarkers

Beyond clinical and laboratory baseline factors, tissular tumoral characteristics may play a role in candidates selection. The first identified biomarker was PDL1 expression by immunohistochemistry, a non-robust test whose interpretation is dependent of the experience of the pathologist that is used to select candidates for antiPD1/PDL1 drugs. Besides, each drug has been developed with a specific assay so different antibodies have been used to measure its expression (Table 1) that may not necessarily be the same used in real life hospitals which leads its credibility under question (64,65).

Table 1: PD-L1 Assay system used in the Blueprint Project (64)

AGENT	NIVOLUMAB	PEMBROLIZUMAB	ATEZOLIZUMAB	DURVALUMAB
Primary antibody clone used in the assay system	28-8 (Dako)	22C3 (Dako)	SP142 (Ventana)	SP263 (Ventana)
Interpretive scoring	Tumor cell membrane	Tumor cell membrane	Tumor cell membrane Infiltrating immune cells	Tumor cell membrane
Instrument and detection systems required	EnVision Flex on AutostainerLink 48	EnVision Flex on AutostainerLink 48	OptiView detection and amplification on Benchmark ULTRA	OptiView detection on Benchmark ULTRA
Therapeutic developer	Bristol Myers Squibb	Merck	Genentech	AstraZeneca

Microsatellite instability (MSI) is another immunohistochemistry based test that has been used to approve antiPD1/PDL1 drugs in tumor agnostic indications (66–68). MSI prevalence varies widely from across tumor types with cancers with prevalence above 15% (endometrial cancers, colon adenocarcinoma o stomach adenocarcinoma) and

other below 1% (for example glioblastoma, pancreatic adenocarcinoma or thyroid carcinoma within others). Special mention merits diagnosis with proved ICI benefit like lung cancer o melanoma whose MSI prevalence is below 2% which highlights the need of biomarkers to identify most of the patients with potential benefit that do not express MSI (69,70).

More reliable biomarkers are the determination of specific genomic alterations. Recently major therapeutic advances have been made in tumors with druggable genomic alterations in specific genes as EGFR (NSCLC, erlotinib/gefitinib), HER2 (breast, trastuzumab/lapatinib), KIT/PDGFRA (GIST, imatinib), BRAF (melanoma, vemurafenib), ALK (NSCLC, crizotinib), IGF1R (Ewing sarcoma, figitumumab), PARP (ovarian/breast, olaparib), BCR-ABL (chronic myeloid lymphoma, imatinib), NTRK (agnostic, entrectinib), RET (Thyroid, NSCLC, selpercatinib) o KRAS (NSCLC o colorectal cancer, adagrasib) within others (19,71–85). Nevertheless, molecular biomarkers to select the tumors sensitive to immunotherapies are still a challenge. Some of these drugs are already in late stages of their development or even approved for different indications like melanoma (ipilimumab, nivolumab, pembrolizumab) or NSCLC (pembrolizumab, nivolumab) without a strong biomarker for candidate selection so a high proportion of those patients do not receive benefit from their immunotherapy.

Tumor mutational burden has also been identified as potential biomarker of response and has been approved for clinical practice in the USA. However, it has been variably successful in predicting responders according to different cancers and its use is limited to specific contexts (24,25).

Gene signatures on RNA may be potential robust and reproducible biomarkers. The Translational Genomic and Targeted Therapeutics in Solid Tumors Groups performed an analysis of 10.462 tumor samples representing 36 cancer types with RNA data available. 547 immune-related genes, including PD1, were explored. A strong association between PD1 mRNA absolute levels and reported overall response rate following anti-PD1 monotherapy was observed with percentile 80th as potential cut off (86,87). These results lead to their validation in ACROPOLI clinical trial that is currently ongoing.

Finally, the outcome of ICI therapy has also been linked to the quality and magnitude of tumor-infiltrating lymphocytes (TILs) responses within the tumor microenvironment, though without current clinical applicability (88,89). Its potential is still under study due to the necessity of standardization of procedures and determination of the role of specific lymphocytes population and their activation status (90–92).

TILs come from peripheral lymphocytes that have previously been activated by APCs. Therefore, the amount and characteristics of this circulating lymphocytes may influence the potential benefit to ICI. In fact, some studies have demonstrated similarities between lymphocytes in tumor microenvironment and in the circulation which serve as a rationale for exploring the role of systemic lymphocytes as ICI biomarkers (93).

ICI Peripheral Lymphocytes Biomarkers

Lymphocytes are a key player in ICI mechanism of action so it seems logical that their decrease may impact their outcomes. Several studies have explored the influence of total lymphocytes in patients treated with ICI, reaching different conclusions. Some studies correlated baseline or 3 months on treatment lymphopenia with worse survival in while others did not so its role as biomarker remains uncertain (94–98). These inconsistent results may be related to the wide range of cells with different functions included in the total lymphocyte count so other studies have focused in specific lymphocyte subpopulations that may be related to the response or toxicity to these therapies (99).

Baseline circulating cells have been explored as potential biomarkers. For example, Zheng et all analyzed samples from 42 lung cancer patients at baseline and differentiated CD4+T cells based on the level of expression of PD1. Seventeen patients were classified as PD1 High (> 12,27 %) and 25 as PD1 Low (< 12,27 %). Significant lower rate of OS and PFS was seen in the PD1 High group. From this population, 5 patients were treated with antiPDL1 therapy. Three of them belonged to the PD1 High group and had long stable disease while the other two belonged to PD1 Low group and had early progression disease and shorter survival (100).

Other studies explored not only baseline circulating cells but also their dynamics along the therapy. For example, Kamphorst et al collected samples from 29 lung cancer patients treated with anti PD1 o PDL1 antibodies at baseline and before each treatment administration. They analyzed CD4 and CD8 T cells and used Ki-67 as activation marker. They observed an increase in Ki67+ T cells mainly related to CD8 lymphocytes within the first 4 weeks after therapy. Besides, they observed that patients who developed partial response also had high frequency of PD1+ cells among ki-67 CD8 T cells, this frequency was variable within those patients that developed an early progression disease (101).

Hwan Kim et all analyzed blood based dynamic biomarkers to predict responses to antiPD1 therapies in solid tumors. They collected serial blood samples before treatment initiation and one week later from two discovery cohorts of thymic epithelial tumors

treated with pembrolizumab (n=33) and NSCLC treated with pembrolizumab or nivolumab (n=33) and finally a validation cohort of NSCLC patients (n=46). They found that the percentage of proliferative (ki-67+) cells among PD1 CD8 T cells significantly increased in the first 7 days after the first dose and decreased in the subsequent 2 weeks. The increase from baseline to day 7 (calculated as fold-change in percentage of Ki-67+ cells amount PD1+CD8 T cells; Ki67D7/D0) was statistically correlated with DCB and PFS (102).

These preliminary results seem promising but PD1 expression by itself does not indicate that the lymphocyte is able to recognize tumor cells so it may need to be combined with other factors as the expressions of other molecules as TIGIT, LAG3 o 4-1BB (93,103–107), T cell activation markers (CD25, HLA-DR, CD40L, CD62L, CD69) o degree of differentiation. After antigen recognition, lymphocytes T differentiate and progressively loose CD27 and CD28 surface expression becoming highly differentiated (CD27neg/CD28 neg) T cells (CD3) (7). Zuazo-Ibarra et all explored the role of CD3 CD4 HD cells in lung cancer patients treated with ICI. They classified patients based on baseline CD3 CD4 HD cells in group 1 (above average) and group 2 (below average). When they correlated these groups with tumoral response found that all patients from group 2 had progression disease as best response while 47% patients of group 1 had an objective response. Besides, they analyzed CD3 CD4 HD cells dynamics differentiating between pattern 1 or THD burst (highly significant increase in systemic CD3 CD4 HD cells) and pattern 2 o THD decrease (significant reduction in systemic CD3 CD4 HD cells). The first pattern was associated with progression disease while the second with tumor responses (108). Finally, they correlated low baseline levels of CD3 CD4 HD cells (group 2) and THD burst pattern to hyperprogression (108,109) Following these results, Arasanz et all explored the relationship between CD3 CD4 HD cells and hyperprogression (110) in a study of 70 NSCLC patients treated with ICI (33 atezolizumab, 28 nivolumab, 9 pembrolizumab). 38 patients presented group 1 baseline profile while 31 had group 2 baseline profile. Hyperprogression was significatively correlated with baseline group 2 profile. THD burst pattern (increase > 1,3 times CD3 CD4 HD) was significatively correlated with hyperprogression, shorter PFS and a trend for shorter OS (110). These studies did not found correlation between CD3 CD8 HD levels and outcomes (108,110).

Most of the studies looking for circulating biomarkers to ICI in blood cells are focused on lymphocytes T, based on short populations of single types of cancer (mostly lung cancer patients) and missing validation cohorts (111,112). To our knowledge, no studies have been done with large multitumor populations exploring baseline lymphocytes

subpopulations and their dynamics during treatment. Therefore, further prospective analysis in a pan-cancer setting treated with ICI is needed in order to identify potential agnostic biomarkers for ICI candidates.

Immunoblood Project

In this context, we designed the Immunoblood project, a prospective observational study with the aim of characterizing the patterns of response to ICI (mainly antiPD1 and antiPDL1) in metastatic solid tumors and exploring patients' clinicopathological, molecular and blood features that can be useful to improve the selection of candidates for this relatively novel therapeutic approach. We carried out the the study at the Clinical Trials Unit of the Hospital Clinic of Barcelona (HCB) in collaboration with Translational Genomics and Targeted Therapies in Solid Tumors lab of IDIBAPs and the HCB Immunology Department (113).

First, we hypothesized that baseline and on treatment lymphocyte subpopulations may correlate with ICI outcomes so we collected blood samples for their analysis at Hospital Clinic of Barcelona immunology department and studied their relationship.

Second, we collected information about already known potential biomarkers when available (PDL1, TMB) and analyzed the presence of TILs and PD1 mRNA expressions when tumor samples were available to explore its correlation with ICI outcomes.

Third, we hypothesized that a more effective prognostic stratification based on clinical and laboratory baseline characteristics might help identifying patients with higher probability of ICI benefit or even patients not responsive that should receive an alternative therapeutic approach. As it remains uncertain which prognosis score better predicts patient outcomes, we propose to perform a direct comparison to pick the best (38,49,60,61,63,114–118).

Chapter 2. Hypothesis and aims

A. PRIMARY OBJECTIVE

- Serial assessment of lymphocytes subpopulation levels over time as biomarker of response or resistance to immunotherapies.

B. SECONDARY OBJECTIVES

- Correlation of baseline clinicopathological factors to response and survival in a comprehensive multitumor population
- Role of PD1 mRNA levels, PD-L1 protein expression and Tumor Infiltrating Lymphocytes (TILs) levels as predictor of response or resistance to immunotherapies.
- Correlation of published prognosis scores and early dynamics with ICI outcomes.

Chapter 3. Methodology

3.1 Ethic Commitee Approval, Documentation and Data Management.

The study was run following the Good Clinical Practice, according to the Helsinki Declaration (Fortaleza, Brasil, October 2013), and to the biomedical research law 14/2007, 3rd of July. This study was evaluated and approved by the Ethic Committee of Hospital Clinic Barcelona on 11th of May 2017.

For European Union member states, the informed consent procedure conformed to the ICH guidelines on Good Clinical Practice. This implied that "the written informed consent form should be signed and personally dated by the patient or by the patient's legally acceptable representative".

The following documents were designed for the study:

- ✓ Protocol (Version 1, 19.04.2017)
- ✓ Informed consent form ("Consentimiento informado para la utilización de datos clínicos y material biológico de proyectos de investigación y excedente del proceso asistencial para investigación biomédica en enfermedades onco-hematológicas y su conservación en la colección biospecimens of patients with solid tumors at Hospital Clinic")
- ✓ Delegation log (version 1, 19.04.2017)
- ✓ Plasma/Buffy coat Lab Manual (Version 2.1, 24.04.2017)
- ✓ Plasma/Buffy coat Lab Manual (Version 2.4, 14.12.2017)
- ✓ Flow citometry Lab manual (version 1, 24.04.2017).
- ✓ Flow citometry Lab manual (version 2, 05.05.2018).
- ✓ IMMUNE-BLOOD Specífic Sample Tracking Log (version 2, 20.01.2017)
- ✓ IMMUNE-BLOOD Specífic Sample Tracking Log (version 2.3, 02.05.2018)
- ✓ PANDORA Blood Sample Tracking Log (version 3. 30/03/2017)
- ✓ PANDORA Blood Sample Tracking Log (version 4. 10/07/2017)
- ✓ PANDORA Blood Sample Tracking Log (version 5. 14/12/2017)

Information related to the samples management, analysis and medical history was reviewed to run this study. Samples management was reviewed as quality control of processing methodology. Samples analysis and medical notes were reviewed to correlate them as specified in the objectives.

This information was managed following the next rules:

- Information collected during the study was kept in a study file whose owner is the site.
- Participants have the right of access their information, rectification, cancellation of their participation and opposition to the study doctor, according to the law 15/99 of personal data protection, contacting the principal investigator (responsible of the study).
- If these data were transferred to a third party (for example an sponsor external to the site), they would be codified, removing any data that could identify the participant. Only principal investigator and research team have access to the list of codes and personal participant information.
- Sponsor/Principal Investigator preserved the privacy of the participant and their confidential information, according to the law 15/1999 of data protection and RD 1720/2007, of 21st of December, which approves the regulation of the organic law 15/1999, 13th of December, of personal data protection through its codification.
- Personal that have access to the source data include investigators. Its institution may give access (but not transference) to documents or source data for monitorization, audit, CEI review and/or inspection of the study by public health authorities.

3.2 Patient recruitment

Candidates were recruited during any of their visits to the hospital. A member of the research team identified potential candidates, approached them and finally offered the consent form.

For this study, candidates that fulfill the following criteria were recruited:

INCLUSION CRITERIA

- 18 years old or older
- Diagnosis of oncologic and/or hematologic disease
- Patient being considered for immune therapy in the near future
- Able to understand the participant information sheet and give written informed consent according to ICH/GCP, and national/local regulations.

EXCLUSION CRITERIA

- Any psychological, familial, sociological or geographical condition potentially hampering compliance with the study protocol; those conditions should be discussed with the patient before registration in the trial

All patients were informed of the aims of the study, the possible adverse events, the procedures and possible hazards to which he/she would be exposed. They were informed as to the strict confidentiality of their patient data, but that their medical records may be reviewed for trial purposes by authorized individuals other than their treating physician. They were informed of the kind of analysis that may be done with their samples, including whole genome sequencing, and that their medical records may be reviewed by members of the study team, always keeping the confidentiality. Afterwards, if patients agreed to participate they were offered the current version of the participant information sheet "*Consentimiento Informado Para La Utilización De Datos Clínicos Y Material Biológico De Proyectos De Investigación Y Excedente Del Proceso Asistencial Para Investigación Biomédica En Enfermedades Onco-Hematológicas Y Su Conservación En La Colección Biospecimens Of Patients With Solid Tumors At Hospital Clinic*". None samples were collected before consenting. This document was signed and personally dated by the patient or by the patient's legally acceptable representative as well as the physician. A copy was provided to the candidate and another one was archived in the study file. This informed consent procedure conformed to the ICH guidelines on Good Clinical Practice.

Only candidates that fulfilled all the inclusion and exclusion criteria of this study were offered to participate. It was emphasized that the participation was voluntary and that the patient was allowed to refuse further participation in the protocol whenever he/she wants. This would not prejudice the patient's subsequent care. Documented informed consent had to be obtained for all patients included in the study before they were registered. If the patient refused further participation during the study, no more samples would be collected. Samples that were already in the collection would be kept and analyzed unless the participant expressed his/her right to destroy them.

Finally, we considered candidates as evaluable and therefore included in the analysis if they fulfilled the following criteria:

- Results of lymphocyte subpopulations from basal sample (C1D1), from second cycle (C2D1) or both had been obtained.
- Patient had a baseline and at least a second radiologic evaluation that allowed as to study the tumoral response by RECIST 1.1 o RANO criteria o did not have a second radiologic assessment but had no doubt of clinical progression disease with fatal outcome.

3.3 Clinical Data

Electronic patient charts were reviewed to collect relevant clinical information, blood test results for collecting specific values and imaging tests for analyzing baseline location of cancer lesions. Treatments and follow-up procedures were decided outside of this project according to clinical trial protocol o standard of care, since patients received ICI in interventional clinical trials o standard treatments chosen by their treating physician.

Clinical data included birth date, age at cycle 1 day 1 (C1D1), diagnosis, date of diagnosis, neoadjuvant radiotherapy (yes/no), neoadjuvant systemic treatment (yes/no), surgery (yes/no), adjuvant systemic treatment, concomitant chemotherapy-radiotherapy (yes/no), adjuvant radiotherapy, number of prior lines of treatment, line of treatment during study participation, if patient had received prior ICI (immune naïve versus previously treated with ICI), treatment schedule (monotherapy versus combination), ICI target (PD1, PDL1, other), treatment within clinical trial versus standard treatment, name of clinical trial (if applicable), date of first treatment administration, baseline ECOG, absence/presence of liver metastasis at baseline, date of death and overall survival.

Full blood count and blood chemistry tests were carried out as part of their standard of care, which usually involves a blood sample collection prior to each treatment prescription including hemoglobin, total leucocytes, neutrophils, lymphocytes, albumin, LDH. These values were collected for C1D1 and C2D1 in order to calculate different prognosis scores in both points and their evolution.

Baseline location of metastasis was also reviewed in order to stablish if the patients had or not visceral, bone and/or central nervous system metastasis, adenopathies (yes/no) or liver metastasis.

Special interest had the potential influence of radiotherapy o antibiotics to the outcome of our ICI so more detailed information was collected regarding them. We collected if the patients have received either of them within 30 days prior to start treatment o during it, and if so, the dates and doses.

Based on the previous information, several prognosis scores were calculated within which we can find the NLR, dNLR, LIPI, RMH, PMH, GRIm and PIPO scores. (38–41,43,44,49,50,54,60,61,63,115,116,119).

3.4 Evaluation of Response

Frequency of radiology procedures were decided outside of this project according to clinical trial protocol o standard of care, since patients received ICI in interventional clinical trials o standard treatments chosen by their treating physician. Their images were reviewed to evaluate in a standardized and objective way the degree of tumoral response. Glioblastoma patients were evaluated by RANO criteria meanwhile the remaining diagnosis were evaluated by RECIST 1.1 criteria. Besides,

Based on these evaluations the following data were collected: time to best response, best response (progression disease [PD], stable disease [SD], partial response [PR] o complete response [CR]), best percentage modification in target lesions from baseline, disease control rate (DCR), overall response rate (ORR), duration of response (DOR), date of PD and progressions free survival.

RECIST 1.1 Criteria (120)

Measurability of tumor at baseline

At baseline, tumor lesions/lymph nodes are categorized as measurable or non measurable as follows:

- Measurable lesion

Tumor lesions: Must be accurately measured in at least one dimension with a minimum size of 10 mm by CT scan, 10 mm caliper measurement by clinical exam o 20 mm by chest X-ray.

Malignant lymph nodes: To be considered pathologically enlarged and measurable, a lymph node must be ≥ 15 mm in short axis when assessed by CT scan.

- Non measurable lesion

All other lesions, including small lesions (longest diameter <10 mm or pathological lymph nodes with ≥10 to <15 mm short axis) as well as truly non-measurable lesions. Lesions

considered truly non-measurable include: leptomeningeal disease, ascites, pleural or pericardial effusion, inflammatory breast disease, lymphangitic involvement of skin or lung, abdominal masses/abdominal organomegaly identified by physical exam that is not measurable by reproducible imaging techniques.

Baseline documentation of ‘target’ and ‘non-target’ lesions

A maximum of five baseline measurable lesions in total (and a maximum of two lesions per organ) representative of all involved organs should be identified as target lesions and will be recorded and measured at baseline.

Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, and their measure must be reproducible.

A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions will be calculated and reported as the baseline sum diameters.

Response Criteria

Evaluation of target lesions

- Complete Response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm.
- Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.
- Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progression).
- Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

Evaluation of non-target lesions

While some non-target lesions may actually be measurable, they need not be measured and instead should be assessed only qualitatively at the time points specified in the protocol.

- Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis).
- Non-CR/Non-PD: Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

- Progressive Disease (PD): Unequivocal progression of existing non-target lesions. (Note: the appearance of one or more new lesions is also considered progression).

Further details can be found in RECIST 1.1 guidelines published by Eisenhauer et al (120).

iRECIST Criteria (Seymour) (121)

iRECIST 1.1 follows the same methodology that RECIST 1.1 in terms of measurable disease, targets and non target lesions and definition of CR, PR, SD and PD (named as iCR, iPR, iSD) and PD (unconfirmed progressive disease [iUPD] or confirmed progressive disease [iCPD]). Unconfirmed progressive disease [iUPD] is equivalent to the RECIST 1.1 PD. Nevertheless, iRECIST criteria require a new evaluation 4-8 weeks later allowing the detection of late responses or pseudoprogressions or its confirmation. Response will be classified as iCPD if the conditions mentioned in table 2 are fulfilled.

Table 2: iRECIST PD Confirmation Criteria (Seymour) (121)

		PD Confirmation Criteria			
UPD Criteria	**UPD Category**	**Target Lesion**	**Non Target Lesion**	**New Target Lesion**	**New Non Target Lesion**
Increase ≥20% y ≥5 mm in target lesion	Target Lesion	Increased ≥ 5 mm	Inequivocal Progression	New	New
Inequivocal Progression	Non Target Lesion	Increased ≥20% and ≥5mm	Increased	New	New
New target lesion	New target lesion	Increased ≥20% y ≥5mm	Inequivocal Progression	New o increased ≥ 5mm	New
New non target lesion	New non target lesion	Increased ≥20% y ≥5mm	Inequivocal Progression	New	New o increased

Legend. UPD: unconfirmed progressive disease.

If the following evaluation fulfills these criteria the PD is considered confirmed (iCPD). Nevertheless, if the lesions remain stable within the PD range it is considered iUPD, and if they decrease the response would be considered iSD, iPR o iCR. In this case, a new increase will be considered iUPD and confirmation would be required again.

Further details can be found in iRECIST guidelines published by Seymour et al (121).

iRECIST criteria was created because of the need to standardized response criteria for immune modulators and to detect late responses. Nevertheless, its implementation has been mainly in clinical trials rather than in standard practice hence non all our patients have a confirmatory CT scan. Therefore, RECIST 1.1 was used as reference for our analysis and the presence of late responses/pseudoprogressions will be explored within those patients with a confirmatory scan.

RANO Criteria (122)

Gliomas are the most common type of malignant primary brain tumor. They have a different behavior than other tumors so specific criteria have been developed to evaluate their tumoral response. The main different with previously mentioned criteria consist in the evaluation of lesion size by both perpendicular diameters.

Measurability of tumor at baseline

The product of the maximal cross-sectional diameters of the enhancing lesions will be used to determine the size of contrast-enhancing lesions.

- Measurable lesion

Measurable disease is defined as contrast-enhancing or non–contrast-enhancing lesions with clearly defined margins by MRI scan, with both perpendicular diameters on a single slice of at least 10 mm, visible on two or more slices.

- Non measurable lesion

Non measurable disease remains defined as either unidimensionally measurable lesions, masses with unclear margins, or lesions with maximal perpendicular diameters <10 mm.

Baseline documentation of 'target' and 'non-target' lesions

Target Lesions

When multiple measurable lesions exist, at least two and no more than three lesions should be identified as target lesions for studies evaluating either enhancing or non enhancing tumors. For patients with multiple lesions, those that are increasing in size should be selected as target lesions, regardless of their relative size. The other lesions will be considered nontarget and should be recorded but not integrated into the total lesion size calculation.

Evaluation of response:

A sum of the perpendicular diameters of the target lesions should be calculated and reported as the baseline sum diameters. Besides of tumor size evolution, we must consider the need of corticosteroids and their symptoms (new o improvements). Based on these factors the tumoral response is classified as mentioned in table 3. CR, PR and SD must fulfill all the criteria but PD only requires one of them.

Further details can be found in RANO 2.0 guidelines published by Wen et al in 2010 (122).

Table 3: Response Evaluation based on RANO Criteria (122)

	Target and Non Target Lesions	Steroids Use	Clinical Status
Complete Response (CR)	Complete disappearance of TL and NTL. No new lesions.	None	Estable or improved.
Partial Response (PR)	TL ≥ 50% decrease sustained for at least 4 weeks Stable/improved NTL. No new lesions.	Estable or decreased.	Estable or improved.
Estable Disease (SD)	TL between PR and PD criteria Stable/improved NTL. No new lesions.	Estable or decreased.	Estable or improved.
Progression Disease (PD)	TL ≥ 25% increase o new lesions.	Increased due to clinical deterioration related to tumor	Clinical deterioration related to tumor

Legend. TL: target lesion. NTL: Non target lesion. CR: complete response. PR: partial response. SD: stable disease. PD: progression disease.

3.5 Blood samples

3.5.1 Sample Collection and y processing

Samples were collected prior to the first drug administration (C1D1), prior to the second administration (C2D1) and as close as possible to the evaluation of response imaging test. At each of these timepoints a 4 mL BD Vacutainer® K2E (EDTA) 7.2mg Ref.368861 tube was collected. These tubes were labeled with the patient identification number, registered in the “Blood Sample Tracking Logs” and transferred for their analysis by the Immunology Department of Hospital Clinic of Barcelona.

Extraction of 4mL of whole blood sample using EDTA tube was performed. Fifty micro liters of blood were transferred from EDTA to “Canto tube” and mixed with 2 ul of specific panel of antibodies described in Table 4, with the exception of FOXP3 panel (5 ul of each antibody were added). In case of CTLA4 and CCR7 50 ul of Buffer (Brilliant Stain Buffer; BD ref 566349) was added. Samples were homogenized for 15 minutes at room temperature in a dark chamber. Two ml of 1:10 lysis buffer were added and homogenized in a dark chamber for 15 minutes and centrifuged at 1500 rpm for 5 minutes at 4ºC. Supernatant was removed and pellet was washed using 3 ml of PBS buffer centrifuged at 1500 rpm for 5 min at 4ºC and supernatant was removed.

Table 4: specific panel of antibodies

TBNK Panel		TACT Panel		FOXP3 Panel	
Antigen	**Antibody**	**Antigen**	**Antibody**	**Antigen**	**Antibody**
CD19	PE_Cy7	HLA_DR	FITC	CD4	FITC
CD16	PE	CD40L (CD154)	PE	CD25	PE
CD56	PE	CD62L	APC	CD3	BV421
CD4	FITC	CD25	PE_Cy5	CD45	PerCPCy5.5
CD8	PerCP_Cy5.5	CD69	PE_Cy7	CD127	PC7
CD45	APC	CD3	APC_Alexa750		
CD3	APC_Alexa750				

CTLA4 Panel			CCR7 Panel	
Antigen	**Antibody**		**Antigen**	**Antibody**
HLA_DR	FITC		CD62L	FITC
CTLA4	PE		CD25	PE
CD25	PE Cy5.5		CCR7	PerCP5.5
CD69	PC7		CD8	PC7
CD274-PD-L1	APC		CD45RO	APC
CD3	APC H7		CD45RA	APC_Cy7
CD19	BV510		CD3	BV421
CD4	BV421		CD45	BV510

Additional steps in FOXP3 panel were performed. Washing with 3mL PBS was performed, and supernatant was removed and the pellet was resuspended using 200uL fixation buffer (Treg Phenotyping Kit Ref 130-122-994 Miltenyi) and homogenized at 4ºC. 3mL of PBS was added, centrifuged at 1500 rpm at 4ºC and supernatant were resuspended using 250uL of fixation buffer 1X. Centrifugation at 1500 rpm for 5 minutes at 4ºC and supernatant was resuspended using 20uL of Fixation buffer 1X and 5uL of FcR Blocking reagent. 10uL of Anti-FOXP3 antibody was added, homogenized and mixed with 250uL of Fixation buffer 1X. Centrifugation at 1500rpm for 5 min at 4ºC was performed.

Pellet was resuspended using 100uL of FACS buffer. Reading of signal using Attune NxT Flow Cytometer was performed. Analysis was done with Attune NxT software.

3.5.2 Analysis of Flow cytometric data

General lymphocytes populations and subpopulations were analyzed by flow cytometry. Lymphocytes were manually gated and the live singlet population was analyzed using Attune NxT software. For the T cell analysis, the cells were additionally gated for CD3+ and CD4+CD8- or CD4-CD8+. Several markers were used in the different panel for clustering after the gating (Table 5). Percentage of each population regarding total T lymphocytes were calculated. Besides, mean fluorescence intensity was used to measure the level of expression of specific markers (CD28, CTLA4, CD1).

Table 5: lymphocyte populations/subpopulations analyzed by flow cytometry

General populations	Lymphocytes T (CD3+) Lymphocytes T (CD3+CD4+) Lymphocytes T (CD3+CD8+) Lymphocytes B (CD19+) NK cells (CD16/56+) CD4+/CD8+ rate
Lymphocytes T subpopulations (% of total Lymphocytes T; %TCD3)	Lymphocytes naive (CD3+CCR7+CD45RA+) (%TCD3+) Central Memory Lymphocytes (CD3+CCR7+CD45RO+) (%TCD3+) Effector Memory Lymphocytes (CD3+CCR7-CD45RO+) (%TCD3+) Regulatory T Lymphocytes (CD3+CD4+CD25+FoxP3+) (%TCD3+) Lymphocytes CD3 PD-L1+ (%TCD3+)
Study of activation markers of lymphocytes T (% of total Lymphocytes T)	Lymphocytes CD3 CTLA-4+ (%TCD3+) Lymphocytes CD3 CTLA4+ PD1+ (%TCD3+) Lymphocytes CD3 PD1+ (%TCD3+) Lymphocytes CD3 PD1 High+ (%TCD3+) Lymphocytes CD3 CD28 (MFI) Lymphocytes CD3 CTLA-4 (MFI) Lymphocytes CD3 PD1 (MFI) Lymphocytes CD3+CD25+ (%TCD3+) Lymphocytes CD3+HLA-DR+ (%TCD3+) Lymphocytes CD3+CD40L+ (%TCD3+) Lymphocytes CD3+CD62L+ (%TCD3+) Lymphocytes CD3+CD69+ (%TCD3+)
CD3+ CD8+ Lymphocytes T subpopulations (% of lymphocytes T CD3+CD8+; % CD8+)	Lymphocytes CD3 CD8 HD (% CD8+)(108,110) Lymphocytes CD3 CD8 CTLA4 (% CD8+) Lymphocytes CD3 CD8 CTLA4+ PD1+ (% CD8+) Lymphocytes CD3 CD8 PD1+ (% CD8+) Lymphocytes CD3 CD8 PD1 High + (% CD8+) Lymphocytes CD3 CD8 CD28 (MFI) Lymphocytes CD3 CD8 CTLA-4 (MFI) Lymphocytes CD3 CD8 PD1 (MFI)
CD3+ CD8+ CD4+ Lymphocytes T subpopulations (double positive)	Lymphocytes CD3+ CD8+ CD4+ (%TCD3+)

CD3+ CD4+ Lymphocytes T subpopulations (% of lymphocytes T CD3+CD4+; %CD4+)	Lymphocytes CD3 CD4 HD (% CD4+) Lymphocytes CD3 CD4 CTLA4 (% CD4+) Lymphocytes CD3 CD4 CTLA4+ PD1+ (% CD4+) Lymphocytes CD3 CD4 PD1+ (% CD4+) Lymphocytes CD3 CD4 PD1 High + (% CD4+) Lymphocytes CD3 CD4 CD28 (MFI) Lymphocytes CD3 CD4 CTLA-4 (MFI) Lymphocytes CD3 CD4 PD1 (MFI)

%TCD3+: % of total lymphocytes CD3+. %CD4+: % of lymphocytes CD3+CD4+; %CD8+: % of lymphocytes CD3+CD8+. MFI: Mean Fluorescence Intensity. HD: highly differentiated.

3.5.3 Sample selection

Higher value samples were those one related to critical timepoints of the ICI treatment as prior to first treatment dose (basal o C1D1), prior to second administration (C2D1), time of best tumoral response (BR) and time of progression disease (PD). Those sample results were selected for the statistical analysis.

Date of best response is defined as the first date in which the tumor reaches the best RECIST 1.1 o RANO category. Therefore, if a patient starts treatment and eight weeks later reaches the partial response that would be the date although the percentage continues decreasing. If the tumor keeps improving and disappear (complete response), he would have reached a better category and this would be the date of best response.

3.6 Tumor Samples

Tumor samples were selected based on the following criteria:

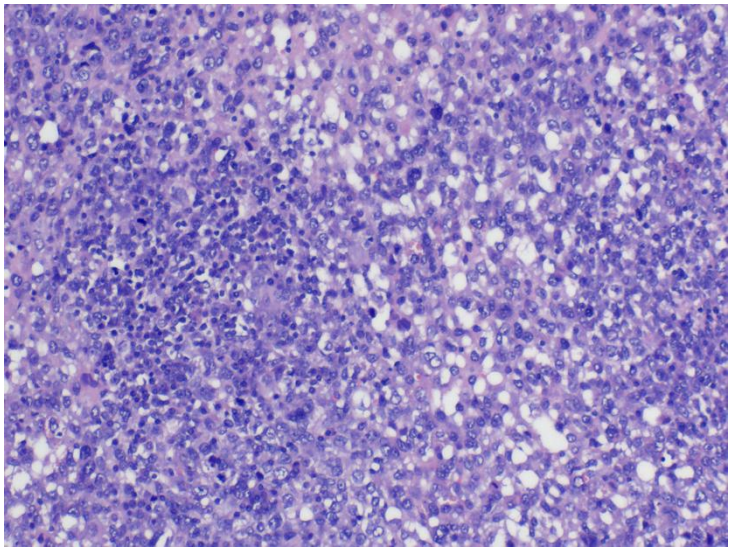
Tumor samples were located at pathological anatomy service of Hospital Clinic of Barcelona. From those available, we selected the most recent sample collected before starting ICI therapy, giving priority to samples from metastatic sites rather than primary tumors.

A minimum of 3 samples were collected from the archive whenever possible. We started working with the top priority sample and reviewed the stromal tumor-infiltrating lymphocytes (TILs) and the percentage of tumor cells present in the sample. In the cases of resection specimens with several slides included, we selected the most appropriated tumor slide.

TILs are a type of immune cells which are located dispersed in the stroma between the carcinoma cells but not directly in contact with them. TILs include all mononuclear cells (lymphocytes and plasmatic cells) and exclude others like granulocytes and polymorfonuclear leukocytes.

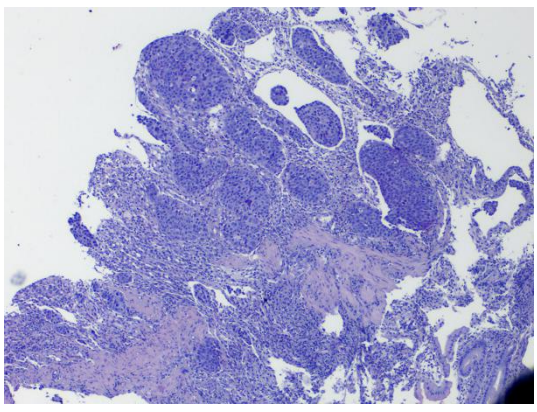
TILs are reported based on stromal compartment (= % stromal TILs). The denominator used to determine the % stromal TILs is the area of stromal tissue (i.e. area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e. fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei).

For our study, we selected a hematoxilin eosin stained section (section of standard thickness of 4–5 μm is often) of formalin fixed paraffin embedded tissues. This slide was reviewed by our pathologist by microscope magnification of x200-400 in light microscopy without the need for immunohistochemical staining. TILs were scored as a continuous variable.

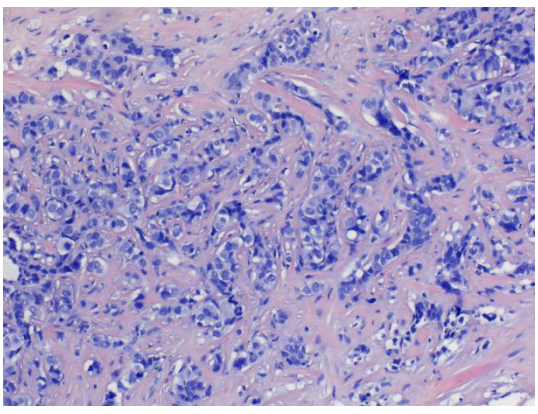


HE staining of poorly differentiated ovarian tumor with some lymphocytes in the tumor stroma around the 30% (20x)

The percentage of tumor cells was also assessed. Its evaluation was carried out by reviewing an hematoxilin eosin slide where an area of the tumor was selected. This area may or may not be the entire surface of the sample. Over this section, the % of tumor cells present was assessed. It consists of an approximation of the amount of tumor cells present, in relation to the stroma and another type of accompanying cellularity in that area.



HE staining of poorly differentiated gastric carcinoma in endoscopy biopsy with around 35% of tumor cells (10x)



HE staining of ductal infiltrating breast carcinoma with around the 60% of tumor cells (20x)

The pathological anatomy reports were reviewed to see if an MSI and PDL1 study had been performed, noting the result in all those cases that was available. Also the clinical history was reviewed to detect some MSI results of external molecular studies.

The internal MSI study had been done with four inmunohistochemical markers: MLH1 (M1, Ventana), MSH2 (G219-1129, Ventana), MSH6 (SP93, Ventana) y PMS-2 (A16-4, Ventana) with Benchmark Ultra (ROCHE) machine, whose results were interpretated as positive or negative nuclear staining in tumoral cells. In other cases, this study had been done with molecular IdyllaMT MSI Test by Biocartis, whose results were “mutation is detected” or not detected. The final conclusion was to identify whether the tumor was stable (MSS) or unstable (MSI).

The PDL1 study was done with primary antibody PDL1 IHQ (22C3) pharmaDX of Dako in the Autostainer link 48 (DAKO) machine. And the interpretation of its result was carried out following the indications of each tumor type, either with TPS (tumor proportion score) or CPS (combined positive score) and their accepted ranges for each organ.

We also studied the expression of PD1 gene in mRNA using the nCounter platform (Nanostring Technologies)(123). The selection of the sample for this part of the study was made following the previously mentioned criteria and also included:

1. Selection of the sample prior to the start of immune therapy
2. Excluding samples from lymph nodes and cell blocks
3. Material confirmation check in the selected paraffin block
4. A section of the FFPE tissue was first examined with hematoxilin eosin staining to confirm presence of invasive tumor cells and determine the tumor area. It was necessary that the samples had a minimum of 10% tumor cellularity in a minimum surface of 4 mm2
5. For RNA purification (Roche® High Pure FFPET RNA isolation kit), between 1 and 8 sections of 10 µ thickness of each of the blocks has been made, and they were introduced into an eppendorf to perform the mRNA extraction study and adequate analysis. Macrodissection was performed, when needed, to avoid normal contamination. The exact number of sections that was necessary for each sample depends on tumor surface present, if the sample was:

Core or small biopsy:

Tumor area (mm^2)	Number of sections
≤ 6 mm^2	8
7-12 mm^2	4
≥ 13 mm^2	2

Resection specimen

Tumor area (mm^2)	Number of sections
4-19 mm2	6
20-99 mm2	3
≥100 mm2	1

A minimum of ~50 ng of total RNA was used to measure the expression of PD1 using the nCounter platform (Nanostring Technologies, Seattle, WA, US). The reaction was performed using DNA oligonucleotides that correspond to this target sequence of PD1: [CTTCTTCCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCTG CAGCTTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGC]. Raw Nanostring counts were subjected to a technical normalization using five housekeeping genes (ACTB2, MRPL19, PSMC4, RPLPO and SF3A1). This normalization is done by dividing all counts of each sample by a normalization factor that is calculated by the geometric mean of the 5 HK genes of each sample and then log base 2 transformed. Samples with 20 or fewer counts in at least 70% of the genes were removed. The entire expression data was scaled from 0 to 10 (123).

3.7 Statistical Analysis

Progression-Free Survival (PFS) was defined as the time from immunotherapy start to disease progression or death from any cause, whichever occurred first. Overall Survival (OS) was defined as the time from immunotherapy start to patients' death from any cause. PFS and OS were estimated by using the Kaplan-Meier method.

Clinical Benefit Rate was defined as the proportion of patients achieving SD, PR or CR as best response, while Overall Response Rate (ORR) was defined as the proportion of patients achieving PR or CR as best response. Durable control benefit was included patients achieving CR+PR+SD retained at 6 months as BR.

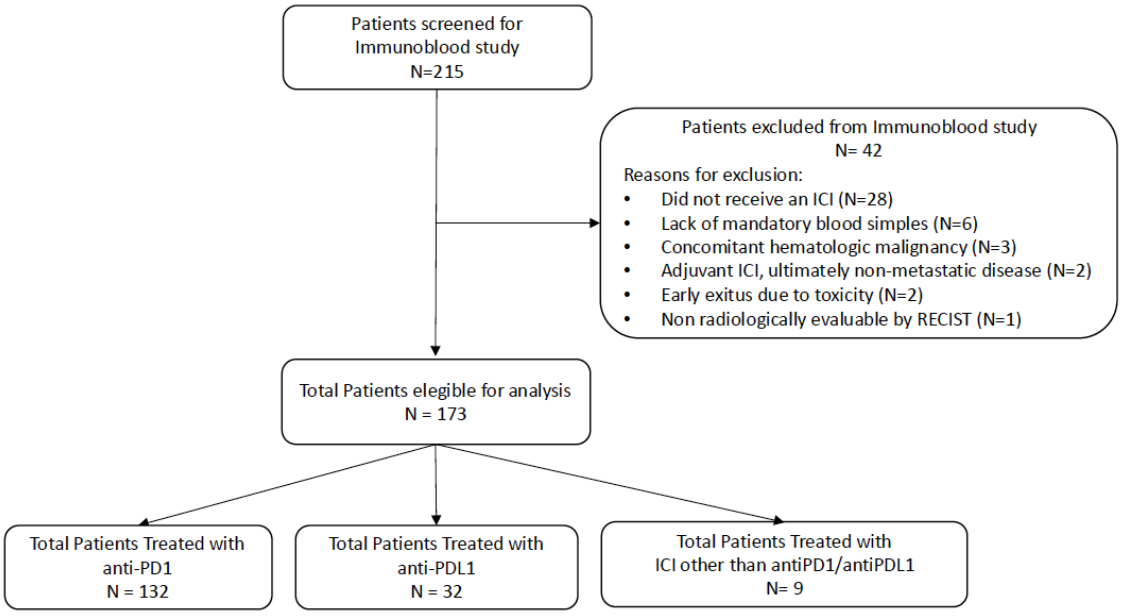
Specific statistical analysis will be mentioned in each objective during the following sections.

Chapter 4. Results

Chapter 4.1 Population Recruited

Between May 2017 and March 2022, 215 patients signed the inform consent form, 173 patients entered the study and 164 received an anti-PD1/anti-PD-L1-based treatment. The selection process for the purpose of this analysis is resumed in Figure 1(124).

Figure 1. STROBE flow-chart.



Legend. ICI: immune-checkpoint inhibitors.

The median follow-up at the data cut-off (23/12/2023) was 38,3 months (95% CI: 36,1-58,6). All patients and tumors characteristics are detailed in Table 6.

Table 6. Population characteristics

		TOTAL - N	TOTAL - %	Anti PD1 - N	Anti PD1 - %	AntiPDL1 - N	Anti PDL1 - %	Other Target - N	Other Target - %
Total Number of patients		173	100	132	100	32	100	9	100
Age	Mean	63,2		64,2		60,3		58,2	
	Standard Deviation	11,5		10,5		15		8,3	
Gender	Female	65	37,6	50	37,9	9	28,1	6	66,7
	Male	108	62,4	82	62,1	23	71,9	3	33,3
Tumor type	Lung	47	27,2	42	31,8	3	9,4	2	22,2
	Prostate	8	4,6	3	2,3	5	15,6	0	0,0
	Renal	6	3,5	4	3,0	2	6,3	0	0,0
	Suprarenal	1	0,6	0	0,0	1	3,1	0	0,0
	Urothelial Cancer	7	4,0	4	3,0	3	9,4	0	0,0
	Breast	15	8,7	11	8,3	1	3,1	3	33,3
	Colorectal cancer	33	19,1	30	22,7	0	0,0	3	33,3
	Head & Neck	10	5,8	4	3,0	6	18,8	0	0,0
	Melanoma	8	4,6	4	3,0	4	12,5	0	0,0
	Esofagous	4	2,3	2	1,5	1	3,1	1	11,1
	Gastric cancer	5	2,9	5	3,8	0	0,0	0	0,0
	Ginecologic cancer	9	5,2	7	5,3	2	6,3	0	0,0
	Sarcoma	1	0,6	0	0,0	1	3,1	0	0,0
	CNS	8	4,6	7	5,3	1	3,1	0	0,0
	Timo	1	0,6	0	0,0	1	3,1	0	0,0
	Cholangiocarcinoma	5	2,9	4	3,0	1	3,1	0	0,0
	Merckell Cells	1	0,6	1	0,8	0	0,0	0	0,0
	Hepatocarcinoma	2	1,2	2	1,5	0	0,0	0	0,0
	Pancreas	1	0,6	1	0,8	0	0,0	0	0,0
	Unknown origin	1	0,6	1	0,8	0	0,0	0	0,0
Metastatic at diagnosis	Yes	97	56,1	79	59,8	14	43,8	4	44,4
	No	76	43,9	53	40,2	18	56,3	5	55,6
ECOG basal	0	54	31,2	23	17,4	31	96,9	0	0,0
	1	100	57,8	91	68,9	1	3,1	8	88,9
	2	17	9,8	16	12,1	0	0,0	1	11,1
	3	2	1,2	2	1,5	0	0,0	0	0,0
Inmune therapy status	Inmunne naive	34	19,7	28	21,2	4	12,5	2	22,2
	No inmunne naive	139	80,3	104	78,8	28	87,5	7	77,8
TILs	0- 10 %	91	52,6	64	48,5	21	65,6	6	66,7
	>10 – 40 %	18	10,4	16	12,1	2	6,3	0	0,0
	>40 %	0	0,0	0	0,0	0	0,0	0	0,0
	Not available	64	37,0	52	39,4	9	28,1	3	33,3
PDL1 (22C3) %	Negative (<1%)	12	6,9	10	7,6	2	6,3	0	0,0
	Positive (≥1%)	40	23,1	33	25,0	5	15,6	2	22,2
	Not available	121	69,9	89	67,4	25	78,1	7	77,8

Microsatellite Instability	MSS	62	35,8	56	42,4	4	12,5	2	22,2
	MSI	6	3,5	5	3,8	1	3,1	0	0,0
	Not available	98	56,6	71	53,8	27	84,4	0	0,0
TMB	0-10	20	11,6	19	14,4	1	3,1	0	0,0
	>10	3	1,7	3	2,3	0	0,0	0	0,0
	Not available	150	86,7	110	83,3	31	96,9	9	100,0
RT in the 30 days before C1D1	No	163,0	94,2	122	92,4	32	100,0	9	100
	Yes	10,0	5,8	10	7,6	0	0,0	0	0
ATB in the 30 days before C1D1	No	164,0	94,8	125	94,7	30	93,8	9	100
	Yes	9,0	5,2	7	5,3	2	6,3	0	0
Corticoid in the 30 days before C1D1	No	156,0	90,2	117	88,6	30	93,8	9	100
	Yes	17,0	9,8	15	11,4	2	6,3	0	0
LNR Basal	0-3	71	41,0	56	42,4	14	43,8	1	11,1
	> 3 - 5	49	28,3	40	30,3	8	25,0	1	11,1
	>= 5	53	30,6	36	27,3	10	31,3	7	77,8
LDH Basal	≤ 450 (ULN)	92	53,2	74	56,1	17	53,1	1	11,1
	> 450 (ULN)	70	40,5	48	36,4	14	43,8	8	88,9
	LDH not available	11	6,4	10	7,6	1	3,1	0	0,0
dNLR Basal	< 3	127	73,4	98	74,2	25	78,1	4	44,4
	≥ 3	46	26,6	34	25,8	7	21,9	5	55,6
LIPI Basal	Good	73	42,2	58	43,9	15	46,9	0	0,0
	Intermediate	68	39,3	51	38,6	12	37,5	5	55,6
	Poor	21	12,1	13	9,8	4	12,5	4	44,4
	Not available due to lack of LDH	11	6,4	10	7,6	1	3,1	0	0,0
RMH_Score	Low risk	84,0	48,6	67	50,8	16	50,0	1	11,1
	High risk	60,0	34,7	40	30,3	12	37,5	8	88,9
	Not available	29,0	16,8	25	18,9	4	12,5	0	0,0
PMH_Score	Low risk	56,0	32,4	42	31,8	14	43,8	0	0,0
	High risk	94,0	54,3	70	53,0	15	46,9	9	100,0
	Not available	23,0	13,3	20	15,2	3	9,4	0	0,0
GRIm_Score	Low risk	133,0	76,9	103	78,0	26	81,3	4	44,4
	High risk	23,0	13,3	14	10,6	4	12,5	5	55,6
	Not available	17,0	9,8	15	11,4	2	6,3	0	0,0
PIPO_Score	Low risk	41	23,7	28	21,2	13	40,6	0	0,0
	Intermediate risk	94	54,3	75	56,8	14	43,8	5	55,6
	High risk	9	5,2	4	3,0	1	3,1	4	44,4
	Not available	29	16,8	25	18,9	4	12,5	0	0,0
Type of regimen during immunoblood study	Monotherapy	92	53,2	61	46,2	23	71,9	8	88,9
	Immunotherapy combination	36	20,8	33	25,0	3	9,4	0	0,0
	Immunotherapy + Other	45	26,0	38	28,8	6	18,8	1	11,1
Line of therapy during immunoblood study	1L	45	26,0	40	30,3	5	15,6	0	0,0
	2L	50	28,9	41	31,1	9	28,1	3	33,3
	3L	33	19,1	25	18,9	8	25,0	0	0,0

	4L	20	11,6	15	11,4	5	15,6	2	22,2
	≥5L	16	9,2	11	8,3	5	15,6	4	44,4
Clinical Trial during immunoblood study	Yes	123	71,1	48	36,4	30	93,8	9	100,0
	No	50	28,9	84	63,6	2	6,3	0	0,0
Best response	PD	86	49,7	69	52,3	11	34,4	6	66,7
	SD	58	33,5	39	29,5	16	50,0	3	33,3
	PR	22	12,7	19	14,4	3	9,4	0	0,0
	CR	7	4,0	5	3,8	2	6,3	0	0,0

Legend. N: number. CNS central nervous system. ECOG: Eastern Cooperative Oncology Group scale. TILs: tumor infiltrating lymphocytes. MSS: microsatellite stable. MSI: microsatellite instable. TMB: tumor mutational burden. RT: radiotherapy. ATB: antibiotic. LNR: lymphocyte neutrophil ratio. dNLR: derive lymphocyte neutrophil ratio. ULN: upper limit of normality. LIPI: lung immune prognosis index. RMH: Royal Marsden score. PMH: Princess Margaret Hospital score. GRIm: Gustave Roussy Immune Score. PIPO: Phase I Prognostic Online score.

A summary of the activity and efficacy outcomes seen in this population is reported in Table 7.

Table 7: Activity and efficacy of ICI in the overall study population

STUDY POPULATION OUTCOMES		
PD timing	**N**	**%**
≤4 months from ICI start	105	60.7
>4 months from ICI start	68	39.3
Best response	**N**	**%**
Complete response	7	4.0
Partial response	22	12.7
Stable disease	59	34.1
Progressive disease	85	49.1
ORR	**%**	**95%CI**
CR+PR	16.8	11.5% - 23.2%
DCB	**%**	**95%CI**
CR+PR+SD≥6 months	50.9	43.2% - 58.5%
Median PFS (months)	**N**	**95%CI**
	2.5	2.0 - 3.7
Median OS (months)	**N**	**95%CI**
	13.3	9.9 - 17.4
12-month PFS	**N (%)**	**95%CI**
	32 (18.5)	13.5% - 25.3%
12-month OS	**N (%)**	**95%CI**
	88 (50.9)	45.0% - 60.0%

Legend. CI: confidence interval; CR: complete response; DCB: durable clinical benefit; ICI: immune-checkpoint inhibitor; N: number; ORR: overall response rate; PD: disease progression; PR: partial response; PFS: progression-free survival; SD: stable disease; OS: overall survival.

Chapter 4.2 PRIMARY OBJECTIVE:

Serial assessment of lymphocytes subpopulation levels over time as biomarker of response or resistance to ICI.

4.2.1 Introduction

Several studies have been performed so far in order to investigate the potential of lymphocytes as biomarker. Preliminary results were found with specific subpopulations at baseline or their dynamics (7,100–102,108,108–112). Nevertheless, most of them were studies with a short number of patients and focused in single cancer diagnosis (mainly NSCLC). To our knowledge, no studies have been done with large multitumor populations exploring baseline lymphocytes subpopulations and their dynamics during treatment. Therefore, we decided to explore this setting in order to identify potential agnostic biomarkers for ICI candidates. Data reported here has not been published yet.

4.2.2 Material y methods

Study design and participants

Full inclusion/exclusion criteria and study procedures have already been reported in chapter 3: Methodology so only specific details related to this analysis are mentioned here. For further details refer to the relevant previous section.

We considered evaluable for this analysis all participants treated with an ICI with radiological data available for an independent assessment of tumor responses according to RECIST 1.1 o RANO criteria (120,122) and lymphocytes subpopulations results available for at least one of the first two simples (C1D1, C2D1). Patients with available baseline imaging experiencing a rapid progression leading to death, hence with no available radiologic reassessment, were also included.

Procedures

Blood samples were collected at C1D1, C2D1 and as close as possible to the evaluation of every evaluation of response and shipped to Immunotherapy Department for their analysis. Collection dates and evaluations of response by RECIST 1.1 / RANO criteria were used to correlate specific samples with the best response (BR) (if no progression at first CT scan) or progression disease (PD) timepoints. Treatments and follow-up procedures were decided outside of this study according to study protocols and clinicians opinion. All data were retrieved from electronic patient charts.

Study endpoints and outcomes

There was no prespecified sample size because of the exploratory nature of this study. The accrual was terminated after 4 years, and the clinical data cut-off was established when a minimum follow-up including at least one reassessment of the disease for every included patient was reached.

This analysis was intended to correlate lymphocyte subpopulation levels to response, in terms of overall response rate (ORR) and disease control benefit (DCB), and survival, in terms of progression-free survival (PFS) and overall survival (OS).

The evaluation of response for the purpose of this study were performed in accordance to RECIST 1.1 and RANO criteria[21]. Tumoral responses were classified as SD, progressive disease (PD), complete (CR) or partial response (PR) independently by the same expert (Javier García Corbacho) from the Clinical Trials Unit of the HCB [21]. For the ORR assessment we considered all patients achieving CR o PR as best response, while for DCB we included all patients achieving CR, PR o SD.

Statistical analysis

Mean value and standard deviation of all lymphocyte subpopulations at each selected timepoint (C1D1, C2D1, BR and PD) were calculated. Trends of each population in terms of mean value were reported by graphs restricted to all the population and to the population achieving a PR o CR (Objective Response Population; ORP). Significant variations in the mean value of all lymphocyte subpopulations at the timepoints of interest were analyzed by ANOVA for total population and ORP. Subpopulations with statistically significant results were analyzed by pairwise comparison to evaluate differences between specific timepoints (C1D1 vs C2D1, C1D1 vs BR, C1D1 vs PD, C2D1 vs BR, C2D1 vs PD, BR vs PD) for both populations. Associations of each subpopulation at C1D1 and C2D1 with ORR and DCB were studied by univariate logistic regressions. Univariate Cox regressions were performed to test the associations of each subpopulation with PFS and OS. Statistically significant results were correlated in order to identify the subpopulations of major interest as potential biomarker.

4.2.3 Results

A total of 989 samples were collected from our population, 475 (48%) of which corresponded to C1D1, C2D1, BR o PD. Out of our 173 patients, 151 (87.3%) had paired blood samples on the first two cycles, while 15 (8.7%) and 7 (4%) only had a sample from C1D1 o C2D1 respectively. Only 31 patients (17.9%) had a sample correlated with the best response which is explained by the fact that a minority of patients had benefit

with ICI therapy and did no progress on the first CT scan. Nevertheless, most of the patients had experienced a progression disease at our cutoff date so the number of PD samples is much higher (120, 69.4%) (Table 8).

Table 8: summary of samples collected.

Nº patients with samples at specified timepoints (% from total patients)	**Patients with paired samples (%)**	**Patients with C1D1 only samples N (%)**	**Patients with C2D1 only samples N (%)**	**TOTAL Patients N = 173 (100%)**
C1D1	151 (87.3%)	15 (8.7%)	0 (0%)	166 (95.9%)
C2D1	151 (87.3%)	0 (0%)	7 (4%)	158 (91.3%)
BR	18 (10.4 %)	8 (4.6%)	5 (2.9%)	31 (17.9%)
PD	107 (61.8%)	8 (4.6%)	5 (2.9%)	120 (69.4%)
TOTAL Nº of samples per timepoint	427	31	17	Total N of samples: 475

Legend. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. N: number. BR: best response. PD: progression disease.

Mean, Standard Deviation, Anova And Pairwise Comparison

Mean value and the standard deviation of each lymphocyte subpopulation at the selected timepoints (C1D1, C2D1, BR, PD) were performed. Afterwards, we run an ANOVA analysis to evaluate significant variations in the mean value of each subpopulation between those timepoints. Finally, statistically significant subpopulations were explored by pairwise comparison to study significant differences between C2D1 and C1D1 without p correction and with Bonferroni p correction. BR and PD timepoints are only identified once the patient has received the treatment and the disease has progressed so exploring dynamics relate those timepoints has no sense from a biomarker perspective.

Lymphocyte subpopulations means and behavior may be different in the general population and in the patients who benefit from ICI so the statistical analysis was performed for the total population and the objective response population (ORP).

Total Population Analysis

First, we calculated the mean value and the standard deviation of each lymphocyte subpopulation at those selected timepoints (C1D1, C2D1, BR, PD) for the whole population (table 9).

Table 9: Mean and standard deviation of each subpopulation at C1D1, C2D1, BR and PD timepoints.

		C1D1		C2D1		BR		PD	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
General populations	Lymphocytes (L) T (CD3+)	68.82	13.42	68.77	12.74	67.82	13.46	66.75	13.75
	LT (CD3+CD4+)	57.55	12.28	57.65	12.60	56.75	13.40	56.07	13.75
	LT (CD3+CD8+)	35.51	11.51	34.95	12.08	36.11	12.66	36.46	13.03
	L B (CD19+)	9.37	6.27	9.03	6.02	10.33	7.30	9.76	6.90
	NK cells (CD16/56+)	17.15	10.47	18.32	15.36	17.00	12.03	16.40	10.67
	CD4+/CD8+ rate	1.91	0.99	1.98	1.19	1.89	1.07	1.90	1.19
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	10.88	7.78	10.61	7.48	11.29	7.98	10.76	7.53
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	1.96	3.26	1.83	2.24	1.77	1.97	1.44	1.53
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	6.22	4.83	6.77	9.04	5.75	5.26	5.04	4.54
	Regulatory T L (CD3+CD4+CD25+ FoxP3+)(%TCD3+)	1.64	2.16	1.47	2.06	1.46	2.03	1.31	1.90
	CD3+PD-L1+ (%TCD3+)	243.08	315.51	277.88	364.20	231.47	326.47	207.83	178.75
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	20.53	11.03	20.47	10.32	19.73	11.47	19.05	11.29
	CD3 CTLA4+ PD1+ (%TCD3+)	23.94	10.85	25.52	11.37	24.64	11.19	24.85	12.03
	CD3 PD1+ (%TCD3+)	1.30	1.03	1.29	1.16	1.15	0.94	1.28	1.06
	CD3 PD1 High + (%TCD3+)	1.82	4.90	2.34	4.65	2.27	4.82	1.43	1.94
	CD3 CD28 (MFI)	0.83	1.20	1.01	1.80	0.83	1.31	0.93	1.41
	CD3 CTLA-4 (MFI)	10.59	11.50	9.58	12.16	8.74	11.71	8.73	11.00
	CD3 PD1 (MFI)	17.21	12.57	18.63	13.37	17.07	12.06	18.24	12.86
Study of activation markers of total lymphocytes T (% of total L T)	CD3+CD25+ (%TCD3+)	2.04	5.44	2.10	4.29	1.70	3.28	1.87	3.59
	CD3+HLA-DR+ (%TCD3+)	42.08	25.22	39.59	23.05	38.77	23.59	38.77	22.82
	CD3+CD40L+ (%TCD3+)	3.14	5.51	3.63	5.95	3.44	8.05	4.72	10.36
	CD3+CD62L+ (%TCD3+)	34.01	13.32	32.31	13.71	33.99	13.74	34.62	13.79
	CD3+CD69+ (%TCD3+)	64.85	24.44	63.19	23.16	64.65	23.87	67.43	23.52
	L CD3 CD8 HD (%TCD8+)	1.52	1.93	1.44	1.54	1.56	2.25	1.55	2.36
	L CD3 CD8 CTLA4 (%TCD8+)	57.45	15.07	59.49	15.39	58.26	15.49	57.07	15.35
	L CD3 CD8 CTLA4+ PD1+ (%TCD8+)	36.59	33.34	32.80	32.73	33.72	33.22	34.94	32.53
	L CD3 CD8 PD1+ (%TCD8+)	9.14	6.89	10.09	7.67	9.07	6.68	8.30	5.49
	L CD3 CD8 PD1 High+ (%TCD8+)	3.25	3.97	3.09	4.07	3.10	2.78	2.86	2.76
	L CD3 CD8 CD28 (MFI)	15.04	6.41	12.83	7.60	14.11	6.20	13.98	6.26
	L CD3 CD8 CTLA-4 (MFI)	4.76	3.67	3.54	2.82	4.43	3.46	4.47	3.81
	L CD3 CD8 PD1 (MFI)	1865.24	842.62	1928.21	917.81	1960.45	949.24	1885.54	757.38
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	627.13	1017.60	705.92	1264.72	444.19	714.32	349.63	498.13

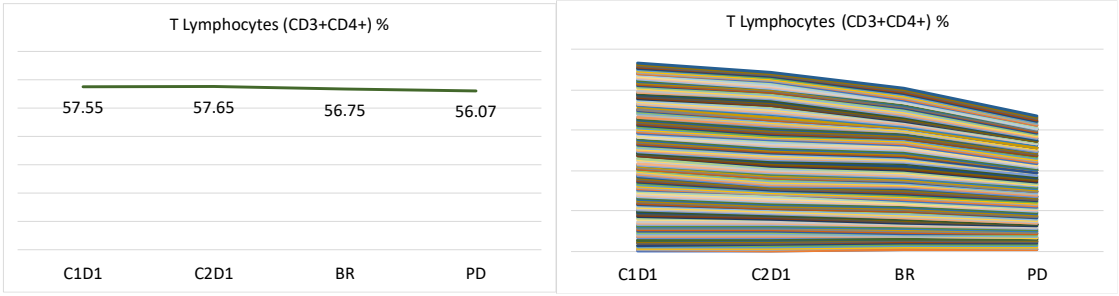
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	65.92	18.74	65.89	17.53	62.77	19.11	65.53	18.58
	CD3 CD4 CTLA4 (%TCD4+)	8.99	6.21	9.35	6.20	8.95	6.27	8.24	5.29
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	2.45	3.07	2.16	1.82	2.27	1.95	1.98	1.76
	CD3 CD4 PD1+ (%TCD4+)	10.53	4.30	9.15	5.28	9.96	3.94	9.32	4.02
	CD3 CD4 PD1 High+ (%TCD4+)	3.20	2.50	2.50	2.03	3.01	2.18	2.88	2.42
	CD3 CD4 CD28 (MFI)	1393.18	642.05	1460.82	685.93	1466.64	653.36	1390.27	579.22
	CD3 CD4 CTLA-4 (MFI)	344.89	460.06	382.79	440.19	313.24	452.34	282.82	257.57
	CD3 CD4 PD1 (MFI)	541.60	477.52	570.12	501.49	548.37	358.32	548.47	310.24

Legend. SD: standard deviation. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. %TCD3+: % of total lymphocytes CD3+. %CD4+: % of lymphocytes CD3+CD4+; %CD8+: % of lymphocytes CD3+CD8+.

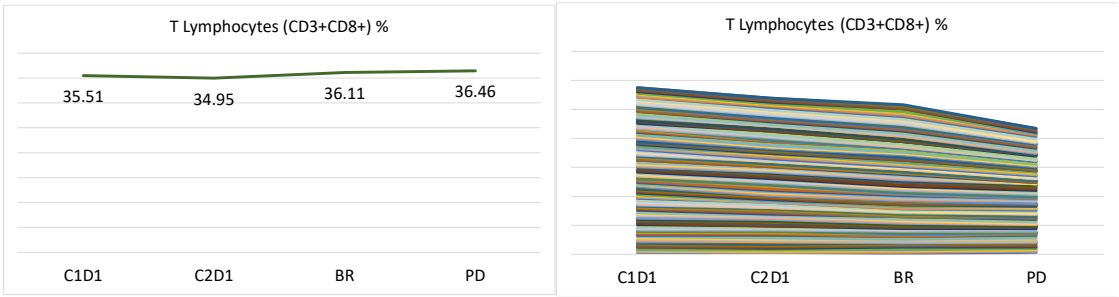
Second, in the ANOVA analysis for the total population means, lymphocytes CD3 PD1+ (p=0.0094), CD3 PD1High+ (p=0.0072), CD3+CD4+ (p=0.03), CD3 CD4 PD1+ (p=0.00612), CD3 CD4 PD1 High+ (p=0.000395), CD3 CD4 CTLA-4 (MFI) (p=0.0104) demonstrated decrease while CD3+CD8+ (p=0.0216) and CD3+ CD8+ PD1+ MFI (p=0.0104) had a significant increase, as shown in Figure 2 / table 10.

Finally, the pairwise comparison analysis showed significant decrease between C1D1 and C2D1 for CD3 PD1+ (%) (p=0.033), CD3 PD1 High+ (%) (p=0.039), CD3 CD8 PD1 (MFI) (p=0.023), CD3 CD4 PD1+ (%) (p=0.019) and CD3 CD4 PD1 High+ (p=0.0093) lymphocyte subpopulations. All other subpopulations showed no significant associations (Figure 2, Table 10).

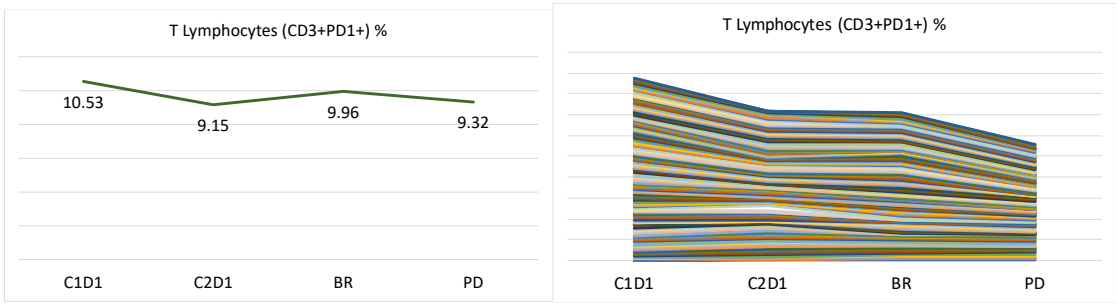
Figure 2: Total population means of specific lymphocyte subpopulations.



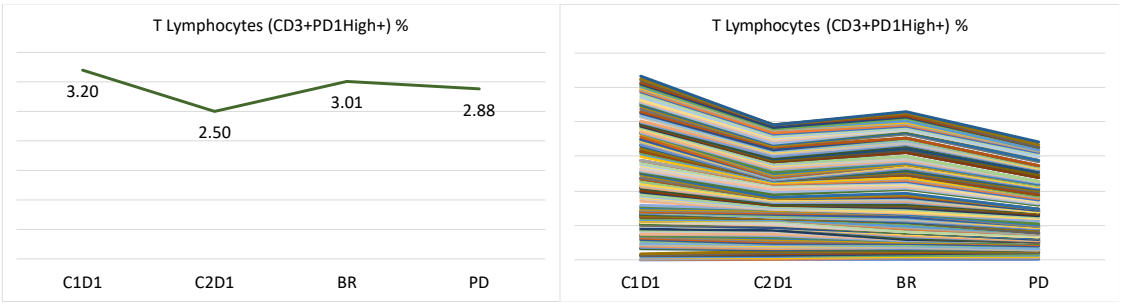
A) T Lymphocytes CD3+ CD4+ % (ANOVA p=0.03)



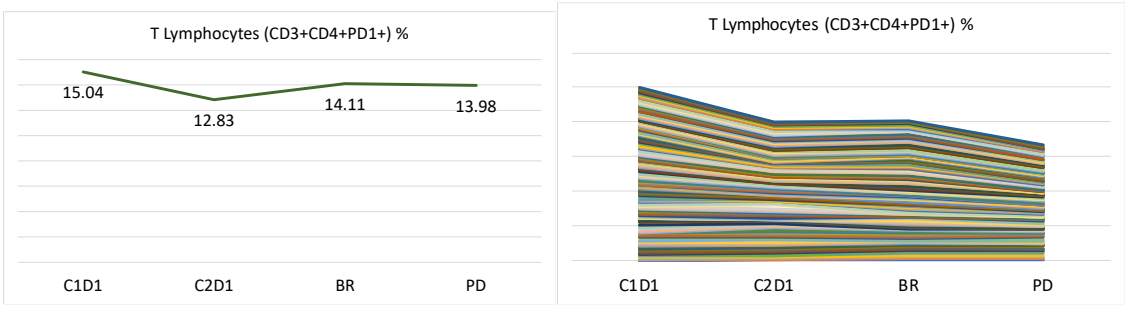
B) T Lymphocytes CD3+ CD8+ (ANOVA p=0.0216)



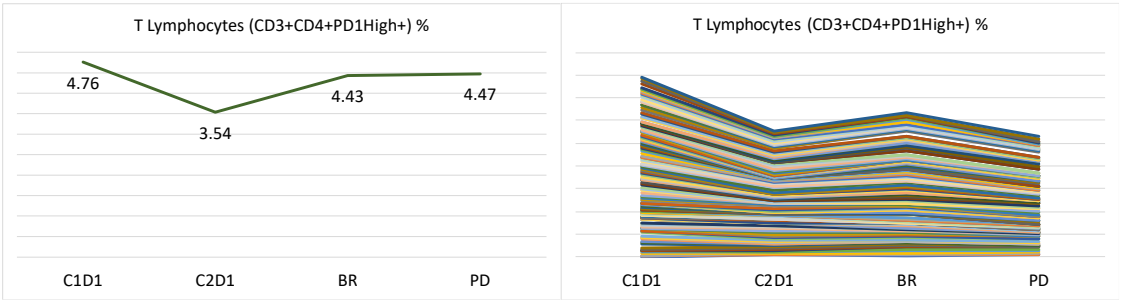
C)T lymphocytes CD3 PD1+ % (ANOVA p=0.0094)



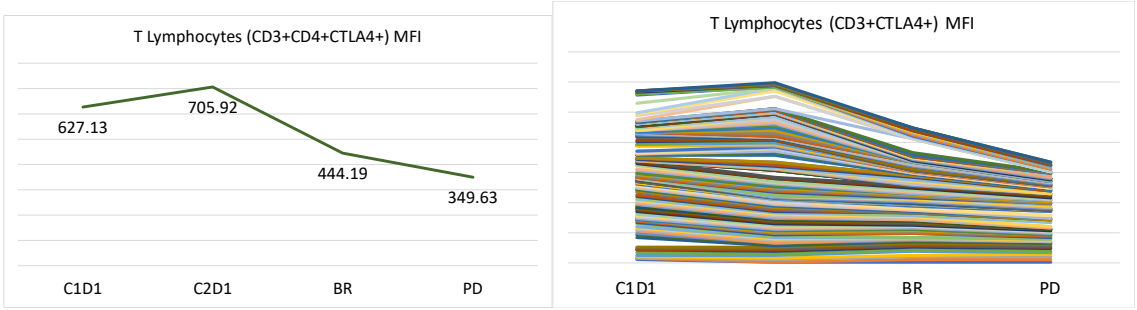
D) T Lymphocytes CD3 PD1 High (ANOVA p=0.0072)



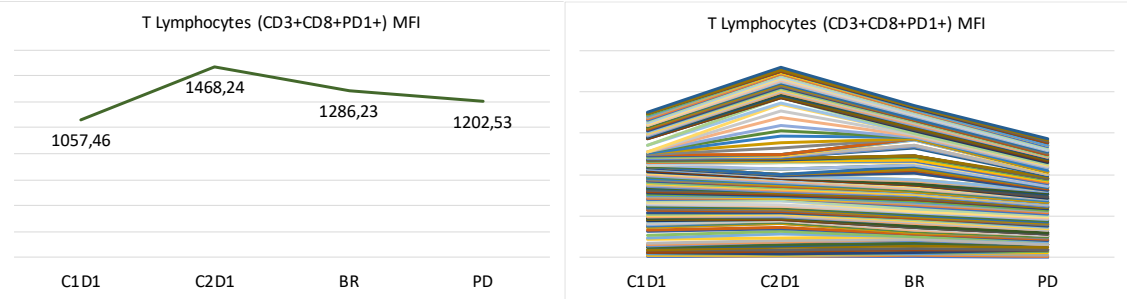
E) T Lymphocytes CD3+ CD4+ PD1+ (ANOVA p=0.00612)



F) T Lymphocytes CD3+ CD4+ PD1 High+ % (ANOVA p=0.000395)



G) T Lymphocytes CD3+ CD4+ CTLA4+ MFI (ANOVA p=0.0104)



H) T Lymphocytes CD3+ CD8+ PD1+ MFI (ANOVA p=0.0104)

Legend. Left graph: mean values. Right graph: single patient values. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity.

Table 10: ANOVA analysis for the total population and pairwise comparison between C2D1 and C1D1 for statistically significant subpopulations.

		ANOVA p value Total Population	Pairwise comparison C2D1 vs C1D1 NC / BC
General populations	Lymphocytes (L) T (CD3+)	0.11	
	LT (CD3+CD4+)	**0.03**	> 0,05 / > 0,05
	LT (CD3+CD8+)	**0.0216**	> 0,05 / > 0,05
	L B (CD19+)	0.0807	
	NK cells (CD16/56+)	0.234	
	CD4+/CD8+ rate	0.517	
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45RA+) (%TCD3+)	0.338	
	Central Memory L (CD3+CCR7+CD45RO+)(%TCD3+)	0.74	
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	0.379	
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	0.298	
	CD3+PD-L1+ (%TCD3+)	0.308	
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	0.698	
	CD3 CTLA4+ PD1+ (%TCD3+)	0.157	
	CD3 PD1+ (%TCD3+)	**0.0094**	**0.0055 / 0.033**
	CD3 PD1 High + (%TCD3+)	**0.0072**	**0.0065 / 0.039**
	CD3 CD28 (MFI)	0.233	
	CD3 CTLA-4 (MFI)	0.348	
	CD3 PD1 (MFI)	0.817	
Study of activation markers of total lymphocytes T (% of total L T)	CD3+CD25+ (%TCD3+)	0.0912	
	CD3+HLA-DR+ (%TCD3+)	0.085	
	CD3+CD40L+ (%TCD3+)	0.804	
	CD3+CD62L+ (%TCD3+)	0.35	
	CD3+CD69+ (%TCD3+)	0.226	
CD3+ CD8+ Lymphocytes T subpopulations	L CD3 CD8 HD (%TCD8+)	0.137	
	L CD3 CD8 CTLA4 (%TCD8+)	0.636	
	L CD3 CD8 CTLA4+ PD1+ (%TCD8+)	0.38	
	L CD3 CD8 PD1+ (%TCD8+)	0.24	
	L CD3 CD8 PD1 High+ (%TCD8+)	0.837	
	L CD3 CD8 CD28 (MFI)	0.267	
	L CD3 CD8 CTLA-4 (MFI)	0.381	
	L CD3 CD8 PD1 (MFI)	**0.0104**	**0.0039 / 0.023**

CD3+ CD8+ CD4+ L T SP (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	0.543	
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	0.783	
	CD3 CD4 CTLA4 (%TCD4+)	0.418	
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	0.771	
	CD3 CD4 PD1+ (%TCD4+)	**0.00612**	**0.0031 / 0.019**
	CD3 CD4 PD1 High+ (%TCD4+)	**0.000395**	**0.0015 / 0.0093**
	CD3 CD4 CD28 (MFI)	0.465	
	CD3 CD4 CTLA-4 (MFI)	**0.0104**	> 0,05 / > 0,05
	CD3 CD4 PD1 (MFI)	0.101	

Legend. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. NC: non corrected p value. BC: Bonferroni corrected p value. L: lymphocytes. CD: cluster of differentiation. SP: subpopulation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

Objective Response Population (ORP) Analysis

We calculated the mean value and the standard deviation of each lymphocyte subpopulation at the selected timepoints (C1D1, C2D1, BR, PD) for the ORP (Table 11).

Table 11: mean and standard deviation of objective response population for each subpopulation at C1D1, C2D1, best response and progression disease timepoints.

		C1D1		C2D1		BR		PD	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
General populations	Lymphocytes (L) T (CD3+)	72.33	13.53	71.92	13.90	68.75	15.42	67.02	15.17
	LT (CD3+CD4+)	59.34	13.98	57.32	13.93	57.63	14.71	59.94	11.82
	LT (CD3+CD8+)	34.53	11.90	35.36	12.82	36.64	14.14	34.04	10.78
	L B (CD19+)	6.74	4.23	6.61	5.09	8.56	6.74	8.26	5.63
	NK cells (CD16/56+)	15.46	10.54	16.15	10.10	16.41	9.88	16.40	8.42
	CD4+/CD8+ rate	2.00	0.92	1.94	0.97	1.91	0.99	2.02	0.92
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+ CD45RA+) (%TCD3+)	8.83	7.67	9.51	8.30	10.45	6.63	9.57	4.25
	Central Memory L (CD3+CCR7+ CD45RO+)(% TCD3+)	1.29	1.22	1.12	1.32	1.88	2.15	1.29	0.88
	Effector Memory L (CD3+CCR7- CD45RO+) (%TCD3+)	6.24	5.02	4.34	4.12	6.12	5.59	5.17	4.05
	Regulatory T L (CD3+CD4+C D25+FoxP3+)(%TCD3+)	1.52	1.47	1.00	1.41	1.79	2.30	1.47	1.59
	CD3+PD-L1+ (%TCD3+)	215.54	211.01	270.66	323.61	162.69	133.50	128.41	99.42
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	20.62	9.72	19.27	8.23	19.89	9.25	24.84	9.16
	CD3 CTLA4+ PD1+ (%TCD3+)	26.48	11.62	29.63	11.80	25.36	9.39	26.05	9.03
	CD3 PD1+ (%TCD3+)	1.67	1.19	1.42	1.12	1.32	0.85	2.03	1.19
	CD3 PD1 High + (%TCD3+)	1.42	2.82	3.28	7.88	1.23	1.62	0.96	0.86

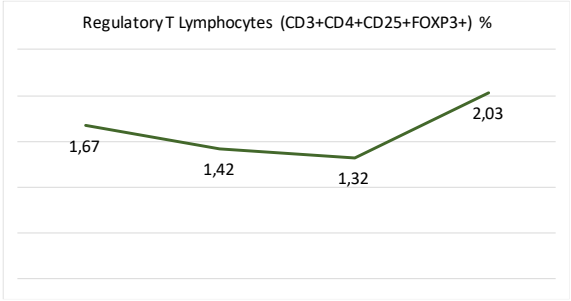
	CD3 CD28 (MFI)	0.71	0.98	0.93	1.15	0.57	0.66	0.46	0.36
	CD3 CTLA-4 (MFI)	12.17	13.91	11.99	16.29	10.46	14.12	8.20	10.16
	CD3 PD1 (MFI)	19.20	10.73	21.50	14.25	15.53	11.20	14.18	6.50
Study of activation markers of total lymphocytes T (% of total LT)	CD3+CD25+ (%TCD3+)	3.96	9.81	1.85	4.29	0.65	1.12	0.25	0.45
	CD3+HLA-DR+ (%TCD3+)	38.23	19.19	38.25	20.79	31.76	24.21	41.15	19.07
	CD3+CD40L+ (%TCD3+)	3.93	7.03	2.17	1.72	1.84	1.67	2.72	3.67
	CD3+CD62L+ (%TCD3+)	32.00	11.95	33.13	13.97	34.90	12.91	32.63	8.60
	CD3+CD69+ (%TCD3+)	62.35	24.26	61.54	25.36	57.82	25.40	61.60	22.51
CD3+ CD8+ Lymphocytes T subpopulations	L CD3 CD8 HD (%TCD8+)	1.71	1.31	1.99	1.59	1.80	1.81	1.37	1.58
	L CD3 CD8 CTLA4 (%TCD8+)	60.76	14.55	59.27	16.29	59.07	13.88	60.85	8.21
	L CD3 CD8 CTLA4+ PD1+ (%TCD8+)	35.11	29.80	29.04	27.82	23.74	27.30	25.28	29.60
	L CD3 CD8 PD1+ (%TCD8+)	7.71	6.34	9.03	7.89	9.24	5.76	9.53	4.10
	L CD3 CD8 PD1 High+ (%TCD8+)	1.95	1.60	2.07	2.39	2.63	1.81	2.59	1.41
	L CD3 CD8 CD28 (MFI)	12.61	6.24	9.66	6.02	13.46	4.37	13.35	4.08
	L CD3 CD8 CTLA-4 (MFI)	3.73	2.79	2.41	2.34	4.58	3.11	4.67	3.28
	L CD3 CD8 PD1 (MFI)	1799.38	753.14	2126.94	1268.57	1906.97	742.92	1871.55	620.24
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	592.45	868.03	827.11	1009.09	320.73	332.62	228.01	147.22
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	65.66	15.86	68.80	16.77	63.60	18.41	62.51	15.41
	CD3 CD4 CTLA4 (%TCD4+)	7.54	6.14	8.38	6.94	9.10	5.33	8.36	4.61
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	1.64	1.38	1.57	1.56	2.18	1.69	1.80	1.01
	CD3 CD4 PD1+ (%TCD4+)	9.77	5.07	7.03	4.19	9.98	3.66	8.53	3.54
	CD3 CD4 PD1 High+ (%TCD4+)	2.87	2.25	1.72	1.67	3.38	2.21	2.76	1.82
	CD3 CD4 CD28 (MFI)	1325.62	622.68	1576.80	1054.99	1408.40	552.77	1396.00	518.43
	CD3 CD4 CTLA-4 (MFI)	276.37	230.19	323.06	353.54	223.21	243.30	156.41	100.30
	CD3 CD4 PD1 (MFI)	436.90	202.03	435.50	248.32	534.50	427.73	458.00	211.44

SD: standard deviation. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

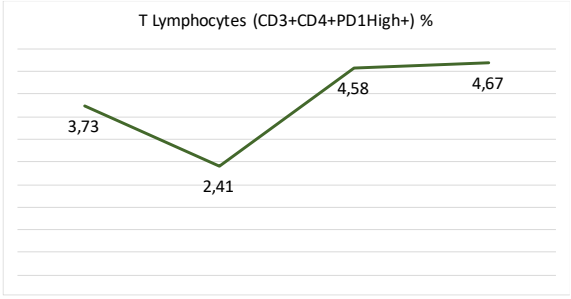
Based on this OR population, the ANOVA analysis showed significant decrease for Regulatory T Lymphocytes (CD3+CD4+CD25+FoxP3+) (p=0.0306), CD3 CD4 PD1 High+ (%) (p=0.0471) and CD3 CD4 CTLA-4 (MFI) (p=0.0236) (Figure 3 /Table 12).

Finally, statistically significant subpopulations were explored by pairwise comparison in the objective response population. Non statistically significant difference was found for any subpopulation between C2D1 and C1D1 (Figure 3, Table 12).

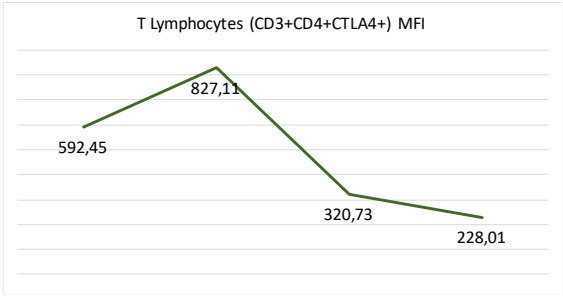
Figure 3: Objective response population values of specific lymphocyte subpopulations.



A) Regulatory T lymphocytes CD3+ CD4+ CD25+ FOXP3+) (ANOVA p=0.0306)



B) T lymphocytes CD3+ CD4+ PD1 High+ (ANOVA p=0.0471)



C) T lymphocytes CD3+ CD4+ CTLA4+ MFI (ANOVA p=0.0236)

Legend. MFI: Mean Fluorescence Intensity.

Table 12: ANOVA analysis for the objective response population.

		ANOVA p value Objective Response Population	Pairwise comparison C2D1 vs C1D1 NC / BC
General populations	Lymphocytes (L) T (CD3+)	0.321	
	LT (CD3+CD4+)	0.806	
	LT (CD3+CD8+)	0.907	
	L B (CD19+)	0.122	
	NK cells (CD16/56+)	0.669	
	CD4+/CD8+ rate	0.707	
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45RA+) (%TCD3+)	0.403	
	Central Memory L (CD3+CCR7+CD45RO+)(%TCD3+)	0.335	
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	0.534	
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	**0.0306**	> 0.05 / > 0.05
	CD3+PD-L1+ (%TCD3+)	0.464	
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	0.633	
	CD3 CTLA4+ PD1+ (%TCD3+)	0.336	
	CD3 PD1+ (%TCD3+)	0.0628	
	CD3 PD1 High + (%TCD3+)	0.0546	
	CD3 CD28 (MFI)	0.313	
	CD3 CTLA-4 (MFI)	0.25	
	CD3 PD1 (MFI)	0.488	
Study of activation markers of total lymphocytes T (% of total L T)	CD3+CD25+ (%TCD3+)	0.944	
	CD3+HLA-DR+ (%TCD3+)	0.148	
	CD3+CD40L+ (%TCD3+)	0.123	
	CD3+CD62L+ (%TCD3+)	0.179	
	CD3+CD69+ (%TCD3+)	0.203	
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	0.686	
	CD3 CD8 CTLA4 (%TCD8+)	0.796	
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	0.257	
	CD3 CD8 PD1+ (%TCD8+)	0.335	
	CD3 CD8 PD1 High+ (%TCD8+)	0.447	
	CD3 CD8 CD28 (MFI)	0.355	
	CD3 CD8 CTLA-4 (MFI)	0.121	
	CD3 CD8 PD1 (MFI)	0.194	
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	0.558	
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	0.225	
	CD3 CD4 CTLA4 (%TCD4+)	0.439	
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	0.337	
	CD3 CD4 PD1+ (%TCD4+)	0.0804	
	CD3 CD4 PD1 High+ (%TCD4+)	**0.0471**	> 0.05 / > 0.05
	CD3 CD4 CD28 (MFI)	0.185	
	CD3 CD4 CTLA-4 (MFI)	**0.0236**	> 0.05 / > 0.05
	CD3 CD4 PD1 (MFI)	0.0871	

C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. NC: non corrected p value. BC: Bonferroni corrected p value. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

Univariate Logistic Regression For Overall Response Rate (ORR)

Univariate logistic regression to test the association of lymphocyte subpopulation levels with ORR were performed at C1D1 and C2D1.

In C1D1, lymphocyte B (CD19+, %) (OR 0.88, 95% confidence interval [CI]: 0.80-0.97, p=0.012), CD3 CD4 CTLA4+ PD1+ (%) (OR 0.77, 95%CI: 0.62-0.95, p=0.02) or CD3 CD4 PD1+ (%) (OR 0.93, 95%CI: 0.87-0.99, p=0.03) showed a statistically significant negative association with the possibility of reaching an objective response. All other subpopulations showed no significant association with ORR (Table 13).

In C2D1, lymphocyte B (CD19+, %) (OR 0.90, 95% CI: 0.83-0.99, p=0.03), CD3 PD1+ (%) (OR 0.89, 95% CI: 0.80-0.98, p=0.02), L CD3 PD1 High+ (%) (OR 0.76, 95% CI: 0.59-0.97, p=0.03), CD3 CD4 PD1 High+ (%) (OR 0.81, 95% CI: 0.68-0.97, p=0.03) and CD3 CD4 PD1+ (%) (OR 0.91, 95% CI: 0.85-0.98, p=0.01) showed a statistically significant negative association with the possibility of reaching an objective response (ORR). All other subpopulations showed no significant association with ORR (Table 13).

Table 13: Univariate logistic regression of each lymphocyte subpopulation levels with ORR at C1D1 and C2D1.

	ORR	**ORR C1D1**				**ORR C2D1**			
		Odds Ratio	**OR - Lower 95CI**	**OR - Upper 95CI**	**P - value**	**Odds Ratio**	**OR - Lower 95CI**	**OR - Upper 95CI**	**P-value**
General populations	Lymphocytes (L) T (CD3+)	1.03	0.99	1.07	0.13	1.03	0.99	1.06	0.17
	LT (CD3+CD4+)	1.01	0.98	1.05	0.40	1.00	0.96	1.03	0.88
	LT (CD3+CD8+)	0.99	0.96	1.03	0.62	1.00	0.97	1.04	0.85
	L B (CD19+)	**0.88**	**0.80**	**0.97**	**0.01**	**0.90**	**0.83**	**0.99**	**0.03**
	NK cells (CD16/56+)	0.98	0.94	1.02	0.35	0.99	0.95	1.02	0.43
	CD4+/CD8+ rate	1.12	0.75	1.67	0.58	0.97	0.67	1.39	0.85
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45RA+) (%TCD3+)	1.00	0.96	1.03	0.79	0.98	0.94	1.02	0.40
	Central Memory L (CD3+CCR7+CD45RO+)(%TCD3+)	1.00	0.96	1.04	0.97	0.99	0.94	1.03	0.53
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	1.03	0.99	1.06	0.19	1.04	1.00	1.07	0.05
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	1.46	0.99	2.15	0.06	1.11	0.79	1.57	0.54
	CD3+PD-L1+ (%TCD3+)	0.89	0.60	1.32	0.57	0.97	0.75	1.26	0.82
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	0.95	0.89	1.02	0.17	0.97	0.90	1.04	0.37
	CD3 CTLA4+ PD1+ (%TCD3+)	0.77	0.59	1.01	0.06	0.76	0.57	1.02	0.07
	CD3 PD1+ (%TCD3+)	0.95	0.86	1.05	0.29	**0.89**	**0.80**	**0.98**	**0.02**
	CD3 PD1 High+ (%TCD3+)	0.94	0.79	1.11	0.44	**0.76**	**0.59**	**0.97**	**0.03**

	CD3 CD28 (MFI)	1.00	1.00	1.00	0.53	1.00	1.00	1.00	0.34
	CD3 CTLA-4 (MFI)	1.00	1.00	1.00	0.39	1.00	1.00	1.00	0.44
	CD3 PD1 (MFI)	1.00	1.00	1.00	0.18	1.00	1.00	1.00	0.13
Study of activation markers of total lymphocytes T (% of total L T)	CD3+CD25+ (%TCD3+)	1.01	0.98	1.05	0.42	1.02	0.99	1.05	0.27
	CD3+HLA-DR+ (%TCD3+)	1.01	0.98	1.05	0.36	1.02	0.99	1.05	0.23
	CD3+CD40L+ (%TCD3+)	1.06	1.00	1.12	0.07	0.98	0.88	1.09	0.75
	CD3+CD62L+ (%TCD3+)	0.99	0.97	1.01	0.37	1.00	0.98	1.02	0.74
	CD3+CD69+ (%TCD3+)	1.03	0.97	1.09	0.42	0.88	0.73	1.05	0.15
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	0.99	0.98	1.01	0.54	1.00	0.98	1.01	0.68
	CD3 CD8 CTLA4 (%TCD8+)	0.96	0.91	1.01	0.12	**0.98**	**0.92**	**1.03**	**0.40**
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	0.83	0.63	1.09	0.17	0.73	0.51	1.03	0.08
	CD3 CD8 PD1+ (%TCD8+)	1.00	0.92	1.09	0.97	0.92	0.84	1.01	0.09
	CD3 CD8 PD1 High+ (%TCD8+)	0.97	0.79	1.18	0.73	0.84	0.63	1.10	0.20
	CD3 CD8 CD28 (MFI)	1.00	1.00	1.00	0.54	1.00	1.00	1.00	0.18
	CD3 CD8 CTLA-4 (MFI)	1.00	1.00	1.00	0.61	1.00	1.00	1.00	0.91
	CD3 CD8 PD1 (MFI)	1.00	1.00	1.00	0.27	1.00	1.00	1.00	0.09
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	1.05	0.88	1.26	0.56	1.25	0.99	1.58	0.06
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	1.00	0.99	1.01	0.79	1.00	0.98	1.01	0.51
	CD3 CD4 CTLA4 (%TCD4+)	0.96	0.91	1.02	0.22	0.98	0.92	1.04	0.43
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	**0.77**	**0.62**	**0.95**	**0.02**	0.83	0.68	1.03	0.09
	CD3 CD4 PD1+ (%TCD4+)	**0.93**	**0.87**	**0.99**	**0.03**	**0.91**	**0.85**	**0.98**	**0.01**
	CD3 CD4 PD1 High+ (%TCD4+)	0.90	0.79	1.02	0.10	**0.81**	**0.68**	**0.97**	**0.03**
	CD3 CD4 CD28 (MFI)	1.00	1.00	1.00	0.64	1.00	1.00	1.00	0.22
	CD3 CD4 CTLA-4 (MFI)	1.00	1.00	1.00	0.84	1.00	1.00	1.00	0.59
	CD3 CD4 PD1 (MFI)	1.00	0.99	1.00	0.33	1.00	0.99	1.00	0.09

ORR: overall response rate. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

Univariate Logistic Regression For Disease Control Benefit (DCB)

Univariate logistic regression to test the association of lymphocyte subpopulation levels with DCB were performed at C1D1 and C2D1.

In C1D1, CD3 PD1 (MFI) (OR 0.998, 95%CI: 0.997-0.999, p=0.04), CD3 CD8 PD1 (MFI) (OR 0.999, 95%CI: 0.998-0.999, p=0.03) and CD3 CD4 PD1+ (%) (OR 0.93, 95%CI: 0.88-0.99, p=0.01), showed a statistically significant negative association with the possibility of reaching a stable, partial o complete response (DCB).

In C2D1, lymphocyte B (CD19+, %) (OR 0.92, 95% CI: 0.86-0.98, p=0.01), CD3 PD1+ (%) (OR 0.92, 95% CI: 0.85-0.99, p=0.03), CD3 CD4 PD1 High+ (%) (OR 0.86, 95% CI: 0.76-0.99, p=0.03), CD3 PD1+ High+ (%) (OR 0.83, 95% CI: 0.69-1.00, p=0.048), CD3 CD4 PD1+ (%) (OR 0.94, 95% CI: 0.89-1.00, p=0.04) and CD3 CTLA4+ PD1+ (%) (OR 0.80, 95% CI: 0.65-1.00, p=0.045) showed a statistically significant negative association with the possibility of reaching a DCB. All other subpopulations showed no significant association with DCB.

Table 14: Univariate logistic regression of each lymphocyte subpopulation levels with DCB at C1D1 and C2D1.

	DCB	**DCB C1D1**				**DCB C2D1**			
		Odds Ratio	**OR - Lower 95CI**	**OR - Upper 95CI**	**P-value**	**Odd Ratio**	**OR - Lower 95CI**	**OR - Upper 95CI**	**OR - P-value**
General populations	Lymphocytes (L) T (CD3+)	1.01	0.98	1.04	0.46	1.00	0.97	1.03	0.97
	LT (CD3+CD4+)	1.00	0.97	1.03	0.89	0.99	0.96	1.02	0.43
	LT (CD3+CD8+)	1.00	0.97	1.03	0.94	1.01	0.98	1.04	0.45
	L B (CD19+)	0.94	0.88	1.00	0.07	**0.92**	**0.86**	**0.98**	**0.01**
	NK cells (CD16/56+)	1.00	0.97	1.03	0.92	1.00	0.98	1.02	0.92
	CD4+/CD8+ rate	0.96	0.68	1.35	0.81	0.79	0.57	1.10	0.17
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45RA+) (%TCD3+)	0.97	0.94	1.01	0.11	0.97	0.94	1.00	0.08
	Central Memory L (CD3+CCR7+CD45RO+)(%TCD3+)	1.01	0.98	1.04	0.67	1.02	0.98	1.05	0.34
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	1.03	0.99	1.06	0.10	1.02	0.99	1.06	0.12
	Regulatory T L (CD3+CD4+CD25+FoxP3+) (%TCD3+)	1.19	0.86	1.65	0.30	1.25	0.94	1.67	0.13
	CD3+PD-L1+ (%TCD3+)	0.81	0.58	1.14	0.23	0.95	0.77	1.18	0.65
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	1.00	0.95	1.06	0.92	0.97	0.92	1.03	0.29
	CD3 CTLA4+ PD1+ (%TCD3+)	0.95	0.82	1.09	0.44	**0.80**	**0.65**	**1.00**	**0.045**
	CD3 PD1+ (%TCD3+)	0.93	0.86	1.01	0.07	**0.92**	**0.85**	**0.99**	**0.03**
	CD3 PD1 High + (%TCD3+)	0.96	0.83	1.09	0.51	**0.83**	**0.69**	**1.00**	**0.048**
	CD3 CD28 (MFI)	1.00	1.00	1.00	0.17	1.00	1.00	1.00	0.81
	CD3 CTLA-4 (MFI)	1.00	1.00	1.00	0.88	1.00	1.00	1.00	0.82
	CD3 PD1 (MFI)	**0.998**	**0.997**	**0.999**	**0.04**	1.00	1.00	1.00	0.12
Study of activation markers of total	CD3+CD25+ (%TCD3+)	1.00	0.97	1.03	0.92	1.01	0.98	1.03	0.65
	CD3+HLA-DR+ (%TCD3+)	1.01	0.98	1.03	0.63	1.01	0.99	1.04	0.42

lymphocytes T (% of total L T)	CD3+CD40L+ (%TCD3+)	1.03	0.97	1.09	0.36	0.95	0.86	1.05	0.31
	CD3+CD62L+ (%TCD3+)	0.99	0.97	1.00	0.13	0.99	0.98	1.01	0.31
	CD3+CD69+ (%TCD3+)	1.01	0.95	1.07	0.78	0.96	0.89	1.04	0.34
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	1.00	0.99	1.01	0.89	1.00	0.98	1.01	0.57
	CD3 CD8 CTLA4 (%TCD8+)	1.00	0.95	1.04	0.84	0.97	0.92	1.01	0.14
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	0.98	0.88	1.10	0.76	0.93	0.78	1.10	0.39
	CD3 CD8 PD1+ (%TCD8+)	1.05	0.98	1.12	0.20	1.01	0.97	1.05	0.58
	CD3 CD8 PD1 High+ (%TCD8+)	1.05	0.90	1.21	0.54	0.94	0.79	1.12	0.52
	CD3 CD8 CD28 (MFI)	1.00	1.00	1.00	0.09	1.00	1.00	1.00	0.49
	CD3 CD8 CTLA-4 (MFI)	1.00	1.00	1.00	0.43	1.00	1.00	1.00	0.69
	CD3 CD8 PD1 (MFI)	**0.999**	**0.998**	**0.999**	**0.03**	1.00	1.00	1.00	0.21
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	1.01	0.85	1.19	0.95	1.03	0.83	1.28	0.77
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	0.99	0.98	1.00	0.31	0.99	0.98	1.00	0.17
	CD3 CD4 CTLA4 (%TCD4+)	1.01	0.96	1.06	0.75	1.00	0.96	1.05	1.00
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	0.94	0.84	1.06	0.32	1.00	0.93	1.09	0.91
	CD3 CD4 PD1+ (%TCD4+)	**0.93**	**0.88**	**0.99**	**0.01**	**0.94**	**0.89**	**1.00**	**0.04**
	CD3 CD4 PD1 High+ (%TCD4+)	0.94	0.86	1.04	0.23	**0.86**	**0.76**	**0.99**	**0.03**
	CD3 CD4 CD28 (MFI)	1.00	1.00	1.00	0.60	1.00	1.00	1.00	0.26
	CD3 CD4 CTLA-4 (MFI)	1.00	1.00	1.00	0.18	1.00	1.00	1.00	0.09
	CD3 CD4 PD1 (MFI)	1.00	0.99	1.00	0.14	1.00	0.99	1.00	0.06

Legend. ORR: overall response rate. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

Univariate Cox Regression For Progression Free Survival (PFS)

Univariate Cox regressions to test the association of lymphocyte subpopulation levels with PFS were performed at C1D1 and C2D1.

In C1D1, Lymphocyte B (CD19+) (Hazard ratio [HR]= 1.03, 1.01 – 1.05, p=0.01) and CD3 CD4 PD1+ (%) (HR= 1.03, 1.01 – 1.05, p=0.01) showed a statistically significant negative association with PFS. All other subpopulations showed no significant association with PFS.

Table 15: Univariate Cox regressions of lymphocyte subpopulation levels with PFS at C1D1

	C1D1 Lymphocyte subpopulation analysis	PFS Patients N	PFS Events N	PFS HR	HR Lower 95%CI	HR Upper 95%CI	P-value
General populations	Lymphocytes (L) T (CD3+)	162	146	1.00	0.98	1.01	0.40
	LT (CD3+CD4+)	162	146	1.00	0.99	1.01	0.95
	LT (CD3+CD8+)	162	146	1.00	0.99	1.01	0.95
	L B (CD19+)	**162**	**146**	**1.03**	**1.01**	**1,05**	**0,01**
	NK cells (CD16/56+)	162	146	1,00	0,99	1,02	0,95
	CD4+/CD8+ rate	162	146	1.01	0.85	1.21	0.90
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	159	144	1.01	1.00	1.03	0.18
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	159	144	1.00	0.98	1.01	0.80
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	159	144	0.99	0.98	1.01	0.21
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	160	145	0.87	0.74	1.03	0.12
	CD3+PD-L1+ (%TCD3+)	163	147	1.07	0.93	1.22	0.37
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	166	149	1.00	0.97	1.02	0.82
	CD3 CTLA4+ PD1+ (%TCD3+)	166	149	1.02	0.97	1.07	0.52
	CD3 PD1+ (%TCD3+)	166	149	1.03	1.00	1.07	0.09
	CD3 PD1 High + (%TCD3+)	166	149	1.02	0.96	1.09	0.45
	CD3 CD28 (MFI)	166	149	1.00	1.00	1.00	0.94
	CD3 CTLA-4 (MFI)	166	149	1.00	1.00	1.00	0.88
	CD3 PD1 (MFI)	166	149	1.00	1.00	1.00	0.22
	CD3+CD25+ (%TCD3+)	163	147	1.00	0.99	1.02	0.87
Study of activation markers of total lymphocytes T (% of total L T)	CD3+HLA-DR+ (%TCD3+)	163	147	0.99	0.98	1.01	0.21
	CD3+CD40L+ (%TCD3+)	163	147	0.98	0.95	1.01	0.18
	CD3+CD62L+ (%TCD3+)	163	147	1.00	1.00	1.01	0.45
	CD3+CD69+ (%TCD3+)	163	147	1.00	0.97	1.03	0.93
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	166	149	1.00	1.00	1.01	0.50
	CD3 CD8 CTLA4 (%TCD8+)	166	149	1.00	0.98	1.02	0.81
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	166	149	1.00	0.95	1.06	0.96
	CD3 CD8 PD1+ (%TCD8+)	166	149	0.99	0.96	1.03	0.68
	CD3 CD8 PD1 High+ (%TCD8+)	166	149	0.99	0.92	1.07	0.88
	CD3 CD8 CD28 (MFI)	166	149	1.00	1.00	1.00	0.64
	CD3 CD8 CTLA-4 (MFI)	166	149	1.00	1.00	1.00	0.73
	CD3 CD8 PD1 (MFI)	166	149	1.00	1.00	1.00	0.16
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	166	149	0.96	0.85	1.08	0.46
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	166	149	1.00	1.00	1.01	0.23
	CD3 CD4 CTLA4 (%TCD4+)	166	149	0.99	0.97	1.02	0.64
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	166	149	1.02	0.98	1.06	0.34

	CD3 CD4 PD1+ (%TCD4+)	**166**	**149**	**1.03**	**1.00**	**1.06**	**0.02**
	CD3 CD4 PD1 High+ (%TCD4+)	166	149	1.03	0.98	1.07	0.22
	CD3 CD4 CD28 (MFI)	166	149	1.00	1.00	1.00	0.92
	CD3 CD4 CTLA-4 (MFI)	162	146	1.00	1.00	1.00	0.57
	CD3 CD4 PD1 (MFI)	166	149	1.00	1.00	1.00	0.44

Legend. HR: hazard ratio. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

In C2D1, lymphocyte B (CD19+) (HR= 1.04, 95%CI: 1.01 – 1.07, p=0.002), CD3 PD1+ (%) (HR= 1.04, 1.01 – 1.08, p=0.01), CD3 CD4 PD1+ (%) (HR= 1.03, 1.00 – 1.06, p=0.02) and lymphocyte CD3 CD4 PD1 (MFI) (HR= 1.0001, 1.00– 1.0002, p=0.03) showed a statistically significant negative association with PFS. Lymphocyte T CD3+HLA-DR+ (HR= 0.99, 0.97 – 1.00, p=0.049) showed a statistically significant positive association with PFS. All other subpopulations showed no significant association with PFS (Table 16).

Table 16: Univariate Cox regressions of lymphocyte subpopulation levels with PFS at C2D1

	C2D1 Lymphocyte subpopulation analysis	**PFS Patients N**	**PFS Events N**	**PFS HR**	**HR Lower 95%CI**	**HR Upper 95%CI**	**P-value**
General populations	Lymphocytes (L) T (CD3+)	154	138	1.00	0.98	1.01	0.57
	LT (CD3+CD4+)	154	138	1.01	0.99	1.02	0.35
	LT (CD3+CD8+)	154	138	1.00	0.98	1.01	0.51
	L B (CD19+)	**154**	**138**	**1.04**	**1.01**	**1.07**	**0.002**
	NK cells (CD16/56+)	154	138	1.00	0.99	1.01	0.90
	CD4+/CD8+ rate	154	138	1.06	0.93	1.21	0.40
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	152	136	1.02	1.00	1.03	0.06
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	152	136	1.00	0.98	1.01	0.65
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	152	136	0.99	0.98	1.00	0.17
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	153	137	0.92	0.78	1.08	0.30
	CD3+PD-L1+ (%TCD3+)	151	135	1.04	0.92	1.17	0.54
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	156	138	1.01	0.98	1.03	0.57
	CD3 CTLA4+ PD1+ (%TCD3+)	157	139	1.07	0.98	1.17	0.11
	CD3 PD1+ (%TCD3+)	**157**	**139**	**1.04**	**1.01**	**1.08**	**0.01**
	CD3 PD1 High + (%TCD3+)	156	138	1.07	0.99	1.17	0.10
	CD3 CD28 (MFI)	156	138	1.00	1.00	1.00	0.85
	CD3 CTLA-4 (MFI)	156	138	1.00	1.00	1.00	0.96
	CD3 PD1 (MFI)	156	138	1.00	1.00	1.00	0.09
	CD3+CD25+ (%TCD3+)	154	138	1,00	0,98	1,01	0,79

Study of activation markers of total lymphocytes T (% of total L T)	**CD3+HLA-DR+ (%TCD3+)**	**154**	**138**	**0.99**	**0.97**	**1.00**	**0.049**
	CD3+CD40L+ (%TCD3+)	152	136	1.00	0.96	1.04	0.88
	CD3+CD62L+ (%TCD3+)	154	138	1.00	1.00	1.01	0.30
	CD3+CD69+ (%TCD3+)	154	138	1.03	1.00	1.05	0.06
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	157	139	1.00	1.00	1.01	0.45
	CD3 CD8 CTLA4 (%TCD8+)	157	139	1.01	0.98	1.03	0.59
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	157	139	1.02	0.95	1.10	0.53
	CD3 CD8 PD1+ (%TCD8+)	157	139	1.00	0.99	1.02	0.85
	CD3 CD8 PD1 High+ (%TCD8+)	157	139	1.03	0.95	1.12	0.52
	CD3 CD8 CD28 (MFI)	156	138	1.00	1.00	1.00	0.75
	CD3 CD8 CTLA-4 (MFI)	156	138	1.00	1.00	1.00	0.62
	CD3 CD8 PD1 (MFI)	156	138	1.00	1.00	1.00	0.10
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	156	138	0.93	0.82	1.06	0.30
CD3+ CD4+ Lymphocytes T subpopulations	CD3 CD4 HD (%)	157	139	1.00	1.00	1.01	0.13
	CD3 CD4 CTLA4 (%TCD4+)	157	139	1.00	0.98	1.02	0.89
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	157	139	1.00	0.97	1.03	0.98
	CD3 CD4 PD1+ (%TCD4+)	**156**	**138**	**1.03**	**1.00**	**1.05**	**0.02**
	CD3 CD4 PD1 High+ (%TCD4+)	157	139	1.05	0.99	1.11	0.14
	CD3 CD4 CD28 (MFI)	156	138	1.00	1.00	1.00	0.42
	CD3 CD4 CTLA-4 (MFI)	152	134	1.00	1.00	1.00	0.31
	CD3 CD4 PD1 (MFI)	**157**	**139**	**1.0001**	**1.00**	**1.0002**	**0.03**

Legend. HR: hazard ratio. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

Univariate Cox Regression For Overall Survival (OS)

Univariate Cox regressions to test the association of lymphocyte subpopulation levels with OS were performed at C1D1, C2D1 and PD.

In C1D1, CD3 CD4 Highly Differenciated, HD (HR= 1.01, 1.00 – 1.01, p=0.03) showed a statistically significant negative association OS. All other subpopulations showed no significant association with OS (Table 17).

Table 17: Univariate Cox regressions of lymphocyte subpopulation levels with OS at C1D1

	C1D1 Lymphocyte subpopulation analysis	OS Patients N	OS Events N	OS HR	HR Lower 95%CI	HR Upper 95%CI	P-value
General populations	Lymphocytes (L) T (CD3+)	162	126	0.99	0.98	1.00	0.14
	LT (CD3+CD4+)	162	126	1.00	0.99	1.02	0.69
	LT (CD3+CD8+)	162	126	0.99	0.98	1.01	0.42
	L B (CD19+)	162	126	1.02	1.00	1.05	0.11
	NK cells (CD16/56+)	162	126	1.01	1.00	1.03	0.12
	CD4+/CD8+ rate	162	126	1.08	0.90	1.30	0.42

Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	159	124	1.01	1.00	1.03	0.17
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	159	124	1.00	0.98	1.01	0.61
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	159	124	1.00	0.98	1.01	0.55
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	160	125	0.91	0.76	1.09	0.30
	CD3+PD-L1+ (%TCD3+)	163	126	1.13	0.97	1.30	0.11
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	166	128	0.98	0.95	1.01	0.19
	CD3 CTLA4+ PD1+ (%TCD3+)	166	128	1.01	0.94	1.08	0.80
	CD3 PD1+ (%TCD3+)	166	128	1.02	0.98	1.06	0.31
	CD3 PD1 High + (%TCD3+)	166	128	1.01	0.94	1.09	0.82
	CD3 CD28 (MFI)	166	128	1.00	1.00	1.00	0.99
	CD3 CTLA-4 (MFI)	166	128	1.00	1.00	1.00	0.83
	CD3 PD1 (MFI)	166	128	1.00	1.00	1.00	0.35
	CD3+CD25+ (%TCD3+)	163	126	1.01	1.00	1.03	0.07
Study of activation markers of total lymphocytes T (% of total L T)	CD3+HLA-DR+ (%TCD3+)	163	126	0.99	0.98	1.01	0.44
	CD3+CD40L+ (%TCD3+)	163	126	0.98	0.95	1.01	0.26
	CD3+CD62L+ (%TCD3+)	163	126	1.00	1.00	1.01	0.56
	CD3+CD69+ (%TCD3+)	163	126	1.00	0.97	1.03	0.94
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	166	128	1.01	1.00	1.01	0.13
	CD3 CD8 CTLA4 (%TCD8+)	166	128	0.99	0.97	1.01	0.35
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	166	128	0.98	0.91	1.06	0.62
	CD3 CD8 PD1+ (%TCD8+)	166	128	0.98	0.94	1.02	0.25
	CD3 CD8 PD1 High+ (%TCD8+)	166	128	0.97	0.88	1.07	0.52
	CD3 CD8 CD28 (MFI)	166	128	1.00	1.00	1.00	0.38
	CD3 CD8 CTLA-4 (MFI)	166	128	1.00	1.00	1.00	0.73
	CD3 CD8 PD1 (MFI)	166	128	1.00	1.00	1.00	0.50
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	166	128	1.01	0.89	1.15	0.87
CD3+ CD4+ Lymphocytes T subpopulations	**CD3 CD4 HD (%)**	**166**	**128**	**1.01**	**1.00**	**1.01**	**0.03**
	CD3 CD4 CTLA4 (%TCD4+)	166	128	0.98	0.95	1.00	0.10
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	166	128	1.02	0.97	1.07	0.50
	CD3 CD4 PD1+ (%TCD4+)	166	128	1.02	0.99	1.05	0.17
	CD3 CD4 PD1 High+ (%TCD4+)	166	128	1.01	0.96	1.06	0.81
	CD3 CD4 CD28 (MFI)	166	128	1.00	1.00	1.00	0.74
	CD3 CD4 CTLA-4 (MFI)	162	125	1.00	1.00	1.00	0.47
	CD3 CD4 PD1 (MFI)	166	128	1.00	1.00	1.00	0.23

Legend. HR: hazard ratio. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

In C2D1, Lymphocyte B (CD19+) (HR= 1.03, 1.01 – 1.06, p=0.02), CD3+CD40L+ (HR= 1.05, 1.01 – 1.09, p=0.02), lymphocyte T CD3+CD69+ (HR= 1.04, 1.02 – 1.07, p=0.002), lymphocyte T CD3+PD-L1+ (HR= 1.14, 1.02 – 1.27, p=0.02), CD3 PD1+ (%) (HR=1.03, 1.00 – 1.07, p=0,04), lymphocyte T CD3+CD62L+ HR=1.01, 1.00 –1.02, p=0.01), CD3 PD1 (MFI) (HR= 1.0005, 1.0001 –1.0008, p=0.04), CD3 CD8 PD1 (MFI) (HR= 1.0001, 1.00 –1.0002, p=0.01) and CD3 CD4 Highly Differenciated, HD (%) HR=1.01, 1.00 – 1.01, p=0.03) showed a statistically significant negative association with OS (table 18).

Table 18: Univariate Cox regressions of lymphocyte subpopulation levels with OS at C2D1

	C2D1 Lymphocyte subpopulation analysis	OS Patients N	OS Events N	OS HR	HR Lower 95%CI	HR Upper 95%CI	P-value
General populations	Lymphocytes (L) T (CD3+)	154	120	1.00	0.98	1.01	0.58
	LT (CD3+CD4+)	154	120	1.01	1.00	1.02	0.17
	LT (CD3+CD8+)	154	120	0.99	0.98	1.00	0.19
	L B (CD19+)	**154**	**120**	**1.03**	**1.01**	**1.06**	**0.02**
	NK cells (CD16/56+)	154	120	1.00	0.99	1.01	0.87
	CD4+/CD8+ rate	154	120	1.12	0.97	1.29	0.13
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	152	119	1.01	0.99	1.03	0.18
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	152	119	1.00	0.98	1.02	1.00
	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	152	119	0.99	0.98	1.01	0.41
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	153	119	0.88	0.74	1.05	0.14
	CD3+PD-L1+ (%TCD3+)	**151**	**117**	**1.14**	**1.02**	**1.27**	**0.02**
Study of activation markers of total lymphocytes (% of total L)	CD3 CTLA4 (%TCD3+)	156	119	0.99	0.96	1.02	0.59
	CD3 CTLA4+ PD1+ (%TCD3+)	157	120	1.03	0.94	1.13	0.51
	CD3 PD1+ (%TCD3+)	**157**	**120**	**1.03**	**1.00**	**1.07**	**0.04**
	CD3 PD1 High + (%TCD3+)	156	119	1.03	0.94	1.12	0.53
	CD3 CD28 (MFI)	156	119	1.00	1.00	1.00	0.80
	CD3 CTLA-4 (MFI)	156	119	1.00	1.00	1.00	0.68
	CD3 PD1 (MFI)	**156**	**119**	**1.0005**	**1.0001**	**1.0008**	**0.01**
	CD3+CD25+ (%TCD3+)	154	120	1.01	1.00	1.03	0.13
Study of activation markers of total lymphocytes T (% of total L T)	CD3+HLA-DR+ (%TCD3+)	154	120	0.99	0.98	1.00	0.18
	CD3+CD40L+ (%TCD3+)	**152**	**118**	**1.05**	**1.01**	**1.09**	**0.02**
	CD3+CD62L+ (%TCD3+)	**154**	**120**	**1.01**	**1.00**	**1.02**	**0.01**
	CD3+CD69+ (%TCD3+)	**154**	**120**	**1.04**	**1.02**	**1.07**	**0.002**
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	157	120	1.01	1.00	1.01	0.18
	CD3 CD8 CTLA4 (%TCD8+)	157	120	0.99	0.97	1.02	0.67
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	157	120	0.99	0.91	1.06	0.72
	CD3 CD8 PD1+ (%TCD8+)	157	120	1.00	0.98	1.02	0.96

	CD3 CD8 PD1 High+ (%TCD8+)	157	120	0.99	0.90	1.08	0.77
	CD3 CD8 CD28 (MFI)	156	119	1.00	1.00	1.00	0.73
	CD3 CD8 CTLA-4 (MFI)	156	119	1.00	1.00	1.00	0.25
	CD3 CD8 PD1 (MFI)	**156**	**119**	**1.0001**	**1.00**	**1.0002**	**0.01**
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	156	119	0.99	0.86	1.13	0.83
CD3+ CD4+ Lymphocytes T subpopulations	**CD3 CD4 HD (%TCD4+)**	**157**	**120**	**1.01**	**1.00**	**1.01**	**0.03**
	CD3 CD4 CTLA4 (%TCD4+)	157	120	0.99	0.97	1.01	0.46
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	157	120	1.00	0.96	1.03	0.92
	CD3 CD4 PD1+ (%TCD4+)	156	119	1.02	1.00	1.04	0.12
	CD3 CD4 PD1 High+ (%TCD4+)	157	120	1.02	0.95	1.08	0.64
	CD3 CD4 CD28 (MFI)	156	119	1.00	1.00	1.00	0.49
	CD3 CD4 CTLA-4 (MFI)	152	115	1.00	1.00	1.00	0.28
	CD3 CD4 PD1 (MFI)	157	120	1.00	1.00	1.00	0.17

Legend. HR: hazard ratio. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

At PD timepoint, lymphocyte T CD3+CD40L+ (HR= 1.12, 1.06 – 1.19, p=0.00002), Lymphocyte T CD3+CD25+ (HR= 1.02, 1.00 – 1.04, p=0.02), CD3 CD4 Highly Differenciated, HD (%) (HR= 1.01, 1.00 – 1.02, p=0.002) and lymphocyte T CD3+PD-L1+ (HR= 1.33, 1.14 – 1.56, p=0.0004) showed a statistically significant negative association. Central Memory L (CD3+CCR7+CD45RO+) (HR= 0.98, 0.96 – 1.00, p=0.02), CD3 CD4 CTLA4 (%) (HR= 0.94, 0.90 – 0.98, p=0.001), CD3 CD4 PD1 High+ (%) (HR= 0.94, 0.89 – 1.00, p=0.04), CD3 CTLA4 (%) (HR= 0.95, 0.92 – 0.99, p=0.02) and CD3 PD1HIGH+ (%) (HR= 0.91, 0.84 – 0.99, p=0.04) showed a statistically significant positive association with PD. All other subpopulations showed no significant association with OS (Table 19).

Table 19: Univariate Cox regressions of lymphocyte subpopulation levels with OS at PD timepoint.

	PD Lymphocyte subpopulation analysis	**OS Patients N**	**OS Events N**	**OS HR**	**HR Lower 95%CI**	**HR Upper 95%CI**	**P-value**
General populations	Lymphocytes (L) T (CD3+)	119	100	1.01	0.99	1.02	0.39
	LT (CD3+CD4+)	119	100	1.00	0.99	1.02	0.81
	LT (CD3+CD8+)	119	100	1.00	0.98	1.02	0.94
	L B (CD19+)	119	100	0.99	0.96	1.02	0.47
	NK cells (CD16/56+)	119	100	1.02	1.00	1.04	0.09
	CD4+/CD8+ rate	119	100	1.06	0.89	1.27	0.49
Lymphocytes T subpopulations (% of total Lymphocytes T)	L naive (CD3+CCR7+CD45 RA+) (%TCD3+)	118	99	1.01	1.00	1.03	0.13
	Central Memory L (CD3+CCR7+CD45 RO+)(%TCD3+)	**118**	**99**	**0.98**	**0.96**	**1.00**	**0.02**

	Effector Memory L (CD3+CCR7-CD45RO+) (%TCD3+)	118	99	1.00	0.99	1.02	0.72
	Regulatory T L (CD3+CD4+CD25+FoxP3+)(%TCD3+)	116	97	0.89	0.73	1.09	0.26
	CD3+PD-L1+ (%TCD3+)	**120**	**101**	**1.33**	**1.14**	**1.56**	**0.0004**
Study of activation markers of total lymphocytes (% of total L)	**CD3 CTLA4 (%TCD3+)**	**119**	**101**	**0.95**	**0.92**	**0.99**	**0.02**
	CD3 CTLA4+ PD1+ (%TCD3+)	119	101	0.90	0.80	1.02	0.09
	CD3 PD1+ (%TCD3+)	119	101	1.00	0.96	1.05	0.95
	CD3 PD1 High + (%TCD3+)	**119**	**101**	**0.91**	**0.84**	**0.99**	**0.04**
	CD3 CD28 (MFI)	119	101	1.00	1.00	1.00	0.77
	CD3 CTLA-4 (MFI)	119	101	1.00	1.00	1.00	0.38
	CD3 PD1 (MFI)	119	101	1.00	1.00	1.00	0.37
	CD3+CD25+ (%TCD3+)	**120**	**101**	**1.02**	**1.00**	**1.04**	**0.02**
Study of activation markers of total lymphocytes T (% of total L T)	CD3+HLA-DR+ (%TCD3+)	120	101	1.01	0.99	1.02	0.25
	CD3+CD40L+ (%TCD3+)	**120**	**101**	**1.12**	**1.06**	**1.19**	**0.00002**
	CD3+CD62L+ (%TCD3+)	120	101	1.01	1.00	1.01	0.25
	CD3+CD69+ (%TCD3+)	120	101	1.01	0.99	1.03	0.25
CD3+ CD8+ Lymphocytes T subpopulations	CD3 CD8 HD (%TCD8+)	119	101	1.01	1.00	1.02	0.14
	CD3 CD8 CTLA4 (%TCD8+)	119	101	0.98	0.95	1.00	0.09
	CD3 CD8 CTLA4+ PD1+ (%TCD8+)	119	101	0.91	0.79	1.04	0.16
	CD3 CD8 PD1+ (%TCD8+)	119	101	0.97	0.93	1.01	0.14
	CD3 CD8 PD1 High+ (%TCD8+)	119	101	0.90	0.81	1.01	0.07
	CD3 CD8 CD28 (MFI)	119	101	1.00	1.00	1.00	0.52
	CD3 CD8 CTLA-4 (MFI)	119	101	1.00	1.00	1.00	0.92
	CD3 CD8 PD1 (MFI)	119	101	1.00	1.00	1.00	0.62
CD3+ CD8+ CD4+ L T subpopulations (double positive)	CD3+ CD8+ CD4+ L T subpopulations (double positive) (%TCD3+)	119	101	1.05	0.96	1.15	0.33
	CD3 CD4 HD (%TCD4+)	**119**	**101**	**1.01**	**1.00**	**1.02**	**0.002**
	CD3 CD4 CTLA4 (%TCD4+)	**119**	**101**	**0.94**	**0.90**	**0.98**	**0.001**
	CD3 CD4 CTLA4+ PD1+ (%TCD4+)	119	101	0.94	0.87	1.01	0.10
	CD3 CD4 PD1+ (%TCD4+)	119	101	1.00	0.97	1.03	0.95
	CD3 CD4 PD1 High+ (%TCD4+)	**119**	**101**	**0.94**	**0.89**	**1.00**	**0.04**
	CD3 CD4 CD28 (MFI)	119	101	1.00	1.00	1.00	0.08
	CD3 CD4 CTLA-4 (MFI)	117	99	1.00	1.00	1.00	0.75
	CD3 CD4 PD1 (MFI)	119	101	1.00	1.00	1.00	0.34

Legend. HR: hazard ratio. OR: odds ratio. CI: Confidence interval. C1D1: cycle 1 day 1. C2D1: cycle 2 day 1. BR: best response. PD: progression disease. L: lymphocytes. CD: cluster of differentiation. HD: Highly Differentiated. MFI: Mean Fluorescence Intensity. Bold Font = statistically significant result.

4.2.4 Discussion

Immune system is composed by a complex network of cells that interact with other circulating cells or tissues, pro or anti-inflammatory soluble factors as well as external environment factors. These interactions determine the balance of activation/inhibition and determine if cancer cells should die o survive. Within this complex system, lymphocytes are critical players so exploring its role as potential biomarker is mandatory. Most of the studies performed in this field so far were focused on non-small cell lung cancer and only included a relatively low number of patients, demonstrating preliminary evidence of their potential either at baseline o their dynamics. Therefore, further research was needed and we decided to explore the potential as ICI biomarker of several circulating lymphocytes subpopulations in a larger and more comprehensive population to improve patient selection for ICI.

In our analysis we included general circulating cells populations, total lymphocytes T subpopulations (CD3+), lymphocytes T CD3 CD4 subpopulations, lymphocytes T CD3 CD8 subpopulations. Within these categories of lymphocytes T, we differentiated lymphocytes subpopulations based on the expression of activation markers (CTLA4, PD1, CD28, CD25, HLA-DR, CD40L, CD62L, CD69). We also included CD3 CD4 HD, CD3 CD8 HD and CD3 CD4 CD8 (double positive) lymphocytes.

Most of the subpopulations that showed statistically significative associations with the selected outcomes were lymphocytes that expressed PD1, a critical transmembrane protein that is expressed on T cells (CD3+) after TCR stimulation, with coinhibitory effect.

We started analyzing general lymphocytes T (CD3) subpopulations. The most relevant subpopulations were CD3 PD1+ and CD3 PD1 high that showed a significant decrease between C1D1 and C2D1 in the total population and shorter ORR, DCB and PFS when measured at C2D1. Unfortunately, none of them showed statistically significant associations at C1D1 for any of our outcomes and therefore not helping us to select our patients.

Within this CD3+ population we can differentiate CD3+ CD4+ (T helper) and CD3 CD8 (T effector) lymphocytes. The second are critical in the mechanism of action of antiPD(L)1 therapies but surprisingly, when we analyzed specifically CD3 CD8 lymphocyte subpopulation the only significative result was an increase between C1D1 and C2D1, ruling out its potential as baseline biomarker to select ICI candidates. Like other previous projects, we observed a significative proliferation of CD3 CD8 PD1+ (MFI) but we were not able to demonstrate any relationship of this subpopulation with ICI benefit. Hwan Kim et all demonstrated an increase of CD8 PD1+ within the first week

after ICI initiation that was followed by a decreased in the subsequent two weeks (102). In our study, most of C2D1 samples were collected three or four weeks after C1D1 so it is possible that our conclusions differ due to the different schedule of sample collection. Although our study does not support CD8 PD1+ as biomarker, it may be worth exploring it further if collecting samples on C1D1 and approximately seven days later.

Among CD4 T lymphocytes, CD3 CD4 PD1+ lymphocytes subpopulation showed a significant decrease in the total population between the basal and second cycle samples, and also an association with shorter ORR, DCB and PFS either in C1D1 or C2D1. When we focused in those CD3 CD4 lymphocytes with higher expression of PD1 (PD1 high) we saw a decrease in the objective response population and we lost the association with ORR, DCB and PFS at C1D1, but it was associated with shorter ORR and DCB at C2D1 as well. Therefore, CD3 CD4 PD1+ lymphocytes subpopulation seems to correlate with worse outcomes, which is in line with previous publications. For example, CD3 CD4 PD1+ lymphocytes subpopulation has been correlated with worse outcome in treatment naïve patients candidate to chemotherapy o tyrosine kinase inhibitors (125) and Zheng et all demonstrated a significant association between CD3 CD4 PD1 high lymphocytes levels and lower rates of OS and PFS (100). These studies recruited a limited number of patients and did not include a validation cohort so their evidence has some limitations (100). In our opinion, CD3 CD4 PD1+ lymphocytes subpopulation levels should be considered as potential biomarker and explored further in a future validation cohort.

Several subpopulations showed statistical relationship with OS, being CD3 CD4 HD subpopulation the most significative. It demonstrated an association with shorter OS when measured at C1D1 and C2D1 timepoints. Zuazo-Ibarra, Arasanz et all explored CD3 CD4 HD in a non-small cell lung cancer cohort and identified that patients with higher baseline levels of CD3 CD4 HD o with a sharp increase in the first days after ICI initiation were associated with shorter PFS and hyperprogression (108,110). Unlike these studies, in our analysis it did not show relationship with shorter PFS o hyperprogression, not supporting its potential role as biomarker. Nevertheless, it seems to be related with worse outcomes including worse survival which highlights its potential as prognosis biomarker.

CD3 CD4 HD levels also demonstrated an association with shorter OS when measured PD timepoints. Obviously, at that time the patient already had received an ICI and could not take any decision regarding this indication but that is the moment in which we must switch his therapy to a new one. Its potential role as prognosis biomarker could help us to take decisions about prescribing another line of therapy, which could include another ICI, or offering best supportive care to our patient.

Lymphocyte B (CD19+) is another population involved in adaptive humoral immunity by producing antibodies and also interacting with T cells which is key for antitumor T cell mediated cytotoxicity (15). B cells can induce antigen specific CD8 and CD4 activation. Low evidence exists regarding the predictor role of peripheral B lymphocytes and ICI benefit. Preliminary results correlated baseline memory B cells with favorable outcomes in solid tumors as lung cancer (126), renal cancer (127) and sarcomas(128). In fact, in our published case report we observed an increase of lymphocytes B during treatment in a UPS sarcoma patient who developed great benefit to ICI (129). These observations differ from another study performed by Barth et all, who collected samples from 39 patients treated with ICI at baseline and at the time of first tumoral response evaluation (8-12 weeks later). No relationship was found between any of the seven lymphocyte B subpopulations and ORR o DCB at baseline (130). In our study lymphocytes B (CD19+) at C1D1 were associated with shorter ORR and PFS and at C2D1 with shorter ORR, DCB, PFS and OS. Therefore, further analysis is recommended to clarify its real predicting value.

Other subpopulations showed sporadic associations with isolated outcomes. These results are not strong enough to consider them as potential candidates of ICI biomarker and therefore we would not encourage further efforts in this direction.

4.2.4 Conclusion

CD3 CD4 PD1+ lymphocytes subpopulations is a promising candidate as biomarker for the selection of ICI candidates. Patients with high levels of CD3 CD4 PD1+ lymphocytes at C1D1 or C2D1 would be associated with shorter ORR, DCB and PFS to ICI and therefore would not be ideal candidates for this therapy.

CD3 CD4 HD levels has been correlated with unfavorable outcomes including shorter overall survival. Although there is no evidence to support its role as biomarker for ICI selection, it may play a role as potential prognosis biomarker.

CD3 CD8 lymphocytes did not show any clinically relevant associations but time of collection could impact their interpretation. Further analysis with serial and early sample collection may be worth exploring in future projects.

Contradictory results were found regarding lymphocyte B (CD19+) levels. While previous publications and our case report support its association with favorable outcomes, the analysis of the total population was against it. Further analysis is recommended to clarify its real predicting value.

Chapter 4.3 SECONDARY OBJECTIVE 1:

Correlation of baseline clinicopathological factors to response and survival in a comprehensive multitumor population.

This objective was explored and published in the Cancer Inmunology, Immunotherapy in 2023. Data data cut-off was 31/08/2021 at that time. Full article is in Appendix 1.

Original article reference is:

García-Corbacho Javier, Indacochea Alberto, González Navarro E. Azucena, Victoria Iván, Moreno Débora, Pesantez David, Angelats Laura, Modrego-Sanchez Andrea, Sanfeliu Esther, Castillo Oleguer, Blasco Paula, Mezquita Laura, Viñolas Nuria, Nogué Miquel, Galván Patricia, Adamo Barbara, Basté Neus, Sauri Tamara, Juan Manel, Prat Aleix, Schettini Francesco. Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study. Cancer Immunology, Immunotherapy. 2023 Jun;72(6):1709-1723.

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Abstract

Background: Immune-checkpoint inhibitors (ICI) have revolutionized the therapeutic landscape of cancer. However, optimal patient selection is still an unmet need.

Methods: One-hundred-forty-six patients with metastatic cancer candidates to ICI at the Hospital Clinic of Barcelona Clinical Trials Unit were prospectively recruited in this observational study. Blood samples were collected at different timepoints, baseline LIPI score calculated and pre-ICI archived tissues retrieved to evaluate PD-L1, tumor-infiltrating lymphocytes (TILs) and PD1 mRNA levels. Tumor assessments were centrally reviewed by RECIST 1.1 criteria. Associations with overall response rates (ORR), durable clinical benefit (DCB), progression-free survival (PFS) and overall survival (OS) were performed with univariable/multivariable logistic and Cox regressions, where appropriate.

Results: At a median follow-up of 26.9 months, median PFS and OS were 2.7 and 12.9 months. Response rates were 17.8% with duration of response (DOR) of 4.4 months. LIPI score was independently associated with PFS ($p = 0.025$) and OS ($p < 0.001$). Immunotherapy-naïve status was independently associated with better PFS ($p = 0.005$). Time-to-best response (TTBR) and ORR ($p < 0.001$ both) were associated with better OS at univariate analysis. PFS and DOR were moderately correlated with OS ($p < 0.001$ both). A PD-L1 10% cut-off detected worse/best responders in terms of ORR (univariate $p = 0.011$, multivariate $p = 0.028$) and DCB (univariate $p = 0.043$). PD1 mRNA levels were strikingly associated to complete responses ($p = 0.021$).

Conclusion: To resume, in our prospective observational pan-cancer study, baseline LIPI score, immunotherapy-naïve status, cancer type and RT before starting ICI were the most relevant clinical factors independently correlated with immunotherapy outcomes. Longer TTBR seemed to associate with better survival, while PD1 mRNA and PD-L1 protein levels might be tumor-agnostic predictive factors of response to ICI and should be furtherly explored.

4.3.1 Introduction

In the last decade, several immune-checkpoint inhibitors (ICI) have revolutionized the therapeutic landscape of many solid tumors. Monoclonal antibodies anti-PD1 o anti anti-PDL1 have become some of the most widely prescribed anticancer therapies. However only a limited proportion of patients seem to benefit (1,2) which may be partially due to clinicopathological factors. The optimal metastatic therapeutic setting (earlier or further lines), the efficacy in immune-pretreated patients, the effects of exposure to immediately previous or concurrent radiotherapy (RT), and the optimal duration of treatment remain questions unanswered. To note, the impact of systemic corticosteroids and exposure to antibiotic (ATB) therapy on response to ICI are another major concern, with only few and/or conflicting data being published so far (28–37). In this analysis we explored the influence of baseline clinicopathological factors, including baseline LIPI score, on ICI outcomes in order to improve patient selection for ICIs.

4.3.2 Materials and methods

Study design and participants

Full inclusion/exclusion criteria and study procedures have already been reported in chapter 3: Methodology so only specific details related to this analysis are mentioned here. For further details refer to the relevant previous section.

We considered evaluable for this analysis all participants treated with an anti-PD1 or anti-PD-L1 ICI with radiological data available for an independent assessment of tumor responses according to RECIST 1.1 o RANO criteria (120,122). Patients with available baseline imaging experiencing a rapid progression leading to death, hence with no available radiologic reassessment, were also included.

Procedures

Apart from the blood samples collected for the primary objective analysis, full blood count and blood chemistry tests were carried out as part of their standard of care, which usually involves a blood sample collection prior to each treatment prescription including hemoglobin, total leucocytes, neutrophils, lymphocytes, albumin, LDH. For this analysis only basal samples were considered. The lung immune prognostic index (LIPI) score was also calculated (56). Treatments and follow-up procedures were decided outside of this study according to study protocol, since patients received ICI in interventional clinical trials. All data were retrieved from electronic patient charts.

Study endpoints and outcomes

There was no prespecified sample size because of the exploratory nature of this study. The accrual was terminated after 4 years, and the clinical data cut-off was established when a minimum follow-up including at least one reassessment of the disease for every included patient was reached.

This analysis was intended to correlate baseline clinicopathological factors to response, in terms of overall response rate (ORR) and durable clinical benefit (DCB), and survival, in terms of progression-free survival (PFS) and overall survival (OS). The primary features of interest were treatment line at which an anti-PD1 or PD-L1 ICI is delivered (1^{st} vs. subsequent lines), patients' immune-naïve status (yes vs. no), the regimen type (ICI monotherapy vs. ICI-based combination), the ICI target (anti-PD1 vs. anti-PD-L1), having received RT, systemic ATB or corticosteroids (>10mg prednisone equivalent dose) within 30 days before, or during ICI treatment, as well as cancer type according to the following groups: NSCLC, genitourinary (GU) tumors, gastrointestinal (GI) tumors, breast cancer/gynecological tumors, other rarer tumors. The effect on OS for the time-to-best response (TTBR) and duration of response (DOR) in patients achieving at least a stable disease (SD), was investigated, as well. The prognostic value of the LIPI score in terms of PFS and OS in a pan-cancer context was also assessed.

The evaluation of response for the purpose of this study were performed in accordance to RECIST 1.1 and RANO criteria. Best responses (BR) were classified as SD, progressive disease (PD), complete (CR) or partial response (PR) independently by the same expert (Javier García Corbacho) from the Clinical Trials Unit of the HCB (131,132). For the ORR assessment we considered all patients achieving CR+PR as BR, while for DCB we included all patients achieving CR+PR+SD retained at 6 months as BR.

Statistical analysis

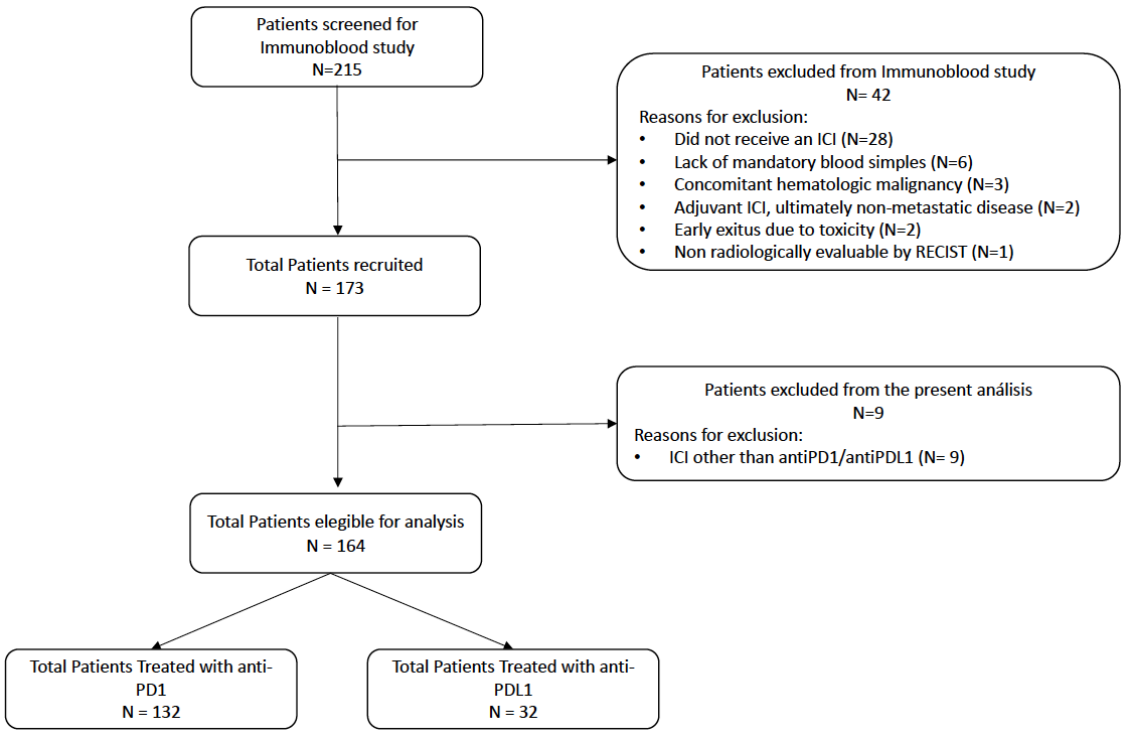
Multiple χ^2 tests and one-way ANOVA were used to calculate differences among poor, best and non-responders with respect to categorical and continuous variables of interest, respectively. For the purpose of this study, we considered as poor responders all patients that achieved SD as their BR, while best responders were those achieving PR or CR as their BR and non-responders were represented by patients with PD as BR. Correlations between continuous variables were evaluated with Pearson's r. Survival curves were estimated by the Kaplan-Meier method and differences between curves were evaluated by the log-rank test. Cox regression models were applied to estimate univariate and multivariate hazard ratios (HR) with their 95% CI to explore the association among clinicopathological/biological variables, TTBR, DOR, PFS and OS. For the primary

endpoint of PFS, the proportional hazard assumption for the univariate and multivariate Cox regression models was previously tested using correlation coefficients between transformed survival times and scaled Schoenfeld residuals and further checked with the smoothed plots of Schoenfeld residuals(133). The clinical data cut-off date for this analysis was 25 August 2021. Patients alive were censored at the date of the last follow-up. A two-sided alfa error of 0.5 was considered for statistical significance. Considering the observational and exploratory nature of the study, we decided not to take into account the multiplicity issue (134,135). All statistical analyses were carried out using R Studio vers.1.0.153 (PBC, Boston, MA) and SPSS vers 24.0 (IBM SPSS Statistics, Armonk, NY: IBM Corp) for MacOSX.

4.3.3 Results

Between May 2017 and June 2021, 156 patients entered the study and 146 received an anti-PD1/anti-PD-L1-based treatment. The selection process for the purpose of this analysis is resumed in **Figure 4** (124).

Figure 4. STROBE flow-chart.



Legend. ICI: immune-checkpoint inhibitors.

The median follow-up at the data cut-off (31/08/2021) was 26.9 months (95% CI: 13.1 – 31.7). All patients and tumors characteristics are detailed in Table 20.

Table 20. Population characteristics at data cut-off (31/08/2021)

Characteristics	Non-responders		Poor responders		Best responders		Overall		*P**
	N	*%*	*N*	*%*	*N*	*%*	*N*	*%*	
	71	48.6	49	33.6	26	17.8	146	100.0	
Age									
Mean	63.3	-	62.6	-	65.0	-	63.3	-	0.69
SD	±12.3	-	±13.1	-	±8.2	-	±11.9	-	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Sex									
Female	27	38.0	15	30.6	7	26.9	49	33.6	0.51
Male	44	62.0	34	69.4	19	73.1	97	66.4	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
ECOG									
0-1	58	87.9	40	93.0	22	88.0	120	89.6	0.67
2-3	8	12.1	3	7.0	3	12.0	14	10.4	
Overall	66	93.0	43	87.8	25	96.2	134	91.8	
Cancer type									
NSCLC	19	26.8	8	11.3	14	28.6	41	28.1	
GI cancers	22	31.0	12	16.9	5	10.2	39	26.7	
GU cancers	7	9.9	11	15.5	3	6.1	21	14.4	**0.03**
CNS, H&N, melanoma and rare cancers	13	18.3	13	18.3	3	6.1	29	19.9	
Breast+gyneco	10	14.1	5	7.0	1	2.0	16	11.0	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic at diagnosis									
Yes	40	56.3	26	53.1	17	65.4	83	56.8	0.59
No	31	43.7	23	46.9	9	34.6	63	43.2	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic treatment line									
1st	16	22.5	12	24.5	12	46.2	40	27.4	
2nd	20	28.2	17	34.7	7	26.9	44	30.1	
≥3rd	35	49.3	20	40.8	7	26.9	62	42.5	0.14
Min-Max	1st - 10th	-	1st - 6th	-	1st - 4th	-	1st - 10th	-	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Immunotherapy-naïve									
Yes	58	81.7	47	95.9	25	96.2	130	89.0	**0.02**
No	13	18.3	2	4.1	1	3.8	16	11.0	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Type of regimen									
Monotherapy	35	49.3	18	36.7	14	53.8	67	45.9	
Immunotherapy combination	20	28.2	11	22.4	4	15.4	35	24.0	0.20
Immunotherapy + other	16	22.5	20	40.8	8	30.8	44	30.1	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	

Immunotherapy target									
PD1	60	84.5	33	67.3	21	80.8	114	78.1	0.08
PD-L1	11	15.5	16	32.7	5	19.2	32	21.9	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Clinical Trial									
Yes	47	66.2	39	79.6	14	53.8	100	68.5	0.06
No	24	33.8	10	20.4	12	46.2	46	31.5	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Number of metastatic sites									
<3	12	16.9	11	22.4	6	23.1	29	19.9	0.68
≥3	59	83.1	38	77.6	20	76.9	117	80.1	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic sites									
Visceral	55	77.5	37	75.5	21	80.8	113	77.4	0.87
Non-visceral	16	22.5	12	24.5	5	19.2	33	22.6	
Bone	15	21.1	13	26.5	4	15.4	32	21.9	
CNS[#]	5	7.0	2	4.1	1	3.8	8	5.5	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
TILs (%)									
Mean	7	-	5	-	7	-	6	-	0.44
SD	±8.9	-	±7.4	-	±8.4	-	±8.4	-	
Overall	54	76.1	29	59.2	19	73.1	102	69.9	
PD-L1									
Positive	15	65.2	9	75.0	11	100.0	35	76.1	0.08
Negative	8	34.8	3	25.0	0	0.0	11	23.9	
Overall	23	32.4	12	24.5	11	42.3	46	31.5	
LIPI Score									
Good	29	44.6	22	45.8	14	63.6	65	48.1	0.46
Intermediate	26	40.0	21	43.8	7	31.8	54	40.0	
Poor	10	15.4	5	10.4	1	4.5	16	11.9	
Overall	65	91.5	48	98.0	22	84.6	135	92.5	
PD1 mRNA									
Mean	-6.34	-	-7.13	-	-6.15	-	-6.5	-	0.14
SD	±1.45	-	±1.76	-	±1.50	-	±1.57	-	
Overall	36	50.7	18	36.7	14	53.8	68	46.6	
RT									
Yes in the 30 days before ICI	6	8.7	2	4.1	1	4.0	9	6.3	0.52
Not in the 30 days before ICI	63	91.3	47	95.9	24	96.0	134	93.7	
Overall	69	97.2	49	100.0	25	96.2	143	97.9	
Yes during ICI	21	30.4	9	18.4	6	24.0	36	25.2	0.33
No during ICI	48	69.6	40	81.6	19	76.0	107	74.8	
Overall	69	97.2	49	100.0	25	96.2	143	97.9	

Corticosteroids									
Yes in the 30 days before ICI	8	11.3	11	22.4	3	12.0	22	15.2	0.22
Not in the 30 days before ICI	63	88.7	38	77.6	22	88.0	123	84.8	
Overall	71	100.0	49	100.0	25	96.2	145	99.3	
Yes during ICI	15	21.1	24	49.0	14	53.8	53	36.3	
No during ICI	56	78.9	25	51.0	12	46.2	93	63.7	**<0.01**
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
sATB									
Yes in the 30 days before ICI	5	7.0	1	2.0	1	3.8	7	4.8	0.44
Not in the 30 days before ICI	66	93.0	48	98.0	25	96.2	139	95.2	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Yes during ICI	12	17.1	18	37.5	11	42.3	41	28.5	
No during ICI	58	82.9	30	62.5	15	57.7	103	71.5	**0.01**
Overall	70	98.6	48	98.0	26	100.0	144	98.6	

Legend. Non-responders: progressive disease as best response; Poor responders: stable disease as best response; Best responders: complete response or partial response as best response; SD: standard deviation; CNS: central nervous system; ICI: immune-checkpoint inhibitors; TILs: tumor-infiltrating lymphocytes; sATB: systemic antibiotics; RT: radiotherapy; GI: gastrointestinal, including colorectal, gastric, esophageal, pancreatic cancer and colangiocarcinoma; GU: genitorurinary, including kidney, bladder urothelial and prostate cancer; Gyneco: gynecological, including ovarian and cervix cancer; CNS tumors includes only glioblastoma; H&N: head & neck tumors; rare tumors include sarcomas, thymic and suprarenal carcinomas; NSCLC: non-small cell lung cancer; *χ^2 test for differences in proportions and unpaired Student's t test for differences in means; #: primary CNS tumors excluded.

A summary of activity and efficacy outcomes at data cut-off (31/08/2021) is reported in Table 21.

Table 21. Overall ICI activity and efficacy

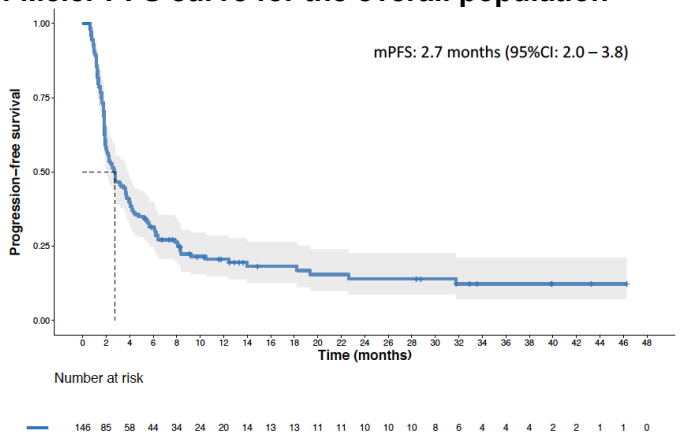
ACTIVITY AND EFFICACY	POPULATION	
	N (146)	% (100.0)
TTBR (months)		
Median (95% CI)	2.5 (2.0 - 2.7)	-
Response		
CR (95% CI)	7	4.8 (2.0 - 9.6)
PR (95% CI)	19	13.1 (8.0 - 19.6)
SD (95% CI)	49	33.6 (26.0 - 41.8)
PD (95% CI)	71	48.6 (40.3 - 57.0)
ORR (95% CI)	26	17.8 (12.0 - 25.0)
DCB (95% CI)	26	17.8 (11.6 - 24.0)
Evaluable patients	146	100.0
DOR (months)		
Median (95% CI)	4.4 (3.3 - 10.5)	-
PFS (months)		
Median (95% CI)	2.7 (2.0 - 3.8)	-
6-month PFS	44 patients at risk	31.5 (24.7 - 40.0)
12-month PFS	20 patients at risk	20.6 (14.8 - 28.6)
OS (months)		
Median (95% CI)	12.9 (9.9 - 17.4)	-
6-month OS	99 patients at risk	72.1 (65.2 - 79.9)
12-month OS	54 patients at risk	50.8 (42.9 - 60.1)

Legend. TTBR: time-to-best response; DOR: duration of response; PFS: progression-free survival; OS: overall survival; CI: confidence interval; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; ORR: overall response rate; DCB: durable clinical benefit.

Progression-free survival

At the time of data cut-off, 120 PFS events had occurred and median PFS was 2.7 months (95% CI: 2.0 – 3.8) (figure 5 and table 21).

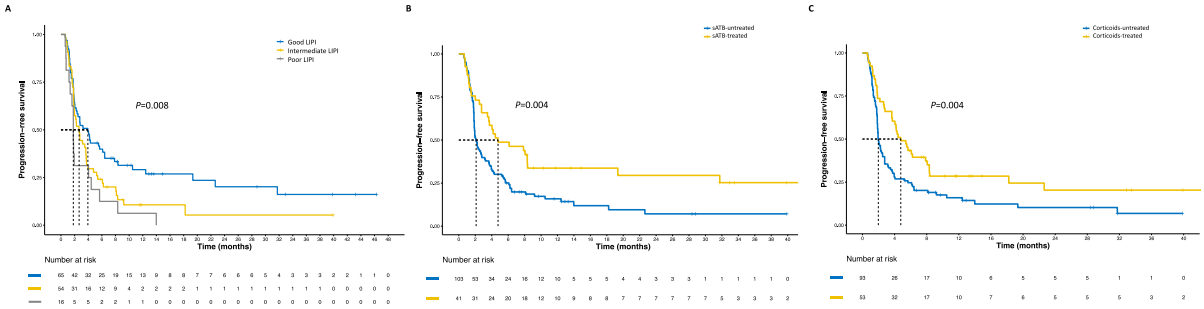
Figure 5 - Kaplan-Meier PFS curve for the overall population



Legend. PFS Kaplan-Meier curves with their 95%CI for the overall population. mPFS: median progression-free survival.

Cancer site showed a significant association with PFS at the univariate analysis (p=0.007) (Figure 6), with NSCLC patients treated with ICI being significantly favored over patients with GI tumors (p=0.011), breast cancer and other gynecological malignancies (p=0.012), melanoma, H&N tumors and other rare malignancies (p=0.003) but not genitourinary cancers (p=0.628). Patients treated in first-line showed better PFS than patients treated in later lines (p=0.037) (Figure 6), and the later the line, the worse the outcome (p=0.001). LIPI score was significantly associated with PFS (p=0.008) (Figure 6), with intermediate (p=0.035) and poor scores (p=0.005) associated with worse PFS than good scores. Immuno-naïve status, systemic ATB and corticosteroids during ICI were also associated with significant PFS improvement (p=0.001, p=0.004 and p=0.004, respectively) (Figure 6). No other clinical or hematological factors were associated with PFS (Table 22).

Figure 6. Progression-free survival curves according to significant population characteristics



Legend. A: PFS according to LIPI score; B: PFS according to sATB administration during ICI treatment; C: PFS according to systemic corticosteroids administration during ICI treatment; PFS: progression-free survival; sATB: systemic antibiotics; ICI: immune-checkpoint inhibitors.

Table 22. Univariate analyses of progression-free and overall survival

Variables	PFS				OS			
	HR	Inf 95%CI	Sup 95%CI	*P*	HR	Inf 95%CI	Sup 95%CI	*P*
Age (continuous)	0.99	0.98	1.01	0.414	1.00	0.98	1.02	0.861
Sex (Male vs. Female)	0.83	0.57	1.22	0.343	0.78	0.50	1.20	0.253
ECOG (0-1 vs. 2-3)	0.93	0.50	1.73	0.822	1.01	0.49	2.10	0.976
Metastatic at diagnosis (Yes vs. No)	0.79	0.55	1.13	0.199	1.02	0.67	1.55	0.927
Cancer site (NSCLC reference)				***0.009***				***0.027***
GI tumors	1.92	1.16	3.18	***0.011***	0.51	0.23	1.12	0.094
Melanoma + H&N + rare tumors	2.26	1.32	3.88	***0.003***	1.19	0.58	2.47	0.636
GU tumors	1.17	0.63	2.17	0.628	1.25	0.60	2.61	0.547
Breast cancer and gynecologic tumors	2.36	1.21	4.62	***0.012***	0.67	0.29	1.55	0.346
ICI treatment line (1st vs. ≥2nd)	0.64	0.42	0.98	***0.037***	0.70	0.43	1.16	0.164

ICI treatment line (continuous)	1.24	1.10	1.40	***0.001***	1.15	1.01	1.31	***0.037***
Immuno-naive status (Yes vs. No)	0.42	0.25	0.72	***0.001***	0.60	0.33	1.11	0.098
Regimen (Monotherapy vs. Combination)	0.92	0.64	1.32	0.644	0.83	0.55	1.26	0.389
ICI target (Anti-PD-L1 vs. Anti-PD1)	0.95	0.62	1.45	0.803	0.91	0.57	1.46	0.697
Visceral disease (Yes vs. No)	0.88	0.58	1.33	0.527	0.85	0.53	1.37	0.510
Number of metastases (≥3 vs. <3)	1.06	0.67	1.67	0.795	0.89	0.53	1.49	0.644
Basal LIPI Score				***0.008***				***<0.001***
Intermediate vs. Good	1.55	1.03	2.32	***0.035***	1.85	1.15	2.97	***0.011***
Intermediate vs. Poor	0.68	0.39	1.12	0.181	0.48	0.26	0.89	***0.019***
Poor vs. Good	2.28	1.29	4.03	***0.005***	3.89	2.03	7.42	***<0.001***
Basal Hb (continous)	1.00	0.99	1.01	0.867	0.99	0.98	1.01	0.362
Basal Albumin (continous)	0.96	0.92	1.01	0.092	0.97	0.92	1.02	0.270
RT within 30 days from ICI start (Yes vs. No)	1.35	0.66	2.77	0.412	2.74	1.24	6.02	***0.009***
RT during ICI (Yes vs. No)	1.11	0.73	1.70	0.625	1.22	0.77	1.93	0.405
sATB within 30 days from ICI start (Yes vs. No)	0.54	0.35	0.83	0.792	1.14	0.42	3.12	0.797
sATB during ICI (Yes vs. No)	0.54	0.35	0.83	***0.004***	0.63	0.39	1.03	0.060
Corticosteroids within 30 days from ICI start (Yes vs. No)	0.87	0.53	1.44	0.582	1.06	0.60	1.89	0.842
Corticosteroids during ICI (Yes vs. No)	0.58	0.39	0.85	***0.004***	0.77	0.50	1.19	0.241
TILs % (continous)	1.00	0.97	1.03	0.730	0.99	0.96	1.02	0.509
PD-L1 % (continuous)	0.99	0.98	1.00	***0.003***	0.99	0.98	1.00	***0.009***
PD-L1 % (>10% vs. ≤10%)	0.32	0.16	0.66	***0.002***	0.36	0.15	0.83	***0.016***
PD1 mRNA (continuous)	0.97	0.82	1.14	0.682	0.89	0.74	1.07	0.208
TTBR (continuous)	-	-	-	-	0.54	0.39	0.76	***<0.001***
ORR (CR+PR vs. SD+PD)	-	-	-	-	0.12	0.05	0.30	***<0.001***

Legend. HR: hazard ratio; inferior; Sup: superior; PFS: progression-free survival; OS: overall survival; ICI: immune-checkpoint inhibitor; TILs: tumor-infiltrating lymphocytes; TTBR: time-to-best response; ORR: overall response rates; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NSCLC: non small cell lung cancer.

At the multivariate analysis, only immunotherapy-naïve status (p=0.005) and LIPI score (p=0.025) were associated with PFS independently from each other, cancer site, treatment line, ATB, corticosteroids and previous RT (**Table 23**). PFS showed a positive moderate correlation with OS: r=0.75, p<0.001.

Table 23. Multivariate Survival analysis

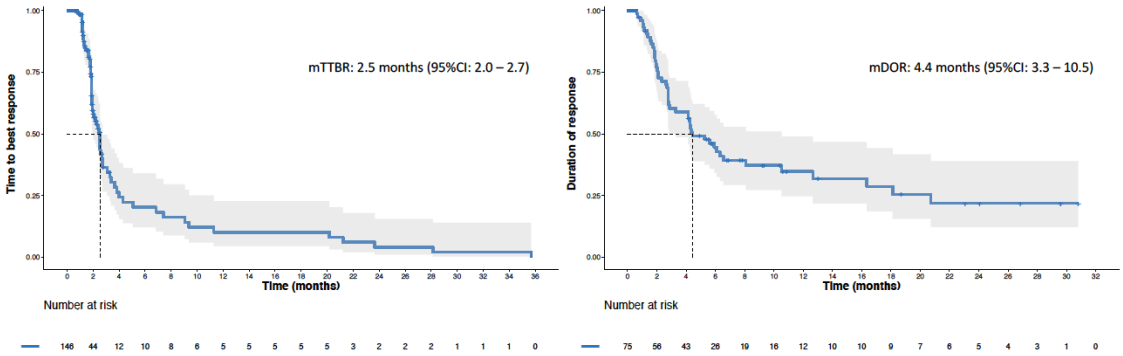
Variables	PFS				OS			
	HR	**Inf 95%CI**	**Sup 95%CI**	***P***	**HR**	**Inf 95%CI**	**Sup 95%CI**	***P***
Cancer site (All others vs. NSCLC+GU)	1.51	0.95	2.39	*0.084*	1.93	1.09	3.43	***0.025***
ICI treatment line (1st vs. ≥2nd)	0.87	0.53	1.41	0.561	0.78	0.43	1.43	0.422
Immunotherapy-naïve status (Yes vs. No)	0.42	0.23	0.78	***0.005***	0.61	0.32	1.18	0.144
Basal LIPI Score				***0.025***				***<0.001***
Intermediate vs. Good	1.32	0.86	2.03	0.211	1.67	1.01	2.77	***0.045***
Intermediate vs. Poor	0.59	0.32	1.07	*0.081*	0.40	0.21	0.77	***0.006***
Poor vs. Good	2.24	1.24	4.03	***0.007***	4.22	2.16	8.23	***<0.001***
sATB during ICI (Yes vs. No)	0.76	0.47	1.23	0.270	0.93	0.54	1.60	0.789
Corticosteroids during ICI (Yes vs. No)	0.71	0.45	1.11	0.136	0.90	0.55	1.50	0.695
Previous RT (Yes vs. No)	1.35	0.52	3.49	0.535	3.10	1.05	9.15	***0.041***
Variables	**ORR**				**DCB**			
	OR	**Inf 95%CI**	**Sup 95%CI**	***P***	**OR**	**Inf 95%CI**	**Sup 95%CI**	***P***
Cancer site (NSCLC+GU vs. all others)	2.19	0.85	5.64	0.105	2.39	0.91	6.29	*0.079*
ICI treatment line (1st vs. ≥2nd)	1.98	0.78	5.05	0.154	0.89	0.32	2.46	0.823
sATB during ICI (Yes vs. No)	1.58	0.62	4.04	0.341	3.89	1.54	9.85	***0.004***
Corticosteroids during ICI (Yes vs. No)	1.82	0.73	4.54	0.198	2.07	0.81	5.27	0.127

Legend. HR: hazard ratio; OR: odds ratio; Inf: inferior; Sup: superior; PFS: progression-free survival; OS: overall survival; ORR: overall response rate; DCB: durable clinical benefit; ICI: immune-checkpoint inhibitor; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NSCLC: non-small cell lung cancer; GU: genitourinary; sATB: systemic antibiotics; RT: radiotherapy.

Activity (TTBR, DOR, ORR, DCR)

The median TTBR was 2.5 months (95%CI: 2.0 – 2.7) (Figure 7), with an ORR of 17.8% (95%CI: 12.0 – 25.0%) (Table 21). Excluding patients who experienced a PD as best response, the median DOR was 4.4 months (95%CI: 3.3 – 10.5) (figure 7), with 17.8% (95%CI: 11.6 – 24.0%) patients experiencing a CR, PR or SD lasting ≥6 months (Table 21).

Figure 7: Kaplan-Meier mTTBR and mDOR curves for the overall population



Legend. Kaplan-Meier curves with their 95%CI for the overall population. mTTBR: median time to best response. mDOR: median Duration of Response.

The DOR showed a positive moderate correlation with OS (r=0.60, p<0.001). Cancer site appeared to be correlated with the achievement of ORR (p=0.044), with NSCLC and GU tumors being associated with better ORR, compared to other cancers (p=0.011) (Figure 8 and 9).

Figure 8: Best response according to tumor site.

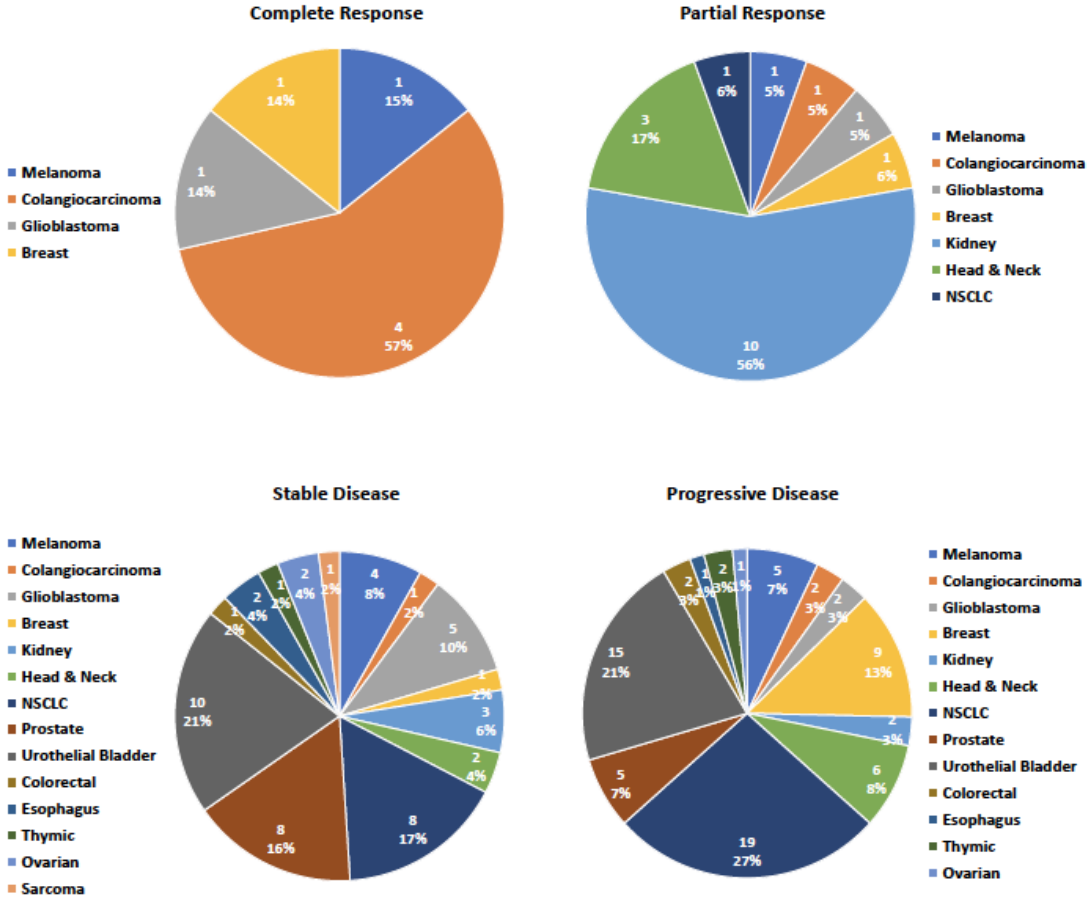
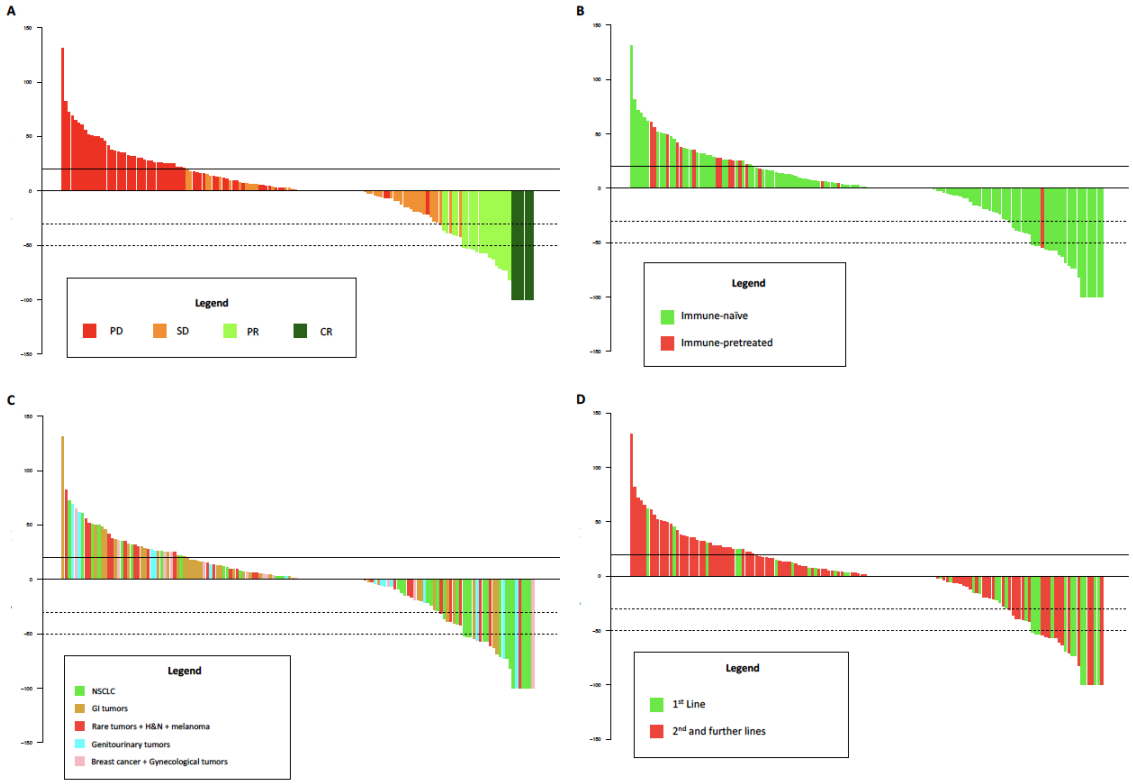


Figure 9: Waterfall plots for responses to immune-checkpoint inhibitors



Legend. A: best responses in the overall population; B: best responses according to immune-naïve status; C: best responses according to tumor site; D: best responses according to treatment line; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NSCLC: non-small cell lung cancer; H&N: head and neck tumors; GI: gastrointestinal.

First-line ICI appeared to be associated with stronger responses, compared to later lines (p=0.021) (Figure 9). Systemic ATB during ICI were associated with increased DCB (p=0.001) but not ORR (p=0.089). Notably, systemic corticosteroids administered during ICI were associated with significantly better ORR (p=0.044) and DCB (p=0.015). There were no other significant associations with ORR and DCB (Table 24).

Table 24. Univariate analyses of overall response rates and durable clinical benefit

Variables	Univariate Analyses							
	ORR				DCB			
	OR	Inf 95%CI	Sup 95%CI	P	OR	Inf 95%CI	Sup 95%CI	P
Age (continuous)	1.02	0.98	1.05	0.439	1.02	0.98	1.06	0.319
Sex (Male vs. Female)	1.46	0.57	3.76	0.431	1.12	0.47	2.63	0.802
ECOG (0-1 vs. 2-3)	1.21	0.31	4.72	0.779	0.50	0.14	1.75	0.279
Metastatic at diagnosis (Yes vs. No)	1.55	0.64	3.74	0.335	1.55	0.64	3.74	0.335
Cancer site (NSCLC+GU vs. all others)	3.15	1.30	7.65	***0.011***	3.15	1.30	7.65	***0.011***
ICI treatment line (1st vs. ≥2nd)	2.82	1.17	6.79	***0.021***	1.52	0.62	3.76	0.365
ICI treatment line (continuous)	0.61	0.40	0.91	***0.016***	0.78	0.56	1.09	0.149
Immuno-naive status (Yes vs. No)	3.57	0.45	28.32	0.228	3.57	0.45	28.32	0.228
Regimen (Monotherapy vs. Combination)	1.47	0.63	3.45	0.371	0.69	0.29	1.65	0.403
ICI target (Anti-PD-L1 vs. Anti-PD1)	0.82	0.28	2.38	0.715	1.40	0.53	3.70	0.498
Visceral disease (Yes vs. No)	1.28	0.44	3.70	0.651	1.23	0.44	3.70	0.651
Number of metastases (≥3 vs. <3)	0.79	0.29	2.19	0.651	2.12	0.59	7.62	0.249
Basal Hb (continous)	1.01	0.98	1.03	0.462	0.98	0.96	1.01	0.216
Basal Albumin (continous)	1.02	0.92	1.13	0.729	1.00	0.91	1.11	1.00
RT within 30 days from ICI start (Yes vs. No)	0.57	0.07	4.80	0.607	0.57	0.07	4.80	0.607
RT during ICI (Yes vs. No)	0.93	0.34	2.54	0.882	1.19	0.45	3.14	0.720
sATB within 30 days from ICI start (Yes vs. No)	0.76	0.09	6.60	0.803	0.76	0.09	6.60	0.803
sATB during ICI (Yes vs. No)	2.15	0.89	5.19	0.089	4.83	1.98	11.77	***0.001***
Corticosteroids within 30 days from ICI start (Yes vs. No)	0.73	0.20	2.66	0.628	0.69	0.19	2.52	0.570
Corticosteroids during ICI (Yes vs. No)	2.42	1.03	5.73	***0.044***	2.94	1.24	7.01	***0.015***
TILs % (continous)	1.01	0.95	1.07	0.788	1.01	0.95	1.07	0.870
PD-L1 % (continuous)	1.03	1.01	1.05	***0.007***	1.03	1.00	1.05	***0.028***
PD-L1 % (>10% vs. ≤10%)	16.92	1.94	147.77	***0.011***	9.63	1.08	86.18	***0.043***
PD1 mRNA (continuous)	1.21	0.82	1.79	0.331	1.08	0.73	1.60	0.686

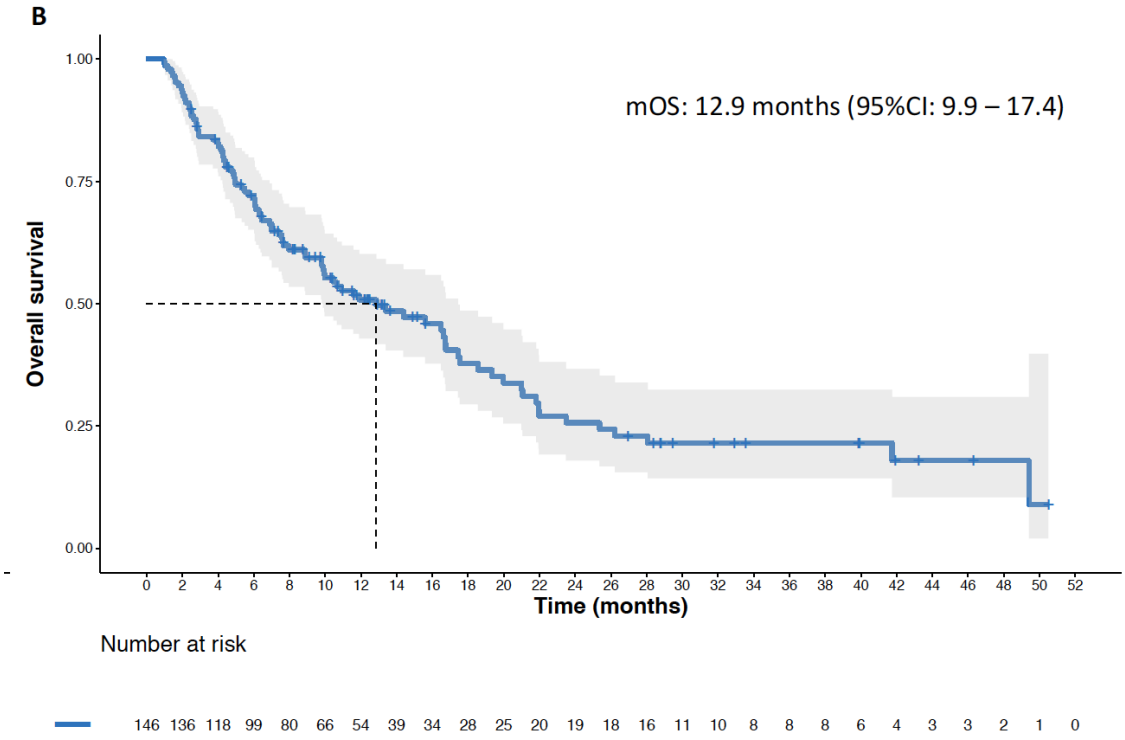
Legend. ORR: overall response rates; DCB: durable clinical benefit; OR: odds ratio; Inf: inferior; Sup: superior; ICI: immune-checkpoint inhibitor; NSCLC: non-small cell lung cancer; GU: genitourinary; TILs: tumor-infiltrating lymphocytes; sATB: systemic antibiotics; RT: radiotherapy.

Overall results were not significant at the multivariate analysis for ORR (Table 23). Conversely, sATB during ICI were independently associated with more favorable DCB (p=0.004) and a trend for better DCB was observed for NSCLC and GU tumors vs. all others (p=0.079) (Table 23).

Overall survival

At the time of data cut-off, 91 deaths had occurred, and median OS was of 12.9 months (95%CI: 9.9 – 17.4) (**figure 10** and **Table 21**).

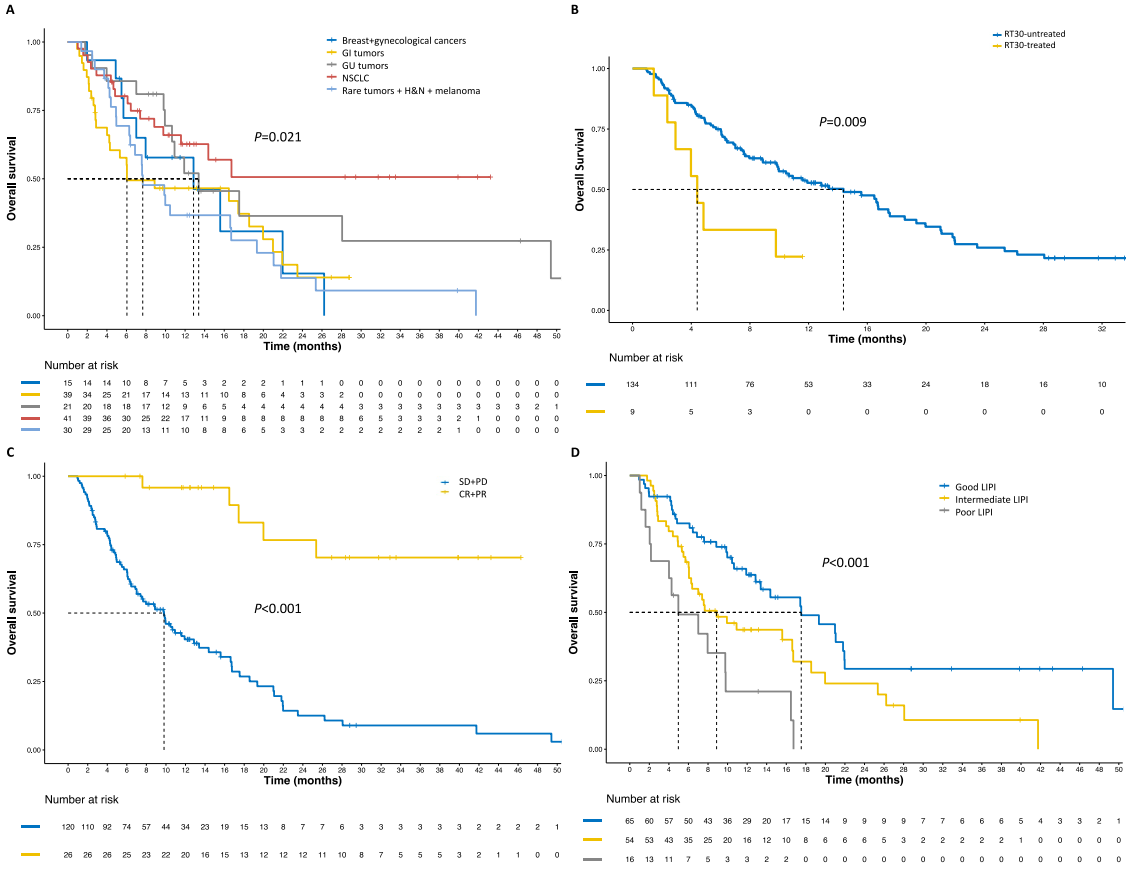
Figure 10: Kaplan-Meier overall survival curve for the overall population



Legend. mOS: median overall survival.

Similarly to PFS, tumor site, number of treatment lines and LIPI score were significantly associated with OS (p=0.021, p=0.037 and p<0.001, respectively) (Figure 11). When RT was administered within 30 days before ICI treatment start, a significantly worse OS was observed (p=0.009). Patients achieving an objective response were also prognostically favored over patients achieving SD or PD as their best response (p<0.001) (Figure 11), with better prognosis for longer TTBR (p<0.001). No other clinical or hematological factors were associated with OS (Table 22).

Figure 11: Overall survival curves according to significant population characteristics



Legend. A: OS according to cancer site; B: OS according to treatment line; C: OS according to best responses; D: OS according to LIPI score; OS: overall survival; NSCLC: non-small cell lung cancer; H&N: head and neck tumors; GI: gastrointestinal; GU: genitourinary; SD: stable disease; PD: progressive disease; CR: complete responses; PR: partial responses; RT30: radiotherapy received within 30 days from ICI start.

At the multivariate analysis, the independent prognostic value of the LIPI score (p<0.001) was confirmed, along with a detrimental effect for RT received within 30 days before ICI was confirmed (p=0.041), as well. Also, compared to NSCLC and GU tumors, all other cancers showed significantly worse OS (p=0.025) (Table 23).

4.3.4 Discussion

Here we assessed the correlation among many clinicopathological and biological factors with activity and efficacy endpoint of ICI treatment, so to identify an easily detectable profile of the patients that might gain the most benefit out of anti-PD1/PD-L1 immunotherapy. Overall, baseline LIPI score, immunotherapy-naïve status, cancer type and RT before starting ICI were the most relevant clinical factors independently correlated with immunotherapy outcomes. Longer TTBR seem to associate with better

survival, suggesting the need for not interrupting ICI therapy unless required for tumor progression, tolerability issues or patient's preference.

We confirmed that roughly 18% of patients treated with anti-PD1/PD-L1 ICI experienced a durable clinical response of at least 6 months, including SD. In patients achieving disease control, the DOR moderately correlated with OS and the longer the DOR, the better the OS. Importantly, the TTBR also seemed to be positively correlated with OS. Considering that no specific factors are currently able to prospectively predict the best response the patient will achieve, nor for how long it will last, these results suggest that anti-PD1/PD-L1 ICI might be preferably discontinued at tumor progression or unacceptable toxicity, justifying maintenance/durable treatment strategies.

Unfortunately, only 17.8% patients were able to achieve a CR or PR, and the type of response was associated with OS, with patients achieving an ORR experiencing an 88% reduction in the risk of death, compared to patients achieving SD as their best response.

We investigated in our study the role of palliative RT administered right before or during anti-PD1/PD-L1 ICI therapy. It has been considered that RT might potentially contribute to determine a stronger systemic immune response (i.e. the abscopal effect) via immunogenic cell death and antigen release, thus enhancing the efficacy of ICI (136,137). However, in our cohort, RT administered during ICI was not associated to PFS, OS or tumor responses. Surprisingly, RT administered within 30 days from ICI treatment start was associated with worse OS, independently from all other clinicopathological factors considered. We have no current explanation for this observation and only 9 patients had received palliative RT immediately before ICI start, making this finding difficult to generalize. Conversely, in line with other findings (138,139), we did not observe any abscopal effect, providing more evidence to debunk a widely postulated, yet scarcely objectivized phenomena(137).

Recently, Pinato et al. showed that systemic ATB administered prior to, but not during ICI monotherapy, are associated with a worse treatment response and OS in solid tumors(140), while ATB treatment in general seems not to impact on chemo-immunotherapy outcomes(141). In our cohort, only ATB during, but not previous to anti-PD1/PD-L1 treatment, were associated with better PFS (univariate analysis) and DCB (univariate and multivariate analysis). To note, considering the very low number of patients (n=7) that received ATB prior to ICI, we cannot completely exclude that an ATB-induced gut microbial dysbiosis might impair ICI efficacy. At the same time, we had no sign of detrimental effect during ICI-based therapy in a wider number of patients (n=41),

in line with recent evidences (140,141), with a significant and independent association to DCB which merits further investigation.

Whether systemic corticosteroids, due to their immunosuppressive effect, might impair or not ICI when administered right before or during treatment is another matter of debate. Several studies led to the conclusion that avoiding or delaying the use of corticosteroids may result in maximizing the potential treatment benefits of immunotherapy (31–33,35,142). However, other evidences highlight that corticosteroids have no detrimental effect on immunotherapy and high doses of steroids might reflect poorer basal conditions (e.g. active brain metastases, concurrent diseases, larger tumor volume), ultimately responsible for the more scarce outcomes observed with ICI (36,37). In our study, systemic administration of corticosteroids during ICI was associated with better PFS, ORR and DCB at the univariate analysis but lost any significant effect when adjusting for other clinicopathological factors. Corticosteroids prior to ICI did not show any significant effect on outcomes. We did not observe any difference when dividing steroid-receiving patients according to dose (above or below an equivalent of 30mg of prednisone; not shown), as well. To note, in 48 out of 61 (78%) cases, systemic corticosteroids were administered to treat immune-related adverse events and in 5 (8%) further cases were administered as premedication to CT scan contrast medium. Thus, in our study corticosteroid use did not reflect a baseline unfavorable condition beyond tumor type and there was no hint that successfully treating ICI immune-mediated toxicities with corticosteroids might ultimately impair anti-PD1/PD-L1 efficacy.

Multiple evidences have highlighted so far the capability of the simple LIPI score, based on the derived neutrophile-to-lymphocyte ratio (dNLR) and LDH, to successfully predict the prognosis of patients with NSCLC treated with immunotherapy (59,143). LIPI score prognostic ability has been also evaluated in patients with various tumor types treated with ICI, like melanoma, bladder cancer or solid tumors harboring MSI (54–56,58,59,144). Our study confirms the capability of the LIPI score to successfully stratify patients with solid tumors treated with anti-PD1/PD-L1 in different prognostic subgroups, independently from all main clinicopathological characteristics, in a tumor agnostic fashion, both in terms of PFS and OS. Patients with poor basal LIPI had a poor benefit from ICI, hence the evaluation of LIPI may identify a subset of patients with no or reduced benefit to anti-PD1/PD-L1 therapy. Considering the evidence available on this score, we strongly encourage its use at least for the selection of patients for clinical trials with ICI or as a stratification factor within such trials.

Noteworthy, an immunotherapy-naïve status was associated to a significantly better PFS, independently from other characteristics. Concordant recommendations regarding

the opportunity to retreat patients already treated with immunotherapy do not exist. Furthermore, these patients are usually excluded from clinical trials that evaluate new ICI drugs or combinations so the evidence of activity in this setting is limited. A recent meta-analysis pooling 49 available studies showed that in patients who had previously discontinued ICI because of PD, ORR and median PFS were inferior to those of patients who had previously discontinued ICI because of toxicity (15.2 % and 2.9 months vs. 44 % and 13.2 months, respectively) (145). Our findings, taken together with current literature, seems to confirm that rechallenges with ICI, at least with anti-PD1/PD-L1, should not be encouraged broadly, although in specific cases this strategy could be considered. Understanding the clinical impact of neo/adjuvant ICI in patients with relapsing metastatic disease candidate for immunotherapy will be of outmost importance considering the rapid expansion of therapeutic indications also in early-stage solid tumors(146,147).

Noteworthy, administering anti-PD1/PD-L1 in earlier lines seemed to be associated with better PFS, OS and ORR at univariate analyses. Although the effect on PFS and OS might have been influenced by a potential lead time bias, it is also true that a less compromised immune system in untreated/less treated patients might favor the elicitation of more potent immune responses. At the same time, it is important to underline that treatment line lost its effect on all endpoints at multivariate analyses. Thus, this finding seems to suggest that treatment line should not be an eligibility criterion for ICI treatment.

Finally, we observed that NSCLC and GU tumors were associated with better survival and activity outcomes compared to the rest of solid malignancies included in our study. This result, for which a specific explanation cannot be provided in the context of this analysis, is somewhat confirmatory of the good sensitivity to immune-checkpoint inhibition observed in the clinical practice scenario. In fact, most ICI are currently approved for NSCLC, prostate, kidney and bladder urothelial cancer.

Our study presents several limitations worth noting. First, its observational nature limited any possibility of control with respect to the administered treatment or for a more homogeneous tumor site distribution or treatment line. Second, there was no control arm. Finally, patients were treated in clinical trials, which means that some agents are not currently approved for the same clinical scenario. At the same time, this potential bias highlights the added value of a Clinical Trials Unit in an Oncology Department, which gives patients real therapeutic possibilities not otherwise or readily available in a pure clinical practice scenario. Despite limitations, our study comprehensively assessed all main clinicopathological characteristics considered in clinical practice. Data were

prospectively collected and there was no specific selection bias related to excessively strict inclusion criteria, which is the typical Achille's heel when generalizing clinical trial results to the "real-life" population(148,149). Furthermore, the sample size was in line with most phase II single arm trials.

4.3.5 Conclusion

To resume, only <20% of patients with solid tumors obtain an objective and durable response with anti-PD1/PD-L1 ICI, with the magnitude and duration of response being directly associated with outcomes. The appropriate selection for patients more likely to achieve a durable response to ICI should be a priority. In this perspective, common clinicopathological factors seem not to be able to identify the best candidates for immunotherapy, except for immunotherapy-naïve status. Systemic corticosteroid administration for treating ICI-related adverse events is a feasible therapeutic strategy which seem not to negatively affect ICI efficacy, as well as systemic ATB administered during treatment. Importantly, none of our RT-treated patients experienced a beneficial abscopal effect, while RT detrimental effect when administered before starting ICI should be further elucidated in wider casuistries. Importantly, our study provides additional evidence to support the use of basal LIPI score at least to select patients for clinical trials with anti-PD1/PD-L1 ICI and/or as stratification factors.

Chapter 4.4 SECONDARY OBJECTIVE 2:

Role of PD1 mRNA levels, PD-L1 protein expression and Tumor Infiltrating Lymphocytes (TILs) levels as predictor of response or resistance to immunotherapies

This objective was explored and published as previously mentioned in the Cancer Inmunology, Immunotherapy in 2023. Data data cut-off was 31/08/2021 at that time. Full article is in appendix 1.

Original article reference is (113):

García-Corbacho Javier, Indacochea Alberto, González Navarro E. Azucena, Victoria Iván, Moreno Débora, Pesantez David, Angelats Laura, Modrego-Sanchez Andrea, Sanfeliu Esther, Castillo Oleguer, Blasco Paula, Mezquita Laura, Viñolas Nuria, Nogué Miquel, Galván Patricia, Adamo Barbara, Basté Neus, Sauri Tamara, Juan Manel, Prat Aleix, Schettini Francesco. Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study. Cancer Immunology, Immunotherapy. 2023 Jun;72(6):1709-1723.

PMID: 36625938. PMCID: PMC10198872. DOI: 10.1007/s00262-022-03360-9

In this publication we explored the role of PD1 mRNA, PDL1 and TILs in our population when tumor samples were available. Besides, we observed an unexpected prolonged complete response in a patient diagnosed of sarcoma so we decided to perform a deeper analysis of that patient that was published as case report in 2023. Full article is in Appendix 2.

Original article reference is (129):

David Pesántez, Alberto Indacochea, Laura Angelats, Marianna Sirico, Iván Victoria, Esther Sanfeliu, Cristina Teixido, Azucena E. González-Navarro, Patricia Galván, Fara Brasó-Maristany, Pedro Jares, Manel Juan, Aleix Prat, Francesco Schettini, Javier Garcia-Corbacho.Unexpected durable complete response with anti-PD-L1 blockade in metastatic undifferentiated pleomorphic sarcoma: a case report with host and tumor biomarker analysis. JCO Precision Oncology

DOI https://doi.org/10.1200/PO.23.00051

4.4.1 FIRST PUBLICATION

Abstract

Background: Immune-checkpoint inhibitors (ICI) have revolutionized the therapeutic landscape of cancer. However, optimal patient selection is still an unmet need.

Methods: One-hundred-forty-six patients with metastatic cancer candidates to ICI at the Hospital Clinic of Barcelona Clinical Trials Unit were prospectively recruited in this observational study. Blood samples were collected at different timepoints, baseline LIPI score calculated and pre-ICI archived tissues retrieved to evaluate PD-L1, tumor-infiltrating lymphocytes (TILs) and PD1 mRNA levels. Tumor assessments were centrally reviewed by RECIST 1.1 criteria. Associations with overall response rates (ORR), durable clinical benefit (DCB), progression-free survival (PFS) and overall survival (OS) were performed with univariable/multivariable logistic and Cox regressions, where appropriate.

Results: At a median follow-up of 26.9 months, median PFS and OS were 2.7 and 12.9 months. Response rates were 17.8% with duration of response (DOR) of 4.4 months. LIPI score was independently associated with PFS ($p = 0.025$) and OS ($p < 0.001$). Immunotherapy-naïve status was independently associated with better PFS ($p = 0.005$). Time-to-best response (TTBR) and ORR ($p < 0.001$ both) were associated with better OS at univariate analysis. PFS and DOR were moderately correlated with OS ($p < 0.001$ both). A PD-L1 10% cut-off detected worse/best responders in terms of ORR (univariate $p = 0.011$, multivariate $p = 0.028$) and DCB (univariate $p = 0.043$). PD1 mRNA levels were strikingly associated to complete responses ($p = 0.021$).

Conclusion: To resume, in our prospective observational pan-cancer study, baseline LIPI score, immunotherapy-naïve status, cancer type and RT before starting ICI were the most relevant clinical factors independently correlated with immunotherapy outcomes. Longer TTBR seemed to associate with better survival, while PD1 mRNA and PD-L1 protein levels might be tumor-agnostic predictive factors of response to ICI and should be furtherly explored.

4.4.1.1 Introduction

In the last decade ICI become part of our therapeutic tools for several solid tumors. Nevertheless, a wide range of the patients who receive a ICI do not benefit from them. The only predictive biomarkers of response that can be used in clinical practice are the assessment of PD-L1 levels by immunohistochemistry (IHC), micro-satellite instability (MSI) and tumor mutational burden (TMB), though the latter only in the USA (27,65,66,150). However, they have been variably successful in predicting responders according to different cancers and their use is limited to specific contexts (65,66,150). The outcome of ICI therapy has also been linked to the quality and magnitude of tumor-infiltrating lymphocytes (TILs)' responses within the tumor microenvironment, though without current clinical applicability(151).

In the previous objective we explored the influence of clinicopathological factors on the outcomes. Here we explore the influence of tumor-based biomarkers in the outcomes of our population, with a data cut off of 31/08/2021.

4.4.1.2 Materials and methods

Study design and participants

Study design, full inclusion/exclusion criteria and evaluable criteria have already been reported in chapter 3: Methodology and chapter 4.3: secondary objective 1.

Procedures

Apart from the blood samples and clinical data previously mentioned, archived tumor sections from the primary or the latest available metastatic biopsy before starting ICI were collected in case of availability and explicit patient consent. An expert pathologist from the HCB carried out an assessment of tumor-infiltrating lymphocytes (TILs) through the evaluation of hematoxylin and eosin (H&E)-stained formalin-fixed paraffin-embedded (FFPE) tumor sections (4-5um thick, magnification x 200-400), according to the methodology proposed by the International Immuno-Oncology Biomarkers Working Group(152).

Dr Prat at Translational Genomics and Targeted Therapies in Solid Tumors Lab previously demonstrated a correlation between PD1 mRNA levels and response to anti-PD1 ICI across cancer types (123). Samples collected were shipped to their lab where we analyzed the expression of PD1 mRNA using the Nanostring® nCounter® platform in FFPE tumor samples across cancer types, as in the previous publication (123).

Methods for RNA extraction, quality assessment and gene expression analysis have been previously described in chapter 3: methodology.

PD-L1 in tumor tissues was analyzed in FFPE tissue sections at the HCB as *per* clinical practice, using the mouse monoclonal antibody 22C3 (Dako) anti-PD-L1 monoclonal antibody on a Dako Autostainer, following manufacturer's recommendations. PD-L1 immunohistochemical (IHC) expression in non small-cell lung cancer (NSCLC) was evaluated in tumor cells to obtain a tumor proportion score (TPS) identifying the % of tumor cells expressing PD-L1, as *per* clinical practice(153). In all other tumors where PD-L1 positivity was tested (14/46 cases, 30%), the combined positive score (CPS) was used to assess PD-L1 status, as *per* clinical practice. The CPS is defined as the total number of tumor cells and immune cells stained with PD-L1, divided by the number of all viable tumor cells, then multiplied by 100(154). It has been previously demonstrated that PD-L1 TPS and CPS are highly concordant in NSCLC (155), therefore we jointed PD-L1 scores for the purpose of this exploratory analysis.

Study endpoints and outcomes

This analysis was intended to correlate baseline clinicopathological factors to response, in terms of overall response rate (ORR) and durable clinical benefit (DCB), and survival, in terms of progression-free survival (PFS) and overall survival (OS). Clinicopathological factors analysis was previously reported. Here we explain the impact of TILs, PD-L1 and PD1 mRNA impact on ORR, DCB, PFS and OS in patients treated with ICI.

Statistical Analysis

Univariate and multivariable logistic regression analyses were performed to investigate the association between PD1 mRNA abundance with tumor response. Odds ratios (OR) with 95% confidence intervals (CI) were used as measure of association with ORR and DCB. The maximally selected rank statistics (MSRS) method was adopted to identify an exploratory optimal cut-off for PD1 mRNA, TILs and PD-L1 protein, considering PFS as the time-dependent end-point(156). Further details regarding outcomes analysis were given in chapter 4.3.

4.4.1.3 Results

Between May 2017 and June 2021, 156 patients entered the study and 146 received an anti-PD1/anti-PD-L1-based treatment. The median follow-up at the data cut-off (31/08/2021) was 26.9 months (95% CI: 13.1 – 31.7). All patients and tumors characteristics were previously detailed in Table 20.

Tissue biomarkers exploratory analysis

PD-L1 protein expression, TILs levels and PD1 mRNA levels could be assessed for 46 (31.5%), 102 (69.9%) and 68 (46.6%) patients, respectively.

Increasing protein levels of PD-L1 were found to be associated with slightly better PFS (HR: 0.987, 95%CI: 0.978 – 0.995, p=0.003). The MSRS method was then applied to detect a potential cut-off of PD-L1 expression to identify patients at better/worse prognosis in terms of PFS. An optimal cut-off of 10% could identify patients with significantly different PFS (≦10% vs. >10% HR: 3.12, 95%CI: 1.53 – 6.36, p=0.002), also when adjusting for cancer site (p=0.030) (**Figure 4, Table 24**).

Table 24. Univariate analyses of progression-free and overall survival

Variables	PFS				OS			
	HR	**Inf 95%CI**	**Sup 95%CI**	***P***	**HR**	**Inf 95%CI**	**Sup 95%CI**	***P***
TILs % (continous)	1.00	0.97	1.03	0.730	0.99	0.96	1.02	0.509
PD-L1 % (continuous)	0.99	0.98	1.00	***0.003***	0.99	0.98	1.00	***0.009***
PD-L1 % (>10% vs. ≤10%)	0.32	0.16	0.66	***0.002***	0.36	0.15	0.83	***0.016***
PD1 mRNA (continuous)	0.97	0.82	1.14	0.682	0.89	0.74	1.07	0.208
TTBR (continuous)	-	-	-	-	0.54	0.39	0.76	***<0.001***
ORR (CR+PR vs. SD+PD)	-	-	-	-	0.12	0.05	0.30	***<0.001***

Legend. HR: hazard ratio; inferior; Sup: superior; PFS: progression-free survival; OS: overall survival; TILs: tumor-infiltrating lymphocytes; TTBR: time-to-best response; ORR: overall response rates; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

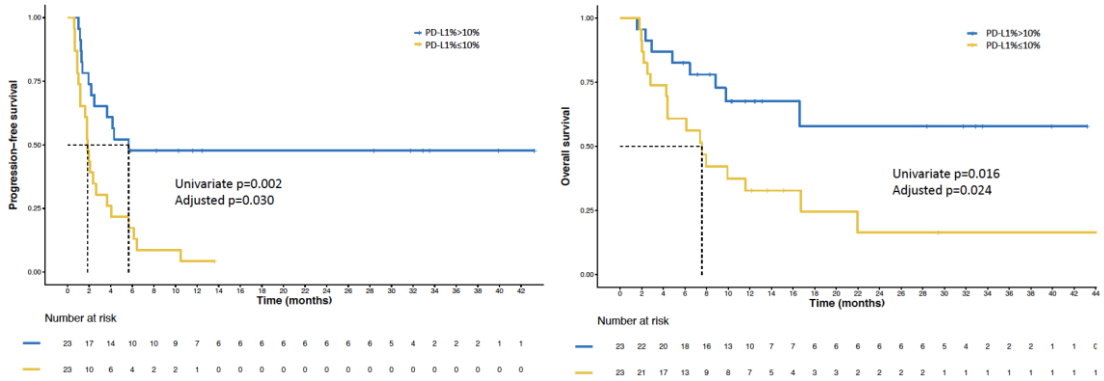
Additionally, higher levels of PD-L1 were associated with significantly better ORR (OR: 1.03, 95%CI: 1.01 – 1.05, p=0.007) and DCB (OR: 1.03, 95%CI: 1.00 – 1.05, p=0.028). The previously established 10% cut-off was able to distinguish between best/worst responders in terms of ORR (p=0.011) and DCB (p=0.043) at univariate analysis, as well (Table 25). When adjusting for cancer site, the cut-off retained its significance in terms of ORR (OR: 11.67, 95%CI: 1.30 – 104.82, p=0.028). Finally, the PD-L1 cut-off was also able to distinguish between patients with worse/better OS at univariate analysis (HR: 2.83, 95%CI: 1.22 - 6.57, p=0.016) and when adjusting for cancer site (p=0.024) (Figure 12, table 24).

Table 25. Univariate analyses of overall response rates and durable clinical benefit

Variables	Univariate Analyses							
	ORR				DCB			
	OR	Inf 95%CI	Sup 95%CI	P	OR	Inf 95%CI	Sup 95%CI	P
TILs % (continous)	1.01	0.95	1.07	0.788	1.01	0.95	1.07	0.870
PD-L1 % (continuous)	1.03	1.01	1.05	***0.007***	1.03	1.00	1.05	***0.028***
PD-L1 % (>10% vs. ≤10%)	16.92	1.94	147.77	***0.011***	9.63	1.08	86.18	***0.043***
PD1 mRNA (continuous)	1.21	0.82	1.79	0.331	1.08	0.73	1.60	0.686

Legend. ORR: overall response rates; DCB: durable clinical benefit; OR: odds ratio; Inf: inferior; Sup: superior; TILs: tumor-infiltrating lymphocytes.

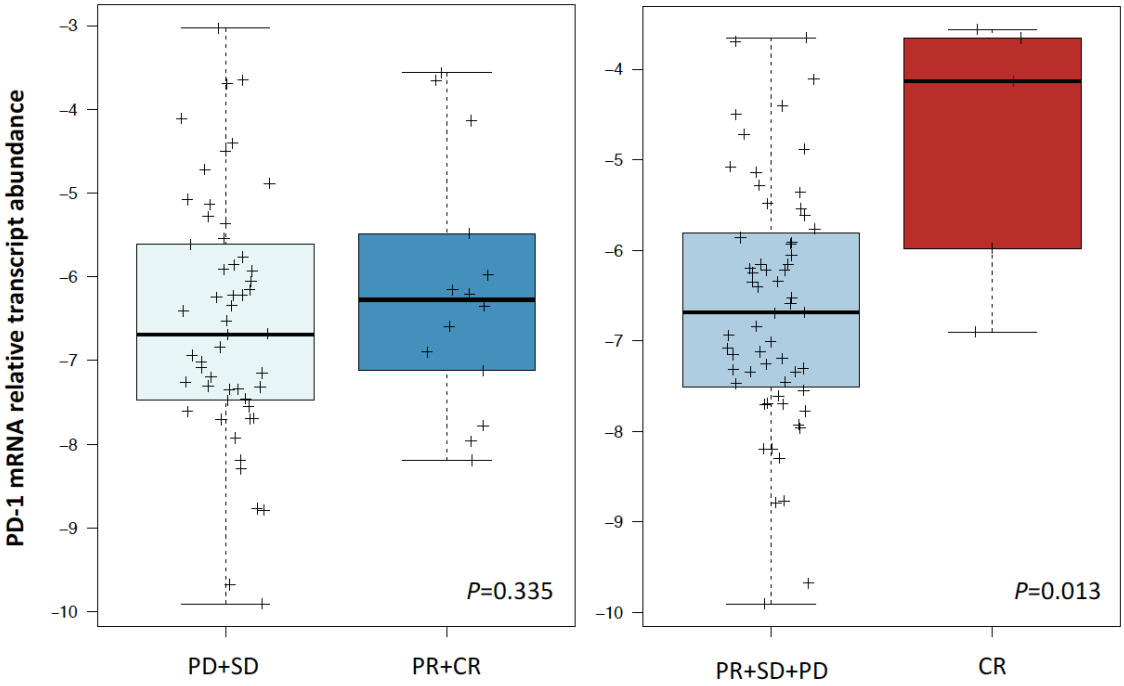
Figure 12. PD-L1 protein associations with Progression Free Survival and Overall Survival.



Legend. Progression-free survival Kaplan-Meier curves according to a PD-L1 cut-off selected with the Maximally Selected Rank Statistics method; Overall survival Kaplan-Meier curves according to the selected PD-L1 cut-off.

Both TILs and PD1 mRNA levels were not significantly associated to PFS (p=0.730 and p=0.682, respectively), ORR (p=0.742 and p=0.331, respectively), DCB (p=0.870 and p=0.352, respectively) and OS (p=0.509 and p=0.208, respectively) (Table 24 and 25). However, PD1 mRNA levels were strikingly associated to the achievement of CR (Figure 13), compared to all other responses (OR: 2.35, 95%CI: 1.14 - 4.87, p=0.021) and achieving an objective response was associated to better OS, as previously reported (HR: 0.12, 95%CI: 0.05 – 0.30, p<0.001).

Figure 13. PD1 mRNA levels' main associations with outcomes.



Legend. PD1 mRNA levels in patients achieving an objective response vs. patient not achieving an objective response in the left box plot and PD1 mRNA levels in patients achieving a complete response vs. patients not achieving a complete response in the right box plot; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; P values in box plots are referred to Student's t tests for differences in mean PD1 mRNA levels.

4.4.1.4 Discussion

Here we assessed the correlation among many clinicopathological and biological factors with activity and efficacy endpoint of ICI treatment, so to identify an easily detectable profile of the patients that might gain the most benefit out of anti-PD1/PD-L1 immunotherapy. Apart from the clinicopathological factors mentioned in the previous section, we observed that PD1 mRNA and PD-L1 protein levels might be tumor-agnostic predictive factors of response to ICI.

Unfortunately, only 17.8% patients were able to achieve a CR or PR, and the type of response was associated with OS, with patients achieving an ORR experiencing an 88% reduction in the risk of death, compared to patients achieving SD as their best response. In this perspective, although the number of cases with tumor tissue available for mRNA detection was too low for introducing the variable in the multivariate logistic regression models, we confirmed the capability of PD1 mRNA to identify patients more likely to achieve an objective response, CR above all (**Figure 13**), as Translational Genomics and Targeted Therapies in Solid Tumors Lab group previously demonstrated (123). Interestingly, while TILs seemed not to correlate with response and survival outcomes in

a pan-cancer context, PD-L1% was positively associated with a slightly higher likelihood of achieving an objective response (OR: 1.03) and a 1% reduction in the risk of progression or death for each unitary increase. Additionally, a cut-off of 10% appeared to be optimal in discriminating between patients at higher likelihood of achieving an objective and durable response and at lower risk of progression or death, similarly to what observed for example, with pembrolizumab in metastatic triple negative breast cancer(157). Nevertheless, a larger casuistry is required to confirm the result independently from other variables and across cancer types, along with a uniform assessment of PD-L1 throughout cancer types.

As our study was a non-interventional trial, we could not realize any tumor biopsy for patients lacking tumor tissues. This prevented us from testing for PD-L1 protein levels and PD1 mRNA in all patients' tumors which is a clear limitation. Nevertheless, we hope that ACROPOLI clinical trial that is currently ongoing will clarify the potential of PD1 mRNA as ICI biomarker. (86)

4.4.1.5 Conclusion

Less than 20% of patients with solid tumors obtain an objective and durable response with anti-PD1/PD-L1 ICI, so the appropriate selection for patients more likely to achieve benefit with ICI remains a priority. Our study provides additional evidence to support the use of PD1 mRNA in tumor tissue at least to select patients for clinical trials with anti-PD1/PD-L1 ICI and/or as stratification factors, while PD-L1%, with a potential 10% cut-off, is a promising tumor-agnostic prognostic and predictive factor. However, it should be further validated in appropriately powered prospective studies and with the same detecting methodology, preferably CPS, potentially more generalizable than TPS.

4.4.2 SECOND PUBLICATION: Case Report (129)

4.4.2.1 Background

Soft tissue sarcomas (STS) are a heterogeneous group of tumors, representing ~1% of adult malignancies (158). Within STS, undifferentiated pleomorphic sarcomas (UPS) are one of the most frequent subgroups (5-15%)(159). UPS are typically deep-seated lesions that enlarge rapidly and painlessly, frequently located in the limbs, followed by the trunk(159). Treatment options for advanced UPS remain limited and the prognosis for patients with metastatic disease is poor, with a median survival of ~12 months (160). UPS is characterized by a high level of genomic instability, as indicated by its complex karyotype with low tumor mutational burden (TMB) but high copy number alterations (CNA) (161,162). This feature can be theoretically associated to higher immunogenicity, due to a potential increase in neoantigens formation (163). For this reason, there is potential role for immunotherapy with immune-checkpoint inhibitors (ICI) in this subset of patients (1).

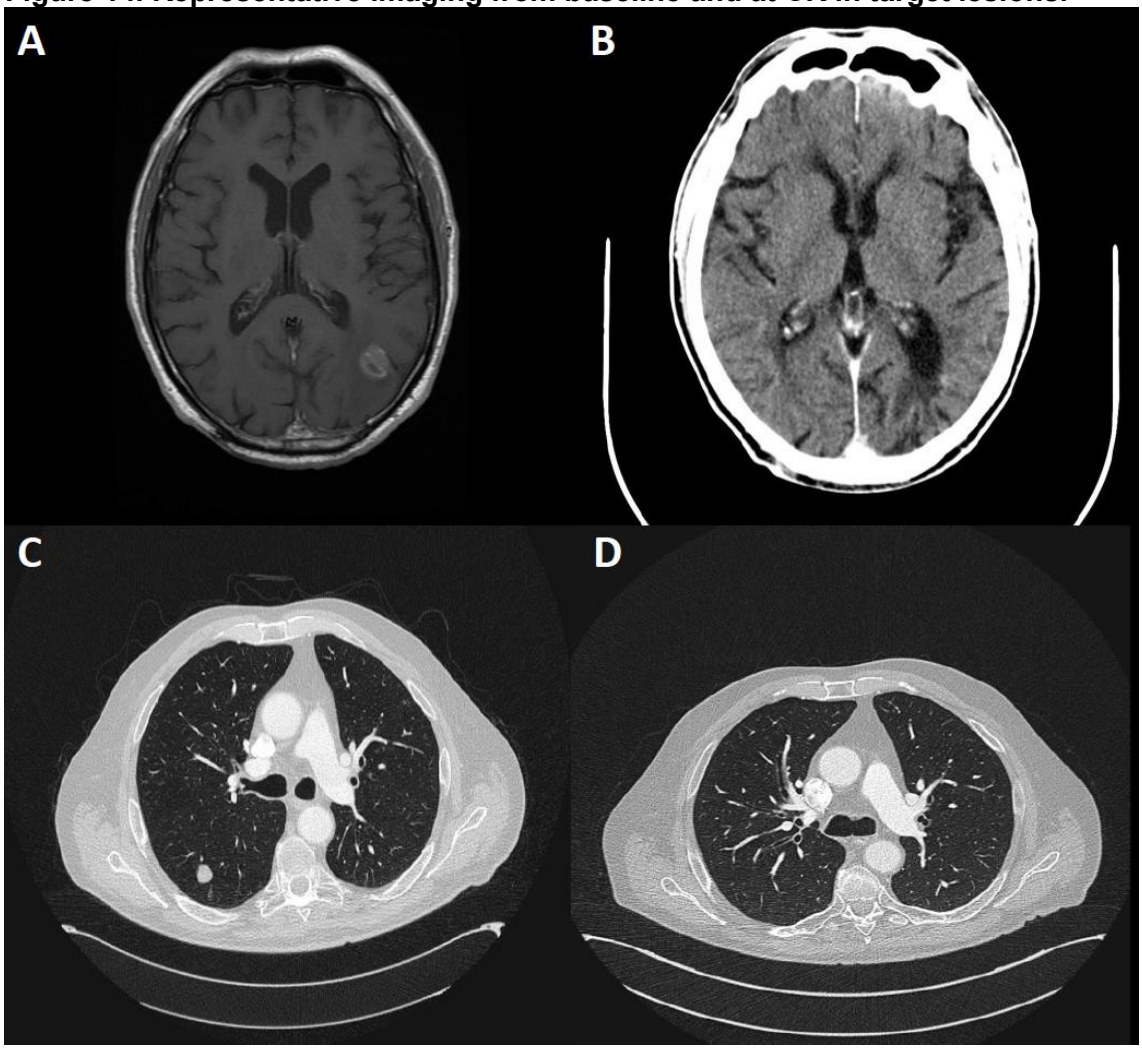
Here, we report the case of a patient with metastatic UPS of the chest wall successfully treated with an anti-PD-L1 ICI at the Clinical Trial Unit (CTU) of the Hospital Clinic of Barcelona (HCB), who experienced a surprisingly prolonged complete response (CR). A molecular profiling to explain this atypical response was carried out and hereby reported.

4.4.2.2 Case Presentation

A 69 year old man without relevant medical history was diagnosed in June 2017 at the HCB with a stage IV UPS of the chest wall with one pulmonary metastasis. As first-line treatment four cycles of doxorubicin 50mg/m^2 in continuous infusion at day 1 plus ifosfamide 2000 mg/m^2 with MESNA uroprotection for three consecutive days, every three weeks were administered. The patient obtained a stable disease (SD) as best response (BR) and a tumorectomy with pulmonary metastasectomy was performed afterwards. However, after 2 months the patient was admitted to our hospital due to seizures. A magnetic resonance imaging (MRI) was performed and showed brain metastasis (Figure 14A-B). The lack of previous symptoms and brain imaging prevents to know if it was already present. A new CT scan showed bilateral lung metastases, as well (Figure 14C). The patient was treated with whole brain radiotherapy (WBRT) with a total of 30 Gy to stabilize the brain lesion; then, after approximately one month, started a second-line treatment with an experimental probody directed against PD-L1, administered every two weeks, in cycles of eight weeks. After eight months of anti-PD-L1 treatment, the patient experienced a CR in extracranial target lesions according to RECIST 1.1 criteria (131) (Figure 14D) and minimal residual changes in brain MRI. After

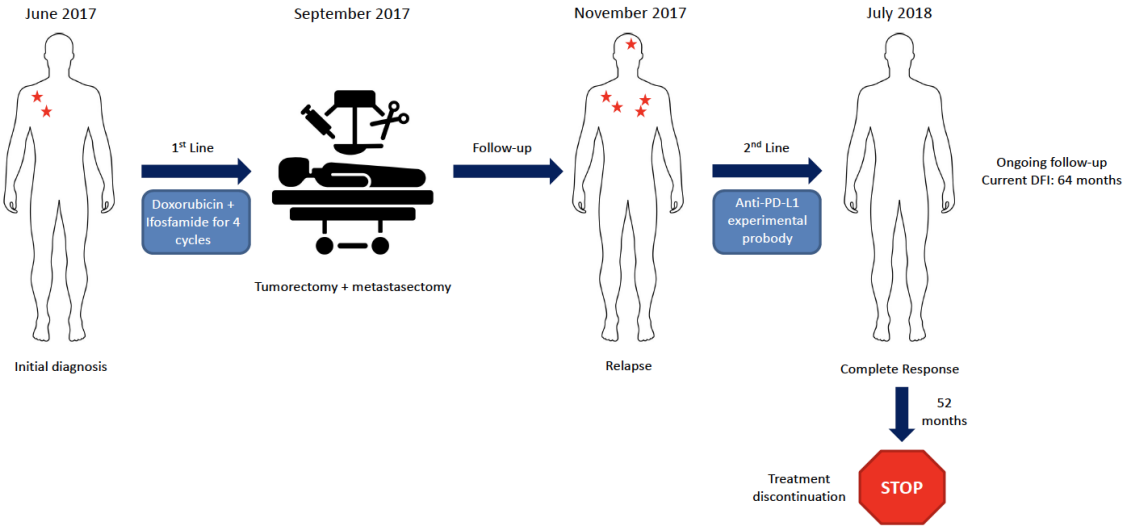
52 months the patient discontinued the treatment because of the lack of production of the study drug. In the last reassessment in November 2022, after 64 months, the patient was still with no evidence of disease progression. The clinical case is resumed in Figure 15.

Figure 14. Representative imaging from baseline and at CR in target lesions.



Legend. (A) CNS metastasis at baseline MRI; (B) CT CNS images at the moment of obtaining a CR (MRI no longer used after baseline); (C) lung metastases at baseline CT scan; the largest lung lesion measured 16 mm in its máximum diameter, with several additional satellite lesions; (D) lung CT scan at the moment of obtaining a CR. CR, complete response; CT, computed tomography; MRI, magnetic resonance imaging.

Figure 15. Case report timeline.



Legend. The first-line scheme consisted in doxorubicin 50 mg/m2 in continuous infusion at day 1 plus ifosfamide 2,000 mg/m2 with MESNA uroprotection for 3 consecutive days, administered once every 3 weeks. Whole-brain radiotherapy was administered to reach a total of 30 Gy to stabilize the brain lesion. The experimental probody directed against PD-L1 was administered once every 2 weeks, in cycles of 8 weeks. DFI, disease-free interval.

4.4.2.3 Molecular assessments

Host Biomarkers

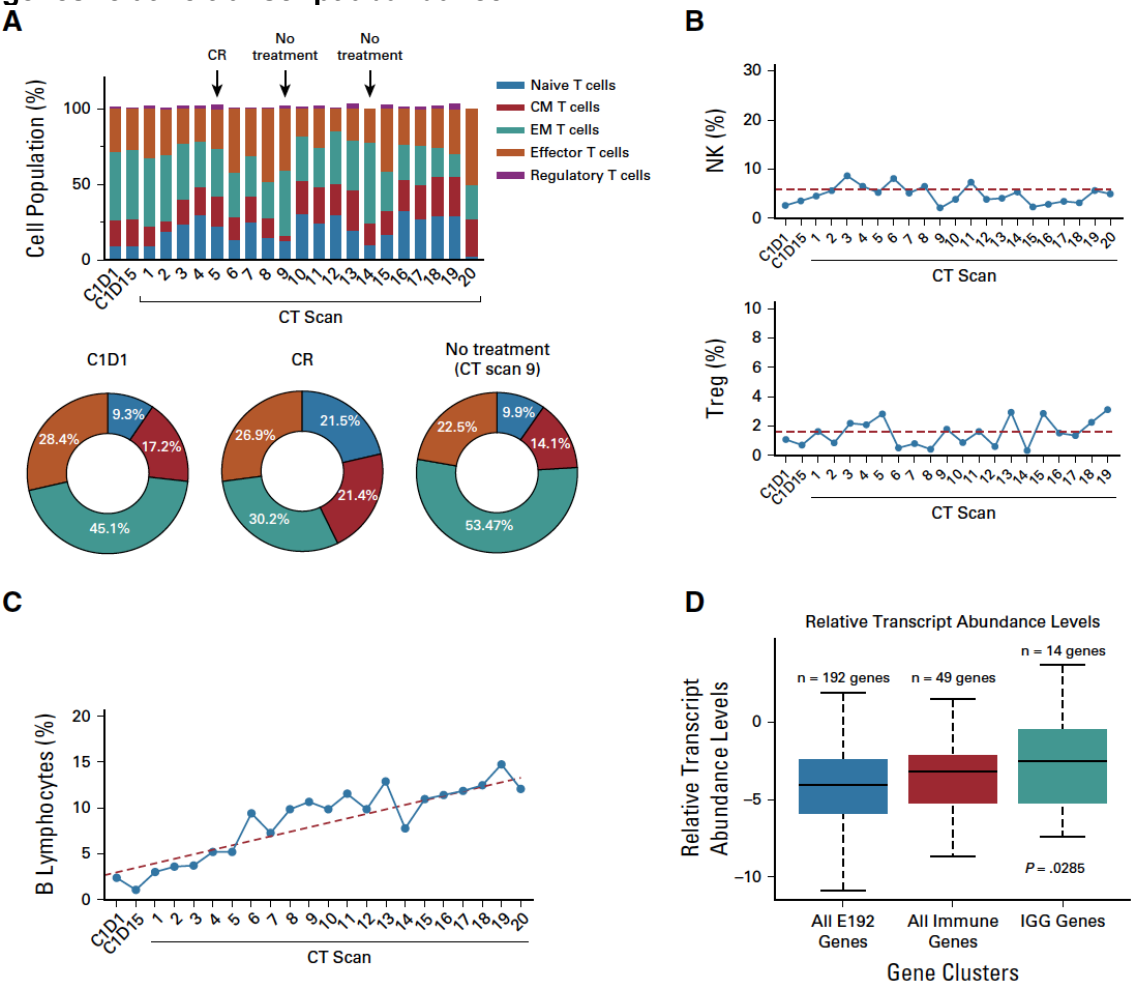
Previously to start the anti-PD-L1 treatment, we calculated the derived neutrophil-to-lymphocyte ratio (dNLR) and the lung immune prognostic index (LIPI), which we proved to be a highly performing tumor-agnostic prognostic scores for ICI-treated patients (164). The dNLR was 1.51 and the LIPI score was 0, both suggesting a good prognosis (49,118). Such scores did not substantially change after the first cycle of ICI.

Also, we performed an exploratory study of the T cells population in peripheral blood. Blood samples were collected at baseline, at cycle 2 and at each radiologic evaluation. Flow cytometry analyses were performed using the lineage and differentiation markers CD25, CD3, FOXP3, CD40L, HLA-DR, CD4, CD62L, CD69, CD8, CTLA4, CD19, CD16/56, CD28, PDL1, PD1, CD45RO/RA and CCR7.

Before cycle 1 of anti-PD-L1 treatment, the patient had high blood levels of effector memory T cells with low naïve T cells. At the time of complete response, the naïve T cell increased from 9.3% to 21.5% with a decrease of 15% in effector memory T cells. When the patient did not receive the anti-PD-L1 treatment at cycle 10, the naïve T cell populations were 9.9%, similar to baseline (Figure 16A). However, T subpopulations levels in blood did not show a clear pattern in relation to treatment response and maintenance. NK lymphocytes and Tregs showed undulatory non-informative patterns,

as well (Figure *16B*). At the same time, B cells levels in blood showed a substantial increase though time (Figure 16C).

Figure 16. Lymphocyte subpopulations levels at different time points and immune genes relative transcript abundance.



Legend. (A) Circulating T lymphocyte subpopulations levels at different time points; (B) circulating NK and Tregs lymphocyte subpopulations levels trends through time; (C) circulating B lymphocyte subpopulations levels trends through time; (D) boxplots of mRNA levels of different gene clusters. The numbers 1-20 (A-C) are referred to the number of TAC evaluation. Red lines (B and C) are representative of the different lymphocytes levels trends. C1, first cycle; CM, central memory; CR, complete response; CT, computed tomography; D1, cycle day 1; D15, cycle day 15; EM, effector memory; IGG, immunoglobulin G; NK, natural killer; Tregs, regulatory T lymphocytes.

Tumor Biomarkers

In order to interrogate genomic alterations of well-known genes altered in cancer that might both potentially explain the unexpected therapeutic response, as well as representing potential future targets, we carried out a molecular testing in pre-treatment formalin-fixed paraffin-embedded (FFPE) tumor tissue through the Oncomine™ Focus assay (ThermoFisher Scientific™, Waltham, Massachussets, USA) (Table 26). This next generation sequencing (NGS)-based assay detected the following pathogenetic hotspot mutations: *BRAF* G469V, *FGFR4* W460Ter, *NRAS* G12S and *PIK3CA* H1047R.

Table 26. List of genes included in the Oncomine™ Focus gene panel

Oncomine™ Gene Panel					
Hotspot mutation target genes		*CNV target genes*		*Pathogenetic fusions involved genes*	
AKT1	*IDH1*	*AKT1*	*MYCN*	*ABL1*	*NTRK3*
ALK	*IDH2*	*ALK*	*PDGFRA*	*AKT3*	*PDGFRA*
AR	*JAK1*	*AR*	*PIK3CA*	*ALK*	*PPARG*
BRAF	*JAK2*	*BRAF*	-	*AXL*	*RAF1*
CDK4	*KIT*	*CCND1*	-	*BRAF*	*RET*
CTNNB1	*KRAS*	*CDK4*	-	*EGFR*	*ROS1*
DDR2	*MAP2K1*	*CDK6*	-	*ERBB2*	-
EGFR	*MAP2K2*	*EGFR*	-	*ERG*	-
ERBB2	*MET*	*ERBB2*	-	*ETV1*	-
ERBB3	*MTOR*	*FGFR1*	-	*ETV4*	-
ERBB4	*NRAS*	*FGFR2*	-	*ETV5*	-
ESR1	*PDGFRA*	*FGFR3*	-	*FGFR1*	-
FGFR2	*PIK3CA*	*FGFR4*	-	*FGFR2*	-
FGFR3	*RAF1*	*KIT*	-	*FGFR3*	-
GNA11	*RET*	*KRAS*	-	*MET*	-
GNAQ	*ROS1*	*MET*	-	*NTRK1*	-
HRAS	*SMO*	*MYC*	-	*NTRK2*	-

Legend. CNV: copy number variation.

FFPE tumor tissue was used to carry out a gene expression-based assay (165) with a Nanostring® nCounter® platform (Nanostring Technologies, Seattle, MA, USA) at our laboratory. The assay included cancer- and immune-related genes, including *PDCD1* (PD1), which expression we considered worthy assessing in this context (Table 27). The PD1 mRNA level detected was -3.657 (relative transcript abundance), meaning high levels of expression according to the cut-off from Paré et al. predicting benefit with anti-PD1 ICI (87). With the same assay we compared the levels of expression of multiple immune genes associated with B cells, T cells, innate immunity cells and cytokines, as well as the established immunoglobulin G (IGG) signature, originally identified in breast tumors (Table 27)(166).

Table 27. List of genes included in the custom 192-gene panel.

PAM50 E192 Gene Panel					
ABCC11	*CD7*	*EOMES*	*IL18R1*	*MKI67*	*RRM2*
ACTG2	*CD79A**	*ERBB2*	*IL23A*	*MLPH*	*S100A9*
ACTR3B	*CD84*	*ERBB3*	*IL2RG**	*MMP1*	*SERPINB5*
AFF3	*CD86*	*ERBB4*	*IL34*	*MMP11*	*SFRP1*
AGR2	*CD8A*	*ESR1*	*IRF1*	*MND1*	*SH2D1A*
AGR3	*CDC20*	*ETFA*	*IRF4*	*MPHOSPH6*	*SIAH2*
ANLN	*CDC6*	*EXO1*	*IRF8*	*MRAS*	*SLAMF1*
AR	*CDCA1*	*F12*	*ISG20*	*MSLN*	*SLC39A6*
ASPM	*CDCA5*	*FA2H*	*ITK*	*MUCL1*	*SPDEF*
AURKA	*CDCA8*	*FGFR1*	*KCTD9*	*MYBL2*	*STARD3*
BAG1	*CDH3*	*FGFR2*	*KIF23*	*MYC*	*STAT1*
BCL2	*CDKN3*	*FGFR4*	*KIF2C*	*NAT1*	*STAT4*
BIRC5	*CENPA*	*FHOD1*	*KLK5*	*NDRG2*	*TCAP*
BLVRA	*CENPF*	*FOXA1*	*KLRB1*	*NECTIN4*	*TFCP2L1*
BOC	*CEP55*	*FOXC1*	*KLRD1*	*NEK2*	*THSD4*
BRCA1	*CLUAP1*	*GABRP*	*KNTC2*	*NFIB*	*TMEM45B*
BRCA2	*CNTNAP2*	*GAPD*	*KRT14*	*NQO1*	*TNFRSF17**
BUB1	*CREB3L4*	*GARS*	*KRT17*	*NTN3**	*TOP2A*
C2orf54	*CRYAB*	*GATA3*	*KRT18*	*ORC6L*	*TROP2*
CCNB1	*CTLA4*	*GNLY*	*KRT5*	*ORMDL3*	*TRPV6*
CCNB2	*CX3CL1*	*GPNMB*	*KRT6B*	*PDCD1*	*TSPAN13*
CCND1	*CXCL13*	*GPR160*	*KYNU*	*PGR*	*TTK*
CCNE1	*CXCL8**	*GRB7*	*LAX1**	*PHGDH*	*TYMS*
CD19	*CXCL9*	*GSDMB*	*LGALS9*	*PIM2**	*UBE2C*
CD2	*CXCR6*	*GZMA*	*LY9*	*PNMT*	*UBE2T*
*CD27**	*CXXC5*	*GZMB*	*MAGED2*	*POU2AF1**	*XBP1*
CD274	*DGKD*	*HLA-C**	*MAPT*	*PSMD3*	*ZNF552*
CD3D	*DNAJC12*	*ID4*	*MDM2*	*PTTG1*	*ACTB*
CD3G	*DNALI1*	*IGJ**	*MELK*	*PUM1*	*MRPL19*
CD4	*E2F1*	*IGKC**	*MFSD2A*	*RAD51*	*PSMC4*
CD40	*EAF2*	*IGL**	*MIA*	*RB1*	*RPLP0*
CD68	*EGFR*	*IGLV3-25**	*MID1*	*RRAGA*	*SF3A1*

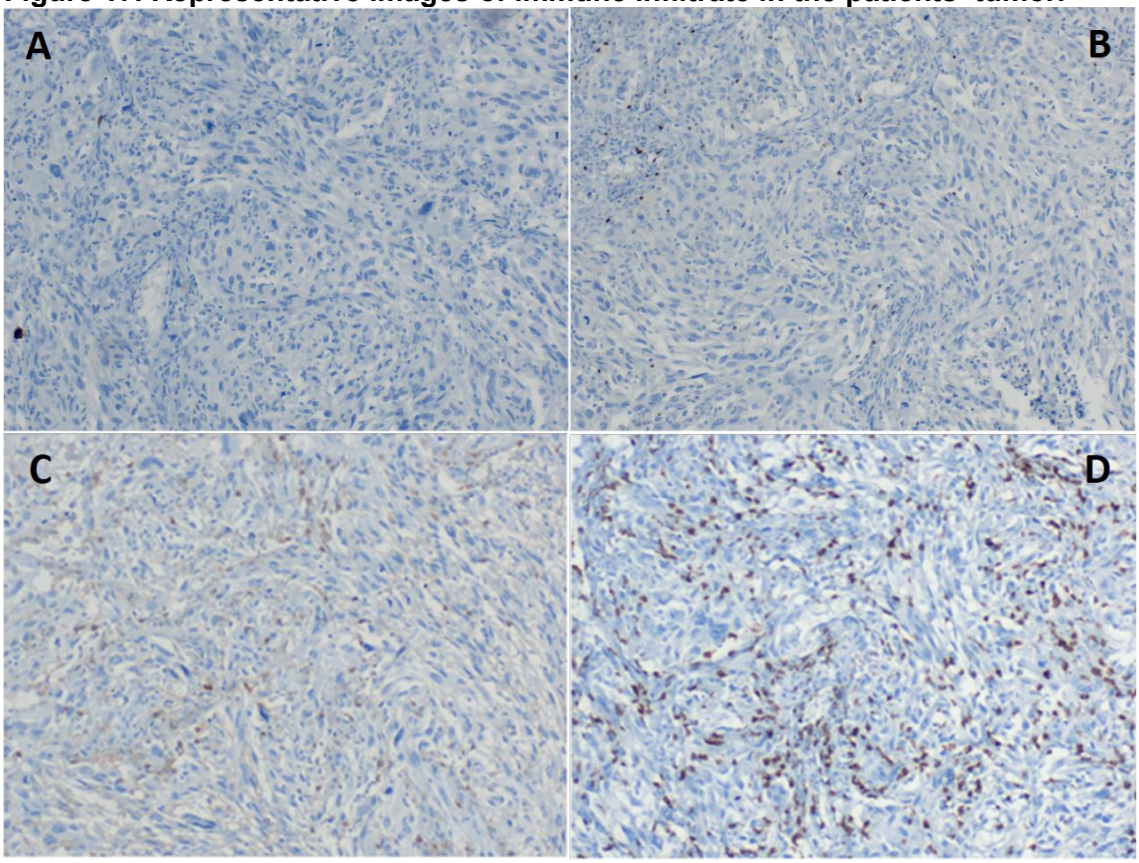
Legend. *: identifies the 14 genes integrating the immunoglobulin G (IGG) signature. These genes are implicated in the maturation of T and B lymphocytes progenitors (*IL2RG*), CD4+ and B lymphocytes activation and survival (*CD27, TNFRSF17, PIM2*), B lymphocytes differentiation in germinal centers (*POU2AF1*), immunoglobulin production (*CD79a, IGJ, IGKC, IGL, IGLV3-25*), chemotaxis (*CXCL8, NTN3*), and regulation of B, T and NK lymphocytes activity (*LAX1, HLA-C*)(66).

The mean mRNA levels for IGG-related genes and of all immune genes taken together were higher than mean mRNA levels of all the 192 genes included in the research-based PAM50 codeset (ANOVA p=0.029) (Figure 16D). The relative transcript abundance of

the IGG signature and of all immune genes together corresponded to the 72nd and 58th percentile of the entire codeset, respectively.

PD-L1 was evaluated using immunohistochemistry (IHC) 22C3 pharmDx (Agilent, Santa Clara, CA, USA). PD-L1 was positive with a combined positive score (CPS) of 70%. Additionally, we explored the tumor microenvironment in the primary tumor through IHC and found a high CD4+ and CD8+ T cell infiltration with low CD20+ (B cells) and FOXP3+ Tregs infiltration (Figure 17). No tertiary lymphoid structures (TLS) were observed.

Figure 17. Representative images of immune infiltrate in the patients' tumor.



Legend. (A) CD4₁ T-helper lymphocytes; (B) CD8₁ cytotoxic T lymphocytes; (C) CD20₁ B lymphocytes; (D) FOXP31 regulatory T lymphocytes (Tregs). All pathology images are magnified at 403. 1, positive; Tregs, regulatory T lymphocytes.

4.4.2.4 Discussion

UPS is a rare and difficult-to-treat solid tumor with limited therapeutic options(160). Recently, several phase 2 studies investigated the outcomes of anti-PD1/PD-L1 ICI in patients with advanced sarcoma(160,167,168). Responses were observed only for some histological subtypes, including UPS, with Tawbi et al. reporting a 40% objective response rate to anti-PD1(167). Other studies showed similar results, suggesting that a subset of UPS patients may respond to immune checkpoint blockade(169,170).

Nevertheless, the PEMBROSARC phase II trial demonstrated that in an unselected population, the clinical benefit of ICI was extremely limited, with a 6-month non-progressing rate of 4.9% (95% confidence interval [CI]:0.6–16.5) and overall response rate of 2.4% (95%CI:0.1–12.9)(168). However, results were impressive when intratumor TLS were observed(168). These evidences suggest that ICI can be beneficial to sarcoma patients, including UPS, but correct biomarker identification is essential(171). In fact, in our case, no TLS was observed at baseline, despite impressive response to ICI.

It has been reported that UPS present with a high expression of genes related to both antigen presentation and T-cell-mediated immunity, and is among the most mutated STS subtypes, suggesting that it may be well suited to treatment with ICI(172). Unfortunately, we could not measure the TMB of our patient's tumor, which is an established biomarker of response to ICI with anti-PD-L1 pembrolizumab(173). However, not many genomic mutations were found, nor were observed alterations clearly associated to immunotherapy benefit. Conversely, the presence of a *BRAF* G469V and *NRAS* G12S mutations suggested a possible hyperactivation of the RAS/MAPK pathway, which usually confers poor prognosis in UPS(174). An *FGFR4* mutation was observed, as well (i.e. *FGFR4* W460Ter), which is a driver gene for rhabdomyosarcomas(175). Despite these potentially unfavorable mutations, our patients showed an unexpected and impressive response to ICI with a progression-free interval of more than 5 years and a durable CR, which is quite unusual for such disease.

Interestingly, our patient received WBRT one month before starting anti-PD-L1 treatment. This approach could have increased the permeability of the blood-brain barrier and improved the brain metastasis response, as suggested from studies conducted in other tumor types. This combined approach might thus merit further evaluation in wider casuistries also in the context of UPS (54,176,177).

To note, the patient had good prognosis according to both basal LIPI score and dNLR, confirming that these scores provide a valuable prognostic information in patients with solid tumors treated with immunotherapy, as we and others reported(49,54,164).

It has to be noted that high PD-L1 protein levels were observed at baseline. This biomarker has been associated to response to ICI directed against the PD1/PD-L1 axis in multiple trials(113,178,179), a predictive potential that seems to find confirmation in our case. However, PD-L1 is a suboptimal biomarker, since different and not interchangeable assays and methodologies for assessment are available, with different indications depending on the tumor and leading to different ICI prescriptions(154,178). Moreover, several meta-analyses led to opposite conclusions (180). Noteworthy, PD1

mRNA levels were also considered high, if taking into account the cut-off for prediction of anti-PD1 ICI benefit recently established in a pan-cancer context(87,113). This biomarker has the advantage over PD-L1 to be detectable with a standardized and high reproducible methodology and might be applied potentially in all solid tumors. In our case, it successfully predicted anti-PD-L1 benefit. Hence, we believe that further confirmation of its predictive potential should be pursued, also in the context of patients treated with anti-PD-L1 ICI.

Interesting from a biologic perspective is the finding that peripheric B lymphocytes levels increased during the treatment and basal levels of the IGG immune signature were higher than overall mean gene expression. This signature seems to reflect adaptive immune response activation mostly associated to B cell response and immunoglobulin production and was associated with more favorable outcomes in the aggressive triple negative breast cancer subtype(181). Interestingly, we recently observed in a publicly available dataset from The Cancer Genome Atlas (TCGA) that the IGG signature was associated to better OS in STS (hazard ratio [HR]: 0.78, 95%CI: 0.62-0.97, p=0.029) (182). Another study showed that TLS enriched in B cells in sarcoma's microenvironment are associated to better prognosis and response to immunotherapy(128), though in our case there were no TLS in baseline tumor tissue. Overall our case, along with these findings, suggest that anti-PD-L1 ICI are an effective treatment option in UPS and that B cell immunity is likely responsible for the antitumoral effect of this therapeutic approach in this disease. Moreover, B cells can contribute to the upregulation of T cell responses. In our patient's tumor microenvironment, high cytotoxic T cell infiltration was observed, with reduced Tregs infiltrates, usually negative regulators of antitumoral immune responses(183), consistently with the recent report from a sub-cohort of the PEMBROSARC trial(184). Whether this might be a proxy for tumor immune sensitivity should be further clarified.

Finally, while B cells infiltrates were not extensive at baseline, circulating B lymphocytes progressively increased throughout the treatment, raising the question of whether B cells levels might represent a good tool to monitor therapeutic response. Unfortunately, we had no available posterior biopsy to evaluate potential treatment-induced modifications in the tumoral immune infiltrate and correlate B lymphocyte levels through time and TLS in the tumor microenvironment, which have been elsewhere associated with response to ICI in sarcoma(128).

4.4.2.5 Conclusions

Despite being a poor prognostic disease, metastatic UPS can be successfully treated with immunotherapy interfering with the PD1/PD-L1 axis. The correct selection of optimal candidates for such a therapeutic approach is imperative, considering the high costs and potential life-threatening toxicities associated to immune-checkpoint blockade(185,186). In this perspective PD-L1 levels or PD1 mRNA at baseline might be useful to identify candidates. Also, the role of WBRT to increase the therapeutic response to ICI in UPS with brain metastasis should be assessed. Considering the prognostic role and the potential association between response to ICI and B cell immunity, the role of baseline IGG signature merits further exploration to define its role as predictor of response to anti-PD1/PD-L1 inhibitors. Similarly, the role of peripheric B lymphocyte levels as a tool to monitor antitumor response also merits further evaluation in prospective wider casuistries.

Chapter 4.5 SECONDARY OBJECTIVE 3: Correlation of published prognosis scores and early dynamics with ICI outcomes.

This objective was explored and published in the Cancer Inmunology, Immunotherapy in 2025. Data data cut-off was 22nd December 2023 at that time. Full article is in Appendix 1.

Original article reference is:

Javier García-Corbacho, Alberto Indacochea, Iván Victoria, Débora Moreno, Laura Angelats, Azucena E. González Navarro, Laura Mezquita, Fara Brasó-Maristany, Patricia Galván, Begoña Mellado, Nuria Viñolas, Tamara Sauri, Miquel Nogué, Barbara Adamo, Joan Maurel, Estela Pineda, Lydia Gaba, Oscar Reig, Neus Basté, Esther Sanfeliu, Manel Juan, Aleix Prat, Francesco Schettini. Blood-based prognostic scores and early dynamics under immunotherapy to select patients with metastatic solid tumors for continuing immune check-point inhibition: a prospective longitudinal study Cancer Immunology, Immunotherapy, 1st February 2025. 74:85

DOI: 10.1007/s00262-024-03933-w

Abstract

Introduction Immune check-point inhibitors (ICI) were a major breakthrough in cancer care, but optimal patient selection remains elusive in most tumors.

Methods Overall 173 adult patients with metastatic solid tumors candidates to ICI in clinical trials at our Institution were prospectively recruited. Blood samples were collected at cycle 1 (C1D1) and 2 (C2D1) and until the occurrence of progressive disease (PD). C1D1 LIPI, RMH, PMHI, NLR, dNLR, PIPO and GRIm prognostic scores were calculated. The primary endpoint was identifying the best score to predict rapid PD (≤ 4 months) with ICI using logistic regressions accounting for tumor type, and receiving operators characteristics (ROC) with area under curve (AUC), accompanied by an extensive comparison of the score performances in the prediction of overall survival (OS), progression-free survival (PFS), overall response rates (ORR) and durable clinical benefit (DCB). Secondary objectives included describing study cohort outcomes and studying the association between the selected score at C1D1, C2D1 and its dynamics with OS and PFS.

Results C1D1 LIPI was the best predictor of rapid PD, OS and PFS, regardless of cancer type, compared to other scores. No score was associated to ORR and only RMH to DCB. Baseline LIPI detected three categories of patients with significantly different OS ($p < 0.001$) and PFS ($p = 0.013$). The same was observed at C2D1 for OS and PFS (both $p = 0.020$). Significant LIPI class shifts were observed in the overall population ($p < 0.001$), rapid progressors ($p = 0.029$) and non-rapid progressors ($p = 0.009$). Retaining a good LIPI or experiencing a shift towards a better prognostic class was associated to improved OS ($p = 0.009$) and PFS ($p = 0.006$). C2D1 LIPI, but not C1D1, remained significantly associated to rapid PD in multivariable analysis.

Conclusions LIPI may improve patient selection for ICI and guide treatment adjustments according to on-treatment dynamics in a pancancer context.

4.5.1 Introduction

Immunotherapy with immune check-point inhibitors (ICI) has represented a major breakthrough for the treatment of solid malignancies in the last decade (5). However, ICI treatment efficacy is extremely heterogeneous and unpredictable (21,187) and may lead to harmful and potentially lethal immune-mediated side effects. Therefore, the identification of proper biomarkers of response is crucial to improve therapeutic outcomes, avoid unnecessary toxicities and optimize resources. In our initial publication we analyzed the impact of clinicopathological characteristics in ICI outcomes and highlighted the potential of LIPI score(113). Following this evidence, we decided to explore the role of the prognosis scores further so we continued recruiting patients and evaluating their tumoral responses and updated the progression free survival and overall survival of our population after a longer follow up period.

In this report, we assessed clinical outcomes in the entire study cohort, compared the most relevant baseline prognostic scores developed for selecting or stratifying candidates to ICI and/or phase I trial across different tumor types (38,49,59–63,117,188,189) to pick the best one in predicting rapid progression to ICI, and further assess the contribution of the most successful score's early dynamics to detect rapid progressors and improve patient selection for continuing immune check-point inhibition.

4.5.2 Materials and methods

Study design and participants

To participate in the study, eligible patients had an advanced solid cancer and a scheduled initiation of an ICI-based treatment. Full inclusion/exclusion criteria were previously reported in chapter 3: Methodology (113). Patients were recruited between November 2016 and March 2022 and followed-up until 22nd December 2023. We considered evaluable for this analysis all participants treated with an ICI with radiological data available for an independent assessment of tumor responses according to the tumor type.

Procedures

A blood sample was collected from each patient at the first day of cycle 1 (C1D1) and 2 (C2D1) prior to receive the treatment and, subsequently, at each radiological evaluation until progressive disease (PD) was determined (113). For the purpose of the present analysis only blood samples at C1D1 and C2D1 were interrogated. Blood chemistry tests were carried out, including the evaluation of albumin, hemoglobin (Hb), lactate dehydrogenase (LDH) and standard leukocyte populations. The use of antibiotics (ATB)

and/or corticoids during ICI was assessed. C1D1 samples were used for the election of the best prognostic predictor among the most commonly used, namely the Lung Immune Prognostic Index (LIPI), Royal Marsden Hospital (RMH), Princess Margaret Hospital Index (PMHI), neutrophil-to-lymphocyte ratio (NLR), derived NLR (dNLR), Phase I Prognostic Online (PIPO) and Gustave Roussy Immune (GRIm) prognostic scores (38,49,59–63,117,188,189). C2D1 samples were also analyzed to assess the best score's dynamics between C1D1 and C2D1. Treatment decisions were made outside of this study according to trial protocol and investigators criteria. All data were retrieved from electronic patient charts.

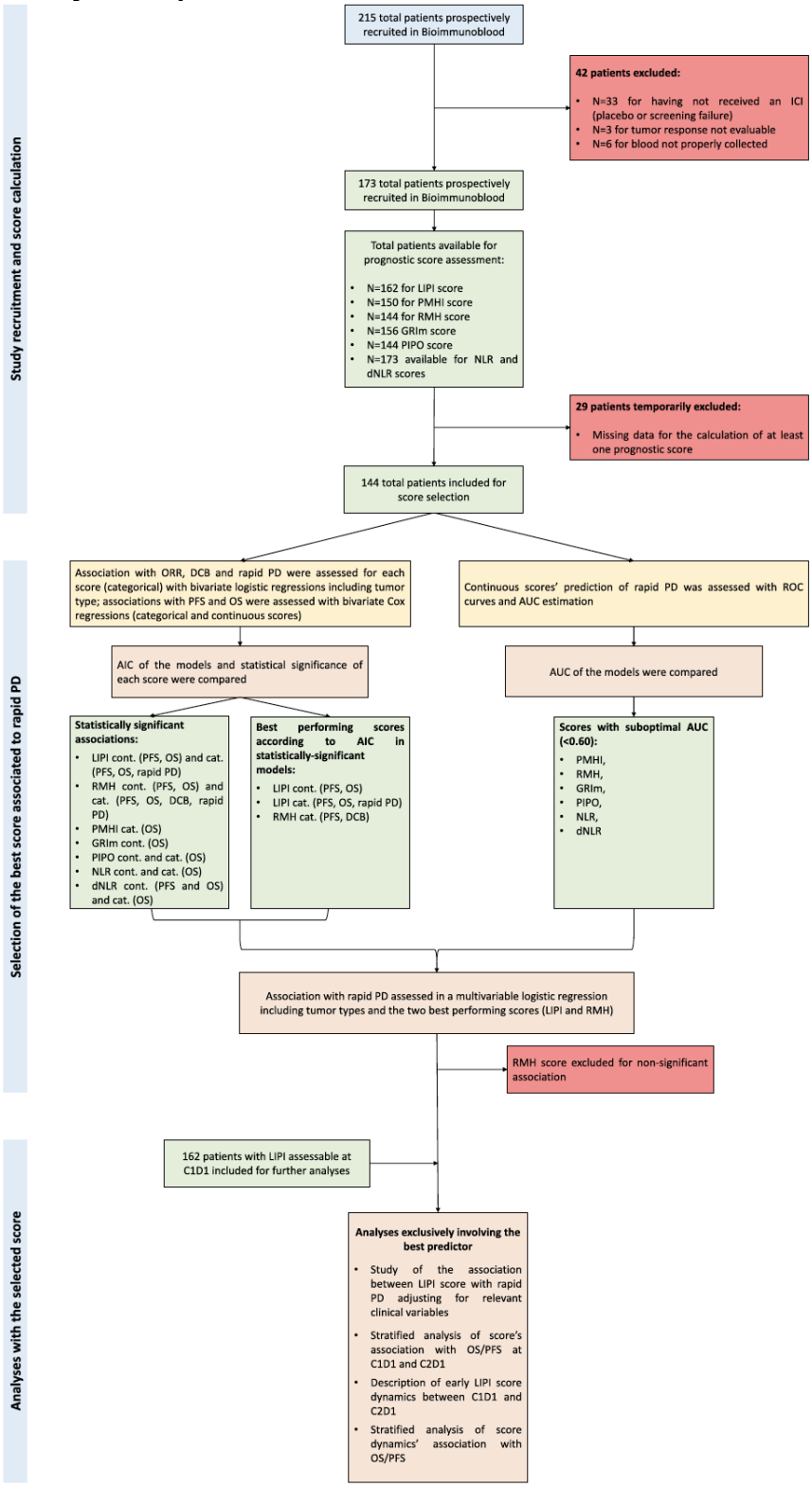
Study endpoints and outcomes

There was no prespecified sample size because of the exploratory nature of this study. The clinical data cut-off was established when a minimum follow-up including at least one reassessment of the disease for every included patient was reached.

The primary objective of this analysis was to provide an extensive comparison of the prognostic scores in terms of prediction of overall survival (OS), progression-free survival (PFS), overall response rates (ORR) and durable clinical benefit (DBC) with ICI, and identify the best score predicting rapid PD to ICI. The key secondary objective was a more refined assessment of the association between the selected best score at C1D1 with OS, PFS, ORR and DBC by accounting for relevant confounding factors. Other secondary objectives were the assessment of the association between the selected score at C2D1 with PFS and at C2D1 with OS, the evaluation of the best score's category changes from C1D1 to C2D1 as well as the evaluation of the association of the best score's dynamics with PFS and OS.

PFS was defined as the time from C1D1 to PD or death from any cause, whichever occurred first. OS was defined as the time from C1D1 to death from any cause. Rapid PD was defined as PFS≤4 months from ICI initiation. This cut-off was determined by the study authors as the minimum clinically acceptable benefit achievable with ICIs, taking into account the balance between the potential treatment toxicity, costs, potential need to detect pseudo-progressions and the prognosis of advanced solid tumors (190). The evaluation of response was performed in accordance to RECIST 1.1 criteria(131), or RANO criteria in the case of glioblastomas (GB) (132). Best overall responses (BOR) were classified as PD, stable disease (SD), complete (CR) or partial response (PR) by the same expert (Dr. García-Corbacho). ORR included all patients achieving CR or PR as BOR. DCB was defined as absence of PD at 6 months(113). The score selection process and main study analyses are resumed in Figure 18.

Figure 18. Study description.



Legend. AIC: Akaike information criterion; AUC: ROC's area under curve; C: treatment cycle; D: day; dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score; N: number of patients; NLR: neutrophil to lymphocyte ratio; LIPI: Lung Immune Prognostic Index; OS: overall survival; PD: progression of the disease; PFS: progression-free survival; PMHI: Princess Margaret Hospital Index; PIPO: Phase I Prognostic Online; RMH: Royal Marsden Hospital; ROC: receiving operator characteristics

Statistical analysis

When appropriate, χ^2, Kruskal-Wallis and Wilcoxon rank-sum tests, for unpaired variables, and McNemar and Wilcoxon signed-rank tests, for paired variables, were used to calculate differences between patients with different prognostic score classes and rapid vs. non-rapid PD. Bivariate logistic regression analyses were performed to estimate the odds ratios (OR) with their 95% confidence intervals (CI) to investigate the association of the prognostic scores at C1D1 with rapid PD, overall response rates (ORR) and durable clinical benefit (DCB). Bivariate Cox proportional hazard models including tumor type and each score at C1D1 were used to estimate hazard ratios (HR) with their 95% CI to explore associations with PFS and OS. Patients alive were censored at the date of the last follow-up. Akaike information criterion (AIC) was used to compare the goodness of fit among multivariable regression models (191). A difference of more than 2 points between models was considered significant, and the model with the lowest AIC was considered to be the best (133). Receiving operator characteristics (ROC) curves and their area under curve (AUC) were then used for each score to assess the capability of predicting rapid PD. AUCs were then compared with the DeLong test. After identifying the score with the best performance on all endpoints and the best capability of predicting rapid PD, Cox proportional hazard models stratified for selected confounders were then used to explore the association of the best score at C1D1, C2D1 and score dynamics between C1D1-C2D1 with PFS and OS. Multivariable logistic regressions accounting for the same confounders were carried out to assess the best score at C1D1, C2D1 and C1D1-C2D1 dynamics with rapid PD. The proportional hazard assumption was properly checked (133) for both OS and PFS. Survival curves were estimated by the Kaplan-Meier method and differences between curves were evaluated by the log-rank test. Landmark analyses to assess 12-month and 24-month OS and PFS according to the best score classes were conducted, as well. No imputation was done for missing data. A two-sided alfa error of 0.05 was considered for statistical significance.

All statistical analyses were carried out using R Studio vers.1.0.153 (PBC, Boston, MA) and SPSS vers. 24.0 (IBM SPSS Statistics, Armonk, NY: IBM Corp) for MacOSX.

4.5.3 Results

Population characteristics, risk stratification and outcomes

A total of 173 patients were included. Population demographics at baseline are reported in Table 28.

Table 28. Study population characteristics

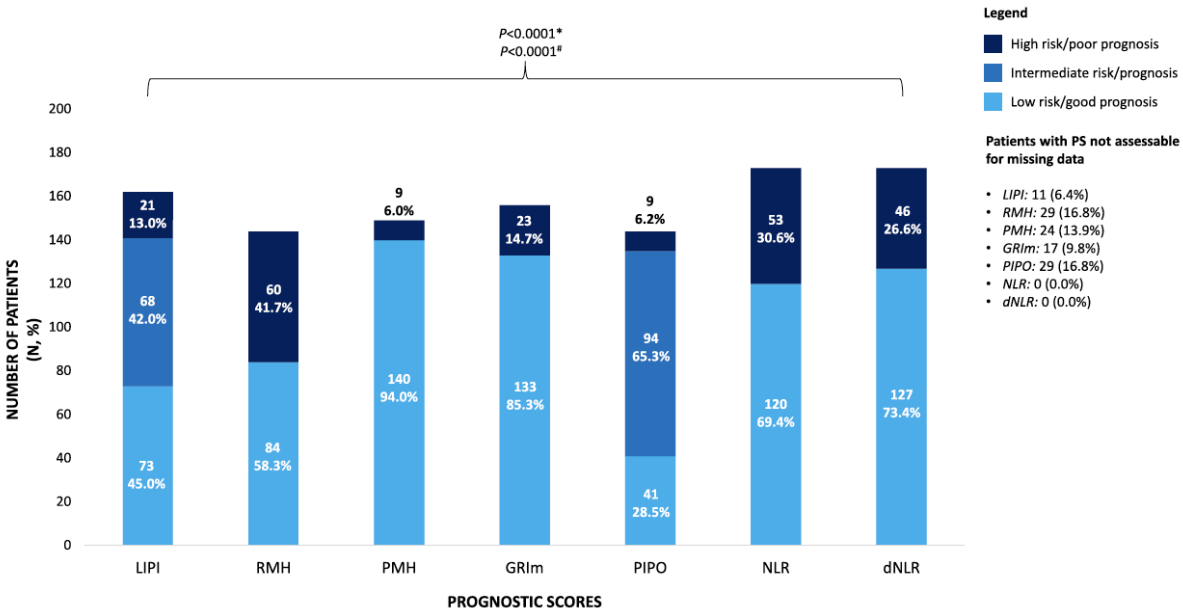
DEMOGRAPHICS	OVERALL	
	N	%
	173	100.0
Age		
Median	64	-
IQR	56.8 - 71.5	-
Sex		
Male	108	62.4
Female	65	37.6
Overall	*173*	*100.0*
ECOG		
0-1	153	88.4
≥2	20	11.6
Overall	*173*	*100.0*
Tumor type		
Breast cancer	15	8.7
Colorectal adenocarcinoma	33	19.1
NSCLC	47	27.2
Head and neck	10	5.8
Gynecologic tumors (Cervix, endometrium, ovary)	9	5.2
Pancreas and biliary tract tumors	6	3.5
Esophageal and gastric carcinoma	9	5.2
Melanoma	9	5.2
Prostate adenocarcinoma	8	4.6
Renal cell carcinoma	7	4.0
Urothelial bladder cancer	6	3.5
Glioblastoma	8	4.6
Other*	6	3.5
Overall	*173*	*100.0*
Number of metastatic sites		
<3	34	19.7
≥3	139	80.3
Overall	*173*	*100.0*
Metastatic involvement		
Visceral	139	80.3
CNS§	10	5.8
Overall	*173*	*100.0*
RT≤30 days from ICI start		
Yes	10	5.8

	No	162	94.2
	Overall	*172*	99.4
Systemic ATB≤30 days from ICI start			
	Yes	9	5.2
	No	164	94.8
	Overall	*173*	100.0
Systemic ATB during ICI			
	Yes	53	30.6
	No	120	69.4
	Overall	*173*	100.0
Systemic corticosteroids ≤30 days from ICI start			
	Yes	23	13.3
	No	150	86.7
	Overall	*173*	100.0
Systemic corticosteroids during ICI			
	Yes	67	38.7
	No	106	61.3
	Overall	*173*	100.0
ICI treatment line			
	1st	46	26.6
	2nd	54	31.2
	≥3rd	73	42.2
	Overall	*173*	100.0
ICI type			
	Anti-PD1	131	75.7
	Anti-PD-L1	32	18.5
	Other	10	5.8
	Overall	*173*	100.0
Regimen type			
	ICI monotherapy	92	53.2
	ICI combination	36	20.8
	ICI+other agent	45	26.0
	Overall	*173*	100.0
Previous immunotherapy in every setting			
	Yes	139	80.3
	No	34	19.7
	Overall	173	*100.0*

Legend. PS: performance status; IQR: interquartile range; NSCLC: non-small cell lung cancer; ATB: antibiotics; RT: radiotherapy; CNS: central nervous system; ICI: immune-checkpoint inhibitor. *: thymic carcinoma, Merkel cell carcinoma, carcinomas of unknown primary site, soft tissue sarcomas, adrenal gland adenocarcinoma, hepatocarcinoma; #: 2 patients received ICI in 1st or 2nd line, but the precise information was not reported in our records; §: excluding glioblastomas.

The baseline prognostic stratification provided by the selected prognostic scores is reported in **Figure 19**. The proportion of patients pertaining to the same prognostic category according to the different predictors varied significantly (p<0.001).

Figure 19. Prognostic stratification of study population according to different prognostic scores at baseline.



Legend. dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score LIPI: Lung Immune Prognostic Index; NLR: neutrophil-to-lymphocyte ratio; PD: progression of the disease; PIPO: Phase I Prognostic Online; PMHI: Princess Margaret Hospital Index; RMH: Royal Marsden Hospital.

At a median follow-up of 38.3 months (95%CI: 36.1-58.6), median PFS (mPFS) was 2.5 months (95%CI: 2.0-3.7) and median OS (mOS) was 13.3 months (95%CI: 9.9-17.4), with rapid PD or death ≤4 months from ICI initiation observed in 60.7% (95%CI: 53.0%-68.0%) cases. Objective responses were scarce, with an ORR of 16.8% (95%CI: 11.5%-23.2%). Main outcomes are detailed in **Table 29**.

Table 29. Activity and efficacy of ICI in the overall study population

STUDY POPULATION OUTCOMES		
PD timing	**N**	**%**
≤4 months from ICI start	105	60.7
>4 months from ICI start	68	39.3
Best response	**N**	**%**
Complete response	7	4.0
Partial response	22	12.7
Stable disease	59	34.1
Progressive disease	85	49.1
ORR	**%**	**95%CI**
CR+PR	16.8	11.5% - 23.2%
DCB	**%**	**95%CI**
CR+PR+SD≥6 months	50.9	43.2% - 58.5%
Median PFS (months)	**N**	**95%CI**
	2.5	2.0 - 3.7
Median OS (months)	**N**	**95%CI**
	13.3	9.9 - 17.4
12-month PFS	**N (%)**	**95%CI**
	32 (18.5)	13.5% - 25.3%
12-month OS	**N (%)**	**95%CI**
	88 (50.9)	45.0% - 60.0%

Legend. CI: confidence interval; CR: complete response; DCB: durable clinical benefit; ICI: immune-checkpoint inhibitor; N: number; ORR: overall response rate; PD: disease progression; PR: partial response; PFS: progression-free survival; SD: stable disease; OS: overall survival.

Identification of the best score predicting PFS, OS, ORR, DCB and rapid PD

We subdivided the study cohort into rapid progressors (RP) (PFS≤4 months from ICI initiation) and non-rapid progressors (NRP) (PFS>4 months from ICI initiation). The groups differed significantly in several baseline clinicopathological factors, as reported in **Table 30**.

Table 30. Baseline clinicopathological features according to rapid tumor progression status

DEMOGRAPHICS		RAPID PROGRESSORS		NON-RAPID PROGRESSORS		*P*
		N	%	N	%	
		105	60.7	68	39.3	
Age						
	Median	62.6	-	67.1	-	*0.030*
	IQR	54.2 - 69.0	-	60.5 - 72.6	-	
Sex						
	Male	61	58.1	47	69.1	
	Female	44	41.9	21	30.9	0.144
	Overall	105	100.0	68	100.0	
ECOG						
	0-1	94	89.5	59	86.8	
	≥2	11	10.5	9	13.2	0.579
	Overall	105	100.0	68	100.0	
Tumor type						
	Breast cancer	12	11.4	3	4.4	
	Colorectal adenocarcinoma	23	21.9	10	14.7	
	NSCLC	22	21.0	25	36.8	
	Head and neck	7	6.7	3	4.4	
	Gynecologic tumors (Cervix, endometrium, ovary)	5	4.8	4	5.9	
	Pancreas and biliary tract tumors	4	3.8	2	2.9	
	Esophageal and gastric carcinoma	6	5.7	3	4.4	*0.031*
	Melanoma	7	6.7	2	2.9	
	Prostate adenocarcinoma	0	0.0	8	11.8	
	Renal cell carcinoma	4	3.8	2	2.9	
	Urothelial bladder cancer	5	4.8	2	2.9	
	Glioblastoma	6	5.7	2	2.9	
	Other*	4	3.8	2	2.9	
	Overall	105	100.0	68	100.0	
Number of M1 sites						
	<3	21	20.0	13	19.1	
	≥3	84	80.0	55	80.9	0.887
	Overall	105	100.0	68	100.0	
Metastatic involvement						
	Visceral	86	81.9	53	77.9	
	Non-visceral	19	18.1	15	22.1	0.522
	Overall	105	100.0	68		
	CNS metastases	6	6.1	4	6.1	
	No CNS metastases	93	93.9	62	93.9	1.000
	Overall§	99	94.3	66	97.1	

RT≤30 days from ICI start						
	Yes	6	5.8	4	5.9	
	No	98	94.2	64	94.1	0.975
	Overall	104	99.0	68	100.0	
Syst ATB≤30 days from ICI start						
	Yes	5	4.8	4	5.9	
	No	100	95.2	64	94.1	0.746
	Overall	105	100.0	68	100.0	
Systemic ATB during ICI						
	Yes	24	22.9	29	42.6	
	No	81	77.1	39	57.4	*0.006*
	Overall	105	100.0	68	100.0	
Syst corticosteroids ≤30 days from ICI start						
	Yes	13	12.4	10	14.7	
	No	92	87.6	58	85.3	0.660
	Overall	105	100.0	68	100.0	
Syst corticosteroids during ICI						
	Yes	28	26.7	39	57.4	
	No	77	73.3	29	42.6	*<0.001*
	Overall	105	100.0	68	100.0	
ICI treatment line						
	1st	21	20.0	25	36.8	
	2nd	33	31.4	20	29.4	*0.044#*
	≥3rd	51	48.6	23	33.8	
	Overall	105	100.0	68	100.0	
ICI type						
	Anti-PD1	78	74.3	54	79.4	
	Anti-PD-L1	18	17.1	14	20.6	*0.044*
	Other	9	8.6	0	0.0	
	Overall	105	100.0	68	100.0	
Regimen type						
	ICI monotherapy	54	51.4	38	55.9	
	ICI combination	28	26.7	8	11.8	*0.043*
	ICI+other agent	23	21.9	22	32.4	
	Overall	105	100.0	68	100.0	
Previous ICI						
	Yes	81	77.1	58	85.3	
	No	24	22.9	10	14.7	0.188
	Overall	105	100.0	68	100.0	

Legend. ICI: immune-checkpoint inhibitors; ATB: antibiotics; NSCLC: non-small cell lung cancer; CNS: central nervous system; IQR: interquartile range. *thymic carcinoma, Merkel cell carcinoma, COD, STS, adrenal gland adenocarcinoma, hepatocarcinoma; M1: metastatic; Syst: systemic; §: excluding glioblastomas; #: chi-square for variable dichotomized in 1st and ≥2nd line.

We explored which was the best prognostic predictor among LIPI, RMH, PMHI, NLR, dNLR, PIPO and GRIm scores in terms of PFS and OS. First, we removed from this analysis all patient with at least one of the scores not assessable, in order to avoid a biased comparison between scores due to their different populations. Following this selection we reached a total population of 144 patients. We run multiple bivariate Cox regression models each one including one of the scores as continuous or categorical variable and tumor types. Only LIPI (p=0.001), dNLR (p=0.024) and RMH (p=0.006) continuous scores, and LIPI (p=0.002) and RMH (p=0.001) categorical scores were significantly associated to PFS, regardless of tumor type (Table 31).

Table 31. Statistically significant bivariate Cox regressions for the association of prognostic scores with PFS and OS

Prognostic scores	Adj PFS HR*	Inf 95%CI	Sup 95%CI	*P*	Prognostic scores	Adj OS HR*	Inf 95%CI	Sup 95%CI	*P*
LIPI continuous score					*LIPI continuous score*				
	1.67	1.25	2.23	0.001		2.06	1.51	2.82	<0.001
RMH continuous score					*RMH continuous score*				
	1.49	1.12	1.98	0.006		1.54	1.13	2.09	0.005
dNLR continuous score					*GRIm continuous score*				
	1.16	1.02	1.33	0.024		1.50	1.07	2.10	0.006
					NLR continuous score				
-	-	-	-	-		1.08	1.01	1.16	0.025
					dNLR continuous score				
-	-	-	-	-		1.32	1.14	1.51	<0.001
					PIPO continuous score				
-	-	-	-	-		1.24	1.02	1.51	0.030
LIPI				0.002	*LIPI*				<0.001
Intermediate vs. good	1.59	1.07	2.37	0.021	Intermediate vs. good	1.84	1.20	2.84	0.006
Poor vs. good	2.87	1.52	5.42	0.001	Poor vs. good	4.69	2.42	9.09	<0.001
Poor vs. intermediate	1.80	0.97	3.35	0.064	Poor vs. intermediate	2.54	1.35	4.79	0.004
RMH					*RMH*				
Poor vs. good	1.92	1.31	2.82	0.001	Poor vs. good	2.10	1.41	3.15	<0.001
					PMHI				
-	-	-	-	-	Poor vs. good	2.61	1.02	6.68	0.046
					NLR				
-	-	-	-	-	High vs. low	1.65	1.07	2.54	0.023
					dNLR				

-	-	-	-	-	High vs. low	2.31	1.44	3.69	<0.001
					PIPO				0.033
-	-	-	-	-	Intermediate vs. low	0.86	0.55	1.34	0.502
					High vs. low	2.46	1.07	5.67	0.034
-	-	-	-	-	High vs. intermediate	2.87	1.30	6.34	0.009

Legend and footnotes. CI: confidence interval; dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score; HR: hazard ratio; Inf: inferior; LIPI: Lung Immune Prognostic Index; NLR: neutrophil-to-lymphocyte ratio; OS: overall survival; PIPO: Phase I Prognostic Online; PMHI: Princess Margaret Hospital Index; PFS: progression-free survival; RMH: Royal Marsden Hospital; Sup: superior. *adjusted for cancer type.

The best AIC was observed for the model including continuous LIPI score (AIC: 1085.70). Similar AIC was observed for the models including categorical LIPI (AIC: 962.59) and RMH (AIC: 968.62). LIPI (p<0.001), NLR (p=0.025), dNLR (p<0.001), RMH (p=0.005), GRIm (p=0.006) and PIPO (p=0.030) continuous scores were significantly associated to OS independently of tumor type. Similarly, LIPI (p<0.001), NLR (p=0.023), dNLR (p<0.001), RMH (p<0.001), PMHI (p=0.046) and PIPO (p=0.033) categorical scores were significantly associated to OS (Table 31). The bivariate model including LIPI showed the best AIC both for the continuous (AIC: 961.12) and categorical (AIC: 962.59) score. No score was significantly associated to ORR (not shown) and only RMH was marginally associated to DCB (adjusted OR [aOR] for high score vs. low: 0.47, 95%CI: 0.22-1.00, p=0.049)

We then assessed the association of all categorical scores with rapid PD in bivariate logistic regression models including tumor types. A significant and independent association was observed with LIPI categorical score (p=0.003); specifically, the poor vs. good (aOR: 6.00, 95%CI: 1.47-24.53, p=0.013) and the intermediate vs. the good categories (aOR: 3.36, 95%CI: 1.48-7.62, p=0.004), without significant differences between the poor and intermediate score categories (p=0.422). RMH categorical score was also associated to rapid PD (aOR: 2.88, 95%CI: 1.29- 6.46, p=0.010), but the best AIC was observed for the LIPI score-containing model (AIC: 192.69 and 189.31, respectively). All other scores were not significantly associated to rapid PD. All statistically significant models' AIC for each endpoint are reported in Table 32.

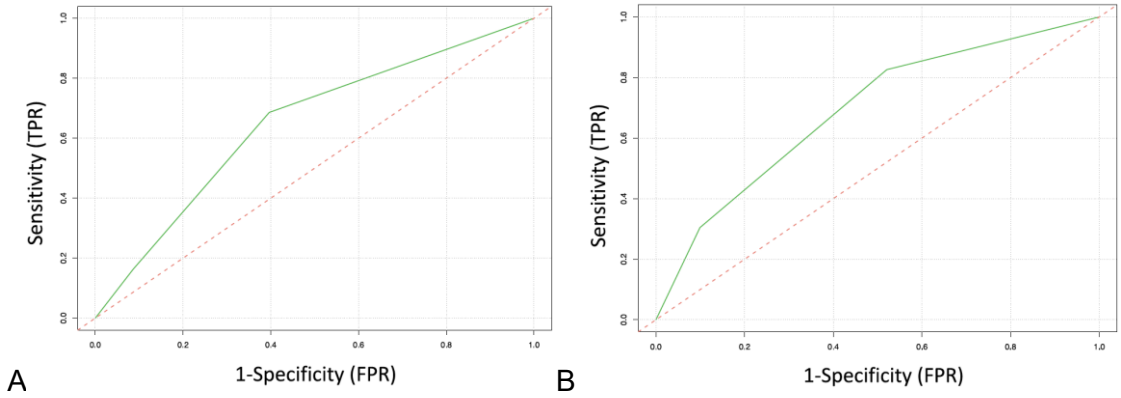
Table 32. AICs of the bivariate Cox and logistic regression models

AICs of the statistically significant bivariate Cox regression models for PFS (increasing order)	
LIPI continuous score model AIC: 1085.70. RMH continuous score model AIC: 1089.90. dNLR continuous score model AIC: 1093.07.	RMH categorical score model AIC: 1086.56. LIPI categorical score model AIC: 1087.59.
AICs of the statistically significant bivariate Cox regression models for OS (increasing order)	
LIPI continuous score model AIC: 961.12. dNLR continuous score model AIC: 968.83. RMH continuous score model AIC: 973.86. GRIm continuous score model AIC: 976.05. PIPO continuous score model AIC: 976.72. NLR continuous score model AIC: 977.10.	LIPI categorical score model AIC: 962.59. RMH categorical score model AIC: 968.62. dNLR categorical score model AIC: 970.40. NLR categorical score model AIC: 976.78. PMH categorical score model AIC: 976.69. PIPO categorical score model AIC: 977.89.
AICs of the statistically significant bivariate logistic regression models for ORR (increasing order)	
None	None
AICs of the statistically significant bivariate logistic regression models for DCB (increasing order)	
None	RMH categorical score model AIC: 204.78.
AICs of the statistically significant bivariate logistic regression models for rapid PD (increasing order)	
None	LIPI categorical score model AIC: 189.31. RMH categorical score model AIC: 192.69.

Legend. AIC: Akaike information criterion; PFS: progression-free survival; OS: overall survival; LIPI: Lung Immune Prognostic Index; RMH: Royal Marsden Hospital. NLR: neutrophil-to-lymphocyte; dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score; ratio; PIPO: Phase I Prognostic Online; PMHI: Princess Margaret Hospital Index.

The majority of the scores were unable to predict rapid PD at the ROC curve analysis, with an AUC ranging 0.51-0.60. LIPI score was the only one significantly able to predict rapid PD, with satisfactory efficacy (AUC: 0.65, 95%CI: 0.56-0.74, DeLong p<0.001) (Figure 20A). The sensitivity and specificity were 68.6% and 60.3%, respectively, for a score class intermediate or poor to be able to detect RP, meaning that out of 100 true RP, 69 could be correctly identified and out of 100 NRP, 60 were correctly detected. Notably, the performance of LIPI score improved when early deaths were the only events considered (AUC: 0.69, 95%CI: 0.56-0.82) (Figure 20B).

Figure 20. ROC curves of LIPI score as a predictor of rapid PD or rapid death.



Legend. A ROC curve of the LIPI score as a predictor of rapid PD; B ROC curve of the LIPI score as a predictor of rapid death, excluding disease progression without survival events. ROC: receiving operator characteristics.

Finally, we built a multivariable model to predict rapid PD including LIPI and RMH categorical scores and tumor types. Only LIPI score retained a significant association with rapid PD (poor/intermediate vs. good score category aOR: 3.10, 95%CI: 1.12-8.60 p=0.029).

Considering the globally superior performance of LIPI score in the previous assessments, it was ultimately selected for further analyses of its dynamics and more refined study of the associations with survival outcomes. To note, LIPI score at C1D1 was available for 162 (93.6%) patients. Baseline clinicopathological features according to LIPI score class are reported in Table 33.

Table 33. Demographics according to baseline LIPI score

	GOOD		INTERMEDIATE		POOR		
DEMOGRAPHICS	**N**	**%**	**N**	**%**	**N**	**%**	***P***
	73	45.0	68	42.0	21	13.0	
Age							
Median	63.4	-	62.5	-	69.0	-	*0.0076*
IQR	59.7 - 72.8	-	52.8 - 68.8	-	64.2 - 73.3	-	
Sex							
Male	46	63.0	42	61.8	10	47.6	
Female	27	37.0	26	38.2	11	52.4	0.4282
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
ECOG							
0-1	65	89.0	62	91.2	18	85.7	
≥2	8	11.0	6	8.8	3	14.3	0.7633
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
Tumor type							
Breast cancer	6	8.2	7	10.3	2	9.5	0.7832

Colorectal adenocarcinoma	10	13.7	17	25.0	5	23.8	
NSLCL	19	26.0	14	20.6	6	28.6	
Head and neck	4	5.5	3	4.4	1	4.8	
Gynecologic tumors (Cervix, endometrium, ovary)	3	4.1	5	7.4	1	4.8	
Pancreas and biliary tract tumors	3	4.1	3	4.4	0	0.0	
Esophageal and gastric carcinoma	4	5.5	2	2.9	3	14.3	
Melanoma	4	5.5	4	5.9	1	4.8	
Prostate adenocarcinoma	4	5.5	2	2.9	2	9.5	
Renal cell carcinoma	2	2.7	4	5.9	0	0.0	
Urothelial bladder cancer	5	6.8	2	2.9	0	0.0	
Glioblastoma	6	8.2	2	2.9	0	0.0	
Other*	3	4.1	3	4.4	0	0.0	
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
Number of metastatic sites							
<3	20	27.4	13	19.1	0	0.0	
≥3	53	72.6	55	80.9	21	100.0	*0.0217*
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
Metastatic involvement							
Visceral	52	71.2	59	86.8	17	81.0	0.0752#
CNS§	4	5.5	4	5.9	1	4.8	0.9742
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
RT≤30 days from ICI start							
Yes	4	5.5	3	4.4	1	4.8	
No	69	94.5	65	95.6	20	95.2	0.9574
Overall	*73*	*100.0*	*68*	100.0	*21*	*100.0*	
Systemic ATB≤30 days from ICI start							
Yes	5	6.8	3	4.4	0	0.0	
No	68	93.2	65	95.6	21	100.0	0.4276
Overall	*73*	100.0	*68*	*100.0*	*21*	*100.0*	
Systemic ATB during ICI							
Yes	26	35.6	17	25.0	7	33.3	
No	47	64.4	51	75.0	14	66.7	0.3813
Overall	*73*	100.0	*68*	100.0	*21*	*100.0*	
Systemic corticosteroids ≤30 days from ICI start							
Yes	11	15.1	7	10.3	4	19.0	0.5225

No	62	84.9	61	89.7	17	81.0	
Overall	*73*	100.0	*68*	100.0	*21*	*100.0*	
Systemic corticosteroids during ICI							
Yes	28	38.4	28	41.2	8	38.1	
No	45	61.6	40	58.8	13	61.9	0.9337
Overall	*73*	100.0	*68*	100.0	*21*	*100.0*	
ICI treatment line							
1st	20	27.4	15	22.1	4	19.0	
2nd	27	37.0	22	32.4	3	14.3	0.7167
≥3rd	26	35.6	31	45.6	14	66.7	
Overall	*73*	100.0	*68*	100.0	*21*	100.0	
ICI type							
Anti-PD1	58	79.5	50	73.5	13	61.9	
Anti-PD-L1	15	20.5	12	17.6	4	19.0	*0.0192*
Other	0	0.0	6	8.8	4	19.0	
Overall	*73*	100.0	*68*	100.0	*21*	100.0	
Regimen type							
ICI monotherapy	39	53.4	33	48.5	11	52.4	
ICI combination	9	12.3	21	30.9	4	19.0	0.0802
ICI+other agent	25	34.2	14	20.6	6	28.6	
Overall	*73*	100.0	*68*	100.0	*21*	100.0	
Previous immunotherapy in every setting							
Yes	60	82.2	53	77.9	15	71.4	
No	13	17.8	15	22.1	6	28.6	0.5432
Overall	73	*100.0*	68	*100.0*	21	*100.0*	
PD timing							
≤4 months from ICI start	35	47.9	49	72.1	15	71.4	
>4 months from ICI start	38	52.1	19	27.9	6	28.6	*0.0079*
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	
Best response							
CR	3	4.1	2	2.9	0	0.0	
PR	11	15.1	7	10.3	2	9.5	
SD	28	38.4	23	33.8	7	33.3	0.7418
PD	31	42.5	38	55.9	12	57.1	
Overall	*73*	*100.0*	*68*	*100.0*	*21*	*100.0*	

Legend. PS: performance status; IQR: interquartile range; NSCLC: non-small cell lung cancer; ATB: antibiotics; RT: radiotherapy; CNS: central nervous system; ICI: immune-checkpoint inhibitor. *: thymic carcinoma, Merkel cell carcinoma, carcinomas of unknown primary site, soft tissue sarcomas, adrenal gland adenocarcinoma, hepatocarcinoma; #: 2 patients received ICI in 1st or 2nd line, but the precise information was not reported in our records; §: excluding glioblastomas.

Association between LIPI at C1D1 and OS/PFS

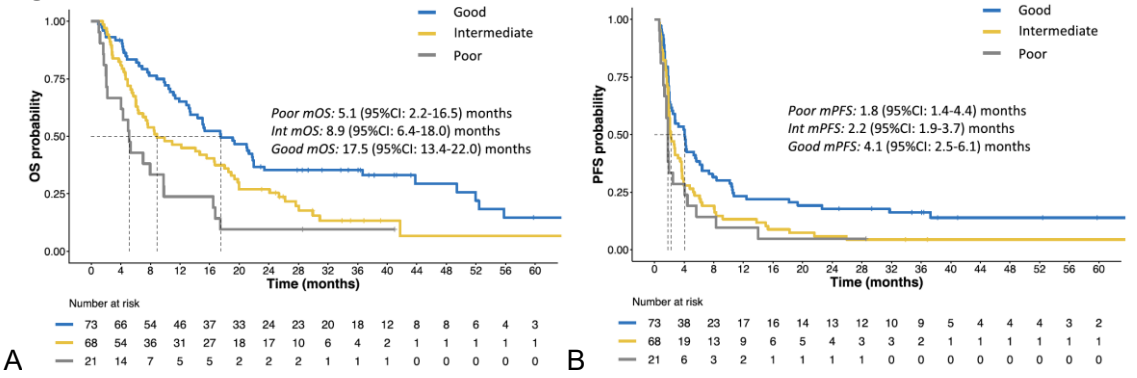
C1D1 LIPI score detected three categories of patients with significantly different OS (p<0.001), where patients with a poor (stratified HR [HR_{st}]: 2.74, 95%CI: 1.56-4.80) and intermediate score (HR_{st}: 1.63, 95%CI: 1.09-2.44) showed significantly worse OS than patients with a good score (Figure 21A) (stratified model's AIC: 719.41). Similarly, LIPI score was also prognostic in terms of PFS (p=0.013). At C1D1, the poor/intermediate scores (HR_{st}: 1.55, 95%CI: 1.10-2.18) score were associated with worse PFS than the good score (Figure 21B) (stratified model's AIC: 807.88). Landmark analyses of 12-month and 24-month OS and PFS provided consistent results (Table 34).

Table 34. Landmark analysis of PFS and OS according to LIPI score classes at different timepoints

TIMEPOINT AND LIPI CLASS			PFS RATES				OS RATES			
LIPI class	**N**	**Time point**	**N. at risk**	**12-month PFS**	**Inferior 95% CI**	**Superior 95% CI**	**N. at risk**	**12-month OS**	**Inferior 95% CI**	**Superior 95% CI**
Good	73	*C1D1*	17	23.3	15.4	35.3	46	65.0	54.8	77.1
Intermediate	68	*C1D1*	9	13.2	7.2	24.3	31	47.1	36.5	60.8
Poor	21	*C1D1*	2	9.5	2.6	35.6	5	23.8	11.1	51.2
LIPI class	**N**	**Time point**	**N. at risk**	**24-month PFS**	**Inferior 95% CI**	**Superior 95% CI**	**N. at risk**	**24-month OS**	**Inferior 95% CI**	**Superior 95% CI**
Good	73	*C1D1*	13	17.8	10.9	29.2	24	35.3	25.7	48.4
Intermediate	68	*C1D1*	4	5.9	2.3	15.2	17	27.4	18.5	40.6
Poor	21	*C1D1*	1	4.8	0.7	32.2	2	9.5	2.6	35.6
TIMEPOINT AND LIPI CLASS			**PFS RATES**				**OS RATES**			
LIPI class	**N**	**Time point**	**N. at risk**	**12-month PFS**	**Inferior 95% CI**	**Superior 95% CI**	**N. at risk**	**12-month OS**	**Inferior 95% CI**	**Superior 95% CI**
Good	54	*C2D1*	17	31.5	21.2	46.7	38	73.2	62.1	86.3
Intermediate	48	*C2D1*	6	13.0	6.2	27.5	22	48.2	35.7	65.0
Poor	12	*C2D1*	0	0.0	0.0	0.0	6	50.0	28.4	88.0
LIPI class	**N**	**Time point**	**N. at risk**	**24-month PFS**	**Inferior 95% CI**	**Superior 95% CI**	**N. at risk**	**24-month OS**	**Inferior 95% CI**	**Superior 95% CI**
Good	54	*C2D1*	12	22.2	13.5	36.6	22	44.4	32.7	60.1
Intermediate	48	*C2D1*	4	8.7	3.4	22.2	13	30.7	19.8	47.4
Poor	12	*C2D1*	0	0.0	0.0	0.0	2	16.7	4.7	59.1

Legend. CI: confidence interval; N. at risk: number of patients alive still at risk of progressing or dying at each given timepoint and according to LIPI score class; PFS: progression-free survival; OS: overall survival; C1D1: day 1 of cycle 1; C2D1: day 1 of cycle 2.

Figure 21. Kaplan–Meier curves of OS and PFS according to LIPI score at C1D1

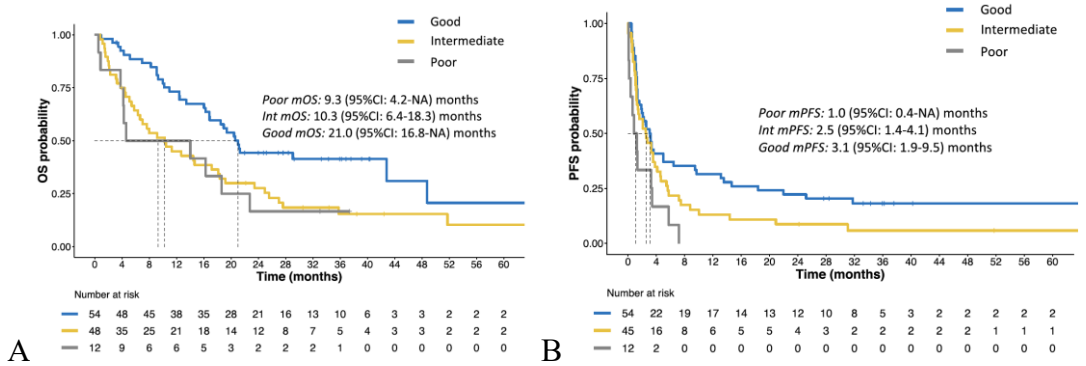


Legend. A Kaplan–Meier curve of OS (A) and PFS (B) according to LIPI score at C1D1. CI confidence interval; C: cycle; D: day; Int: LIPI score intermediate class; m: median; OS: overall survival; PFS: progression-free survival. Good, Intermediate and Poor are referred to LIPI score classes

Association between LIPI at C2D1 and OS/PFS

LIPI at C2D1 was assessable for 114 (65.9%) patients. When considering LIPI score at C2D1 and stratifying also for LIPI at C1D1, a global significant difference in OS between the three score classes was observed (p=0.020) (stratified model's AIC: 60.57). The intermediate (HR_{st}: 8.34, 95%CI: 1.03-67.42) and poor (HR_{st}: 40.75, 95%CI: 1.59-1045.04) scores performed worse than the good (Figure 22A). In terms of PFS, the intermediate (HR_{st}: 1.64, 95%CI: 1.01-2.67) and poor (HR_{st}: 2.61, 95%CI: 1.24-5.51) score classes showed a significantly worse outcome than the good score class (p=0.020), stratifying also for baseline LIPI (Figure 22B) (stratified model's AIC: 453.49). Landmark analyses of 12-month and 24-month OS and PFS provided consistent results (table 34).

Figure 22. Kaplan–Meier curves of OS and PFS according to LIPI score at C2D1

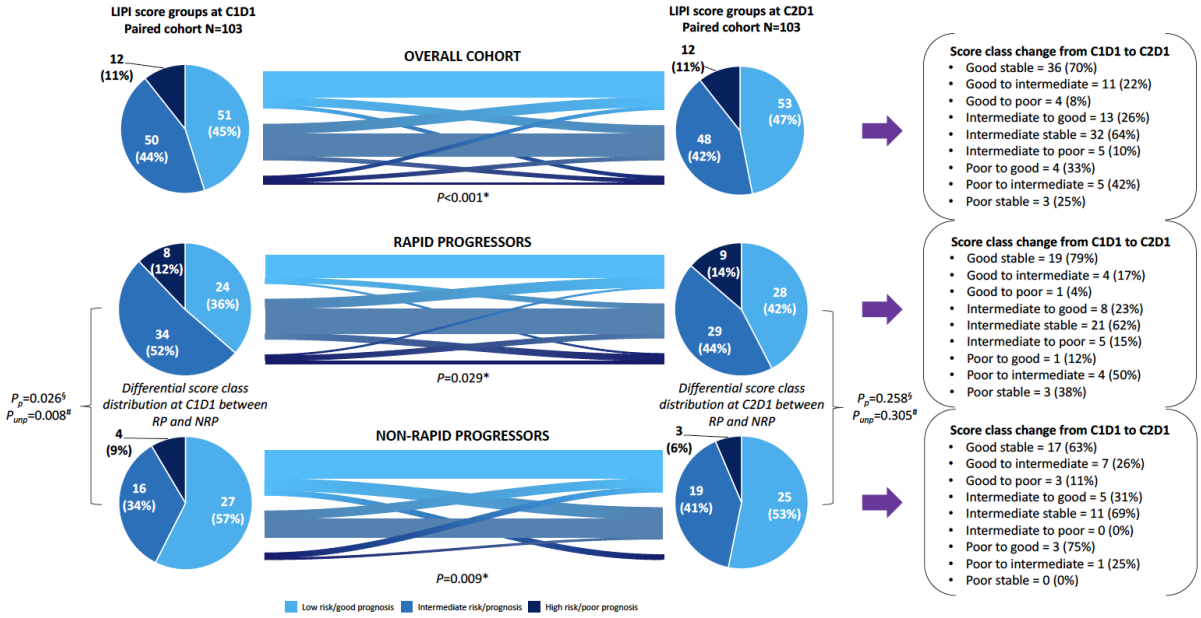


Legend. A Kaplan–Meier curve of OS (A) and PFS (B) according to LIPI score at C2D1. CI confidence interval; C: cycle; D: day; Int: LIPI score intermediate class; m: median; OS: overall survival; PFS: progression-free survival. Good, Intermediate and Poor are referred to LIPI score classes.

LIPI score dynamics and association with rapid PD, OS and PFS

Paired LIPI scores at C1D1 and C2D1 were assessable for 113 (65.3%) patients. We explored potential LIPI score changes from C1D1 to C2D1. A significant number of patients changed their LIPI class from C1D1 to C2D1. It happened in the overall population (p<0.001), RP (p=0.029) and NRP (p=0.009). Despite those changes, the proportion of good, intermediate and poor classes between the two timepoints remained similar (Figure 23). When separating the study population into RP vs. NRP, by taking into account all patients (not only those with paired C1D1-C2D1 samples), we observed that NRP had 38 (60.3%) good, 19 (30.2%) intermediate and 6 (9.5%) poor LIPI score class cases, as compared to 35 (35.4%) good, 49 (49.5%) intermediate and 15 (15.1%) poor score cases in RP at C1D1 (p=0.008). Conversely, no significant difference was observed at C2D1 (p=0.305). Similarly, when restricting the comparison only to NRP and RP patients with paired LIPI score at C1D1 and C2D1, a significantly higher proportion of LIPI good cases for NRP vs. RP was still observed, with more intermediate/poor cases in the RP group (p=0.026) and a similar proportion of LIPI score classes at C2D1 (p=0.258) (Figure 23). Similarly to what previously done for LIPI at C1D1, we built a bivariate logistic regression model adjusting for tumor type, so to test the association of LIPI at C2D1 with rapid PD. A significant association was observed, as well (p=0.049), especially for the poor vs. good score (aOR: 9.91, 95%CI: 1.58-62.04) and the poor vs. intermediate (aOR: 6.83, 95%CI: 1.06-43.87). Other comparisons did not reach statistical significance. A shift towards poorer score classes or stability of the intermediate and poor classes was not formally associated to rapid PD (OR: 2.28, p=0.096).

Figure 23. LIPI score early dynamics.

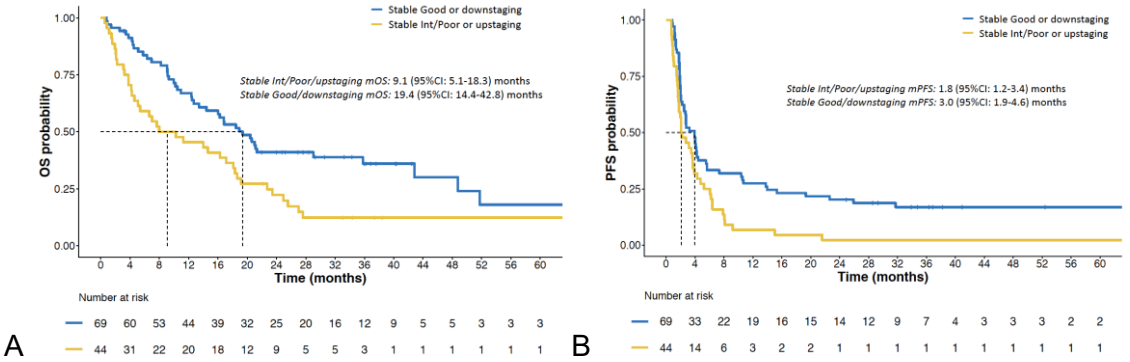


Legend. A LIPI score distribution at C1D1 and C2D1 and dynamics. CI: confidence interval; Int: LIPI score intermediate class; m: median; NRP: non-rapid progressors; OS: overall survival; PD: progression of the disease; PFS: progression-free survival, P_p: p-values for the cohort with available C1D1 and C2D1 paired samples; P_{unp}:p values for the total cohort with LIPI scores available at C1D1 and/or C2D1; RP: rapid progressors; #: p values from χ_2 tests comparing LIPI Good vs. LIPI intermediate/poor between RP and NRP; Åòp values from χ_2 tests comparing LIPI good vs. intermediate vs. poor between RP and NRP; *p values from McNemar tests to assess LIPI dynamics in paired samples. Good, Intermediate and Poor are referred to LIPI score classes

When assessing the association of rapid PD with either LIPI at C1D1 or LIPI at C2D1 in a multivariable model accounting for all clinicopathological features differently distributed between RP and NRP (Table 30), as well as ECOG performance status (PS), LIPI at C1D1 was no longer associated (p=0.593) whereas LIPI at C2D1 was. More specifically, the poor score vs. the good (OR_{adj}: 6.95, 95%CI: 1.19-40.51, p=0.031) or intermediate (OR_{adj}: 9.63, 95%CI: 1.47-63.22, p=0.018) scores retained a strong significant association with rapid PD, independently of baseline LIPI, age, tumor type, treatment line at which the ICI was delivered, ICI regimen type, the ICI target and the use of systemic ATB or corticosteroids during ICI treatment and ECOG. In addition, the multivariable model including both LIPI at C1D1 and C2D1 presented an AIC of 135.16, similar to the AIC of 133.07 exhibited by the model including only LIPI at C2D1, while the model including only LIPI at C1D1 showed an AIC of 191.65, supporting the value of assessing early LIPI dynamics to predict rapid PD.

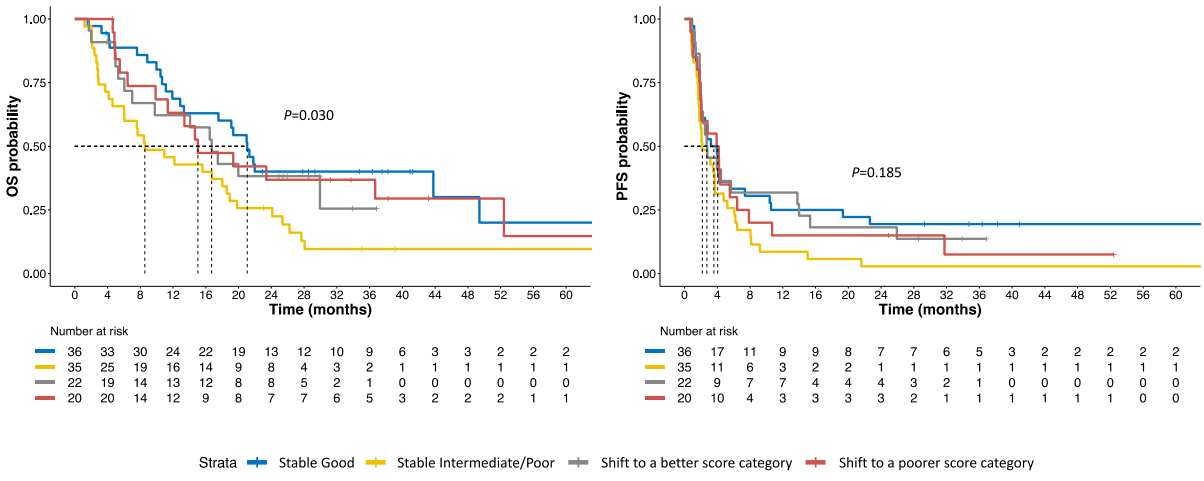
In terms of prognosis, retaining a good prognostic score class between the C1D1 and C2D1 or experiencing a downstaging from poor to intermediate or good, or from intermediate to good was associated to significantly better OS (HR_{st}: 1.90, 95%CI: 1.17-3.09, p=0.009) than remaining in the same intermediate or poor class or experiencing an upstaging (Figure 24A and Figure 25) (stratified model's AIC: 394.08). Consistently, in terms of PFS, those retaining a good prognostic score class between C1D1 and C2D1 or experiencing a downstaging experienced significantly better outcomes than all other cases (HR_{st}: 1.86, 95%CI: 1.18-2.92, p=0.006) (Figure 24B and Figure 25) (stratified model's AIC: 452.14). We checked also if numerical score variations between the first two ICI treatment cycles, without considering the score class, could impact on prognosis.

Figure 24. LIPI score early dynamics association with survival outcomes.



Legend. OS and PFS curves of LIPI dynamics between C1D1 and C2D1. Int: LIPI score intermediate class; m: median; OS: overall survival; PD: progression of the disease; PFS: progression-free survival. Good, Intermediate and Poor are referred to LIPI score classes.

Figure 25. Kaplan-Meier curves of OS and PFS according to LIPI score dynamics between C1D1 and C2D1



Legend. OS: overall survival; PFS: progression-free survival; C: cycle; D: day. Good, Intermediate and Poor are referred to LIPI score classes.

4.5.4 Discussion

In this prospective cohort of patients with advanced solid tumors treated with ICI in a clinical trial at our Institution, almost 61% of patients progressed or died within 4 months from ICI initiation, with roughly 17% achieving an objective response and 18% completed at least one year on ICI treatment. These findings are in line with current evidence about immunotherapy outcomes (192,193). Several prognostic scores have been developed to improve ICI and/or phase I clinical trial candidates selection (38,49,59–63,117,188,189). Nevertheless, these scores' capability of detecting rapid PD under ICI has not been tested and they have never been directly compared to predict objective responses and prognosis. Here, we performed a direct comparison of the most common scores in terms of PFS, OS, ORR, DCB and assessed the best predictor of a meaningful clinical benefit with ICI, considered to be a progression-free interval of more than 4 months. LIPI proved to be the best prognostic score, with the best performance in the identification of rapid progressions, with a solid association with OS and PFS beyond tumor type and relevant clinical features. In addition, LIPI score early dynamics between C1D1 and C2D1 were investigated and seemed to help improving prognostic prediction in terms of both PFS and OS beyond baseline score. Interestingly, the dNLR at baseline combined with its determination at C2D1 improved prediction of ICI outcomes in the context of advanced NSCLC, further suggesting that the early assessment of inflammatory biomarker dynamics can be a valuable prognostic tool beyond baseline determination(50).

At present, only PD-L1 positivity, high microsatellite instability (MSI-H)/mismatch repair deficiency (dMMR) and high tumor mutational burden (TMB-H) are clinically-approved biomarkers for ICI patient selection (27,65,66,150). However, all of these biomarkers present several limitations, including low frequency(67), heterogeneity among cancer types(150,154,194,195) and costs. Other immunohistochemical or transcriptomic-based biomarkers are currently under investigation by our group and others (e.g. PD1 mRNA levels, IGG signature, tumor-infiltrating lymphocytes, tertiary lymphoid structures, LORIS)(86,87,89,113,129,151,196–198). Nonetheless, they are still far from reaching the clinical practice scenario. In this context, LIPI score emerges as a cheap and easy-to-detect blood-based biomarker which effectively stratified patients in three prognostic groups, independently of main clinicopathological factors. This score, assessed by detecting blood levels of LDH and dNLR (absolute neutrophil count/[white blood cell concentration − absolute neutrophil count]) (59), accounts for peripheral pro-inflammatory status (119,199,200), a known poor prognostic factor in patients with cancer(201,202). Higher categories of LIPI score are the reflection of higher LDH and dNLR blood levels, suggesting more peripheral chronic inflammation. Our experience is

coherent with the results of a large meta-analysis involving almost 10,000 patients in 35 studies, where LIPI score robustly stratified patients receiving ICI into three groups with different survival outcomes (42).

Focusing on rapid progressions, we considered 4 months as an acceptable cut-off to detect a minimum clinically meaningful benefit by ICI treatment, taking into account the need for radiologic re-assessments for pseudo-progressions, toxicity, costs and life expectancy of these pretreated population (40% received the ICI in ≥3rd line and several prognostically unfavorable cancers included). LIPI score, with a sensitivity of 68.6%**,** was reasonably good at detecting RP, differently from all other tested scores, also independently of cancer type and relevant clinical factors differentially distributed between RP and NRP, as well as ECOG PS. To note, when accounting for the same clinical factors along with baseline LIPI, LIPI at C2D1 showed a strong and independent prediction of rapid PD beyond the baseline score, especially for what concerns the poor score category. In fact, a poor LIPI at C2D1 was associated to an 595% and a 863% increase in the odds of achieving rapid PD in comparison to a good or intermediate score. Furthermore, when stratifying for relevant clinical factors and LIPI score at C1D1, the Cox model including C2D1 showed a higher goodness of fit than the stratified Cox model including only baseline LIPI, suggesting a more refined prognostic accuracy, as also supported by landmark analysis of 12- and 24-month PFS/OS.

From a biological perspective, a downstaging in LIPI score category might be related to either a reduction in LDH and/or dNLR, which is associated to a lowering of neutrophils and/or an increase in lymphocytes levels. On one hand, LDH has been classically linked to tumor burden and cancer metabolism, but important immunosuppressive effects were also described in more recent years(203,204). Thus, its reduction might both directly reflect tumor response to treatment, as well as a reduction in immunosuppression, favoring response to ICI. On the other hand dNLR reduction due to lymphocytes increase suggests an effective ICI-promoted activation of adaptive immune response against the tumor (205). This combination of factors could thus explain not only the baseline prognostic role of the score, but also the association of its dynamics and levels at C2D1 with better long-term outcomes and less rapid progressions. Importantly, we assessed the prognostic impact of score dynamics by considering the numerical variations instead of LIPI score class change, we did not detect any meaningful impact on prognosis. This leads to the conclusion that it is more about “quality over quantity”, meaning that the numerical change is not impactful in itself. Rather, it is the change in the score class that matters.

Our study is not exempt from limitations. First, missing hematologic parameters, especially LDH at C2D1, reduced the number of patients that could be tested for paired analyses, as well as the number of overall patients included in the multivariable and stratified regression models accounting for both LIPI at C1D1 and C2D1. We had no possibility of thoroughly assessing molecular features of included tumors and PD-L1 status was mostly unknown. However, most patients were treated in a ≥2nd-line setting and in tumors in which PD-L1 status was not mandatory for prescribing the therapy in Europe. Also, the kind of ICI was decided in sponsored trials. Additionally, being a single-center study, our findings require validation in an external cohort. Finally, some patients from the poor LIPI group achieved clinical benefit, possibly because automated neutrophil counts do not discriminate between the different subpopulations of neutrophils that could have protumor or antitumor functions (59,206,207). Besides these limitations, our study confirms the effective prognostic role of baseline LIPI in ICI-treated patients in a pancancer context. LIPI might be especially useful to detect patients more or less likely to derive a clinically meaningful benefit from ICI, potentially serving either as a critical stratification factor or as an inclusion/exclusion criteria in ICI trials and with very limited costs; though further improvements in this research area are advisable. Moreover, early dynamics between C1D1 and C2D1 were able to identify patients with more favorable outcomes beyond baseline score. As far as we are concerned, this is the first study assessing LIPI score early dynamics and its association with survival. Only another study assessed LIPI dynamics, but evaluated the association with side effects and was exclusively conducted in a cohort of patients with NSCLC (208). These results merit further validation, as LIPI dynamics might become a useful inexpensive on-treatment tool to identify patients either benefiting from continuing immune-checkpoint inhibition or candidates to escalated or alternative therapeutic strategies in the context of adequately designed clinical trials.

Chapter 5. Discussion

A few years ago immune check-point inhibitors appeared in our clinics and revolutionized the therapeutic protocols of most solid tumors. These novel drugs interact with our immune system to release its potential and enhance tumor cells recognition. During the oncogenesis, neoantigens are released and captured by dendritic cells that act as APCs that will display fragments of those antigens to T cells. Depending on the balance of costimulatory (CD28, ICOS, …) and inhibitory signals (PD1, CTLA4, …) antigen recognition may result in the activation of effector T cells against cancer specific antigens, cell destruction and new antigen release or tolerance(3). Nevertheless, this immune cycle may fail in oncology patients. Tumor antigens may not be detected o considered self-antigens instead of foreign, T cells may not infiltrate tumors, o tumor microenvironment factors may inhibit T cells (16). In fact, a wide percentage of patients who receive ICIs do not benefit from them, but are exposed to their potential side effects and create an economic burden to the national health care system. Therefore, it is critical to find a way to improve patient selection, that was precisely the main focus of this thesis.

Between May 2017 and March 2022, 173 evaluable patients entered the study and 164 of them received an anti-PD1/anti-PD-L1-based treatment(124). Our population had a mean age of 63,2, a majority of patients were male (62,4%), had ECOG 0-1 (89%), had not received corticosteroids, radiotherapy o antibiotics within 30 days of starting the ICI and had received previously another ICI (80,3%). A wide range of solid tumors were included being lung, colorectal, breast and head and neck cancers the most frequent diagnosis. Most of the patients were participating in a clinical trial, which explains the fact of the prevalent good performance status and the representation of diagnosis in which ICIs are not approved yet.

As primary objective of this thesis we decided to explore the role of peripheral lymphocytes subpopulations as ICI biomarker. Previous evidence links the presence of lymphocytes T in tumor microenvironment (TILs) with ICI outcomes (88,89) as well as preliminary studies correlate peripheral lymphocytes o their early dynamics with ICI outcomes. Nevertheless, these studies included a small number of patients without validation cohorts so the question remains open (100–102,108,111,112).

Oncology treatments are usually prescribed as lines of therapy, that keep going until the disease shows a progression and we switch to another therapy. This is the critical moment in which we decide the treatment strategy for the following months so ideally our biomarker should help us to decide if ICIs are an appropriate strategy before starting it. In our analysis of baseline samples, CD3+CD4+PD1+ was the most relevant

subpopulation. Higher values of CD3+CD4+PD1+ were associated with lower rates of responses (ORR, DCB) and shorter times until progression (PFS) discouraging ICI prescription. Similar conclusions were seen when analyzing CD3+CD4+PD1+ levels prior to the second infusion. These results are in line with previous publications. For example, CD3 CD4 PD1+ lymphocytes subpopulation has been correlated with worse outcome in treatment naïve patients candidate to chemotherapy o tyrosine kinase inhibitors (125) and Zheng et all demonstrated a significant association between CD3 CD4 PD1 high lymphocytes levels and lower rates of OS and PFS (100). These studies recruited a limited number of patients and did not include a validation cohort so their evidence has some limitations (100). Our study included a higher number of patients supporting its role as potential biomarker but these results must be validated before applying it in our clinics. Therefore, further analysis will be done in a future new cohort of patients to validate our results.

CD3 CD8 (T effector) lymphocytes are critical in the mechanism of action of antiPD(L)1 therapies but baseline o early dynamics of CD3+CD8+ didn´t predict ICI outcomes. Similarly, to other previous projects, we observed a significative proliferation of CD3 CD8 PD1+ (MFI) but we were not able to demonstrate any relationship of this subpopulation with ICI benefit. Hwan Kim et all demonstrated an increase of CD8 PD1+ within the first week after ICI initiation that was followed by a decreased in the subsequent two weeks (102). In our study, most of C2D1 samples were collected three or four weeks after C1D1 so it is possible that our conclusions differ due to the different schedule of sample collection. Although our study does not support CD8 PD1+ as biomarker, based on the mechanism of action rational and the previous results of other authors, it may be worth exploring it further if collecting samples on C1D1 and approximately seven days later.

Apart from the potential responses o the duration of the therapy, the potential survival of the patient is another factor to take into account when selecting the new treatment. In terms of OS several subpopulations showed statistical relationship, being CD3+CD4+HD subpopulation the most significative, being associated with shorter OS when measured at C1D1 and C2D1 timepoints. Zuazo-Ibarra, Arasanz et all explored CD3+CD4+HD in a non-small cell lung cancer cohort and identified that patients with higher baseline levels of CD3 CD4 HD o with a sharp increase in the first days after ICI initiation were associated with shorter PFS and hyperprogression (108,110). Unlike these studies, in our analysis it did not show relationship with shorter PFS o hyperprogression, not supporting its potential role as biomarker. Nevertheless, it seems to be related with worse outcomes including worse survival which highlights its potential as prognosis biomarker.

CD3 CD4 HD levels also demonstrated an association with shorter OS when measured PD timepoints. Obviously, at that time the patient already had received an ICI and could not take any decision regarding this indication but that is the moment in which we must switch his therapy to a new one. Its potential role as prognosis biomarker could help us to take decisions about prescribing another line of therapy, which could include another ICI, or offering best supportive care to our patient.

Low evidence exists regarding the predictor role of peripheral lymphocytes B (CD19+) and ICI benefit but we included them because they can induce antigen specific CD8 and CD4 activation (15). Preliminary results correlated baseline memory B cells with favorable outcomes in solid tumors as lung cancer (126), renal cancer (127) and sarcomas(128). In fact, in our published case report we observed an increase of lymphocytes B during treatment in a UPS sarcoma patient who developed great benefit to ICI (129). These observations differ from another study performed by Barth et all, who collected samples from 39 patients treated with ICI at baseline and at the time of first tumoral response evaluation (8-12 weeks later). No relationship was found between any of the seven lymphocyte B subpopulations and ORR o DCB at baseline (130). In our study lymphocytes B (CD19+) were associated with worse outcomes (shorter ORR/PFS at C1D1, shorter ORR, DCB, PFS and OS at C2D1). Therefore, further analysis is recommended to clarify its real predicting value.

Baseline clinical/hematological characteristics and previous medical history may influence the chances of benefit of our candidates (28–37) but the ideal patient profile is unknown. As secondary objective we assessed the correlation among many clinicopathological and biological factors with activity and efficacy endpoint of ICI treatment, so to identify an easily detectable profile of the patients that might gain the most benefit out of anti-PD1/PD-L1 immunotherapy.

We investigated the role of palliative RT administered right before or during anti-PD1/PD-L1 ICI therapy. It has been considered that RT might potentially contribute to determine a stronger systemic immune response (i.e. the abscopal effect) via immunogenic cell death and antigen release, thus enhancing the efficacy of ICI (136,137). However, in our cohort, RT administered during ICI was not associated to PFS, OS or tumor responses. Surprisingly, RT administered within 30 days from ICI treatment start was associated with worse OS, independently from all other clinicopathological factors considered. We have no current explanation for this observation and only 9 patients had received palliative RT immediately before ICI start, making this finding difficult to generalize. Conversely, in line with other findings(138,139), we did not observe any abscopal effect,

providing more evidence to debunk a widely postulated, yet scarcely objectivized phenomena(137).

Recently, Pinato et al. showed that systemic ATB administered prior to, but not during ICI monotherapy, are associated with a worse treatment response and OS in solid tumors(140), while ATB treatment in general seems not to impact on chemo-immunotherapy outcomes(141). In our cohort, only ATB during, but not previous to anti-PD1/PD-L1 treatment, were associated with better PFS (univariate analysis) and DCB (univariate and multivariate analysis). To note, considering the very low number of patients (n=7) that received ATB prior to ICI, we cannot completely exclude that an ATB-induced gut microbial dysbiosis might impair ICI efficacy. At the same time, we had no sign of detrimental effect during ICI-based therapy in a wider number of patients (n=41), in line with recent evidences(140,141), with a significant and independent association to DCB which merits further investigation.

Whether systemic corticosteroids, due to their immunosuppressive effect, might impair or not ICI when administered right before or during treatment is another matter of debate. Several studies led to the conclusion that avoiding or delaying the use of corticosteroids may result in maximizing the potential treatment benefits of immunotherapy(31–35). However, other evidences highlight that corticosteroids have no detrimental effect on immunotherapy and high doses of steroids might reflect poorer basal conditions (e.g. active brain metastases, concurrent diseases, larger tumor volume), ultimately responsible for the more scarce outcomes observed with ICI (36,37). In our study, systemic administration of corticosteroids during ICI was associated with better PFS, ORR and DCB at the univariate analysis but lost any significant effect when adjusting for other clinicopathological factors. Corticosteroids prior to ICI did not show any significant effect on outcomes. We did not observe any difference when dividing steroid-receiving patients according to dose (above or below an equivalent of 30mg of prednisone), as well. To note, in 48 out of 61 (78%) cases, systemic corticosteroids were administered to treat immune-related adverse events and in 5 (8%) further cases were administered as premedication to CT scan contrast medium. Thus, in our study corticosteroid use did not reflect a baseline unfavorable condition beyond tumor type and there was no hint that successfully treating ICI immune-mediated toxicities with corticosteroids might ultimately impair anti-PD1/PD-L1 efficacy.

Noteworthy, an immunotherapy-naïve status was associated to a significantly better PFS, independently from other characteristics. Concordant recommendations regarding the opportunity to retreat patients already treated with immunotherapy do not exist. Furthermore, these patients are usually excluded from clinical trials that evaluate new

ICI drugs or combinations so the evidence of activity in this setting is limited. A recent meta-analysis pooling 49 available studies showed that in patients who had previously discontinued ICI because of PD, ORR and median PFS were inferior to those of patients who had previously discontinued ICI because of toxicity (15.2 % and 2.9 months vs. 44 % and 13.2 months, respectively) (145). Our findings, taken together with current literature, seems to confirm that rechallenges with ICI, at least with anti-PD1/PD-L1, should not be encouraged broadly, although in specific cases this strategy could be considered. Understanding the clinical impact of neo/adjuvant ICI in patients with relapsing metastatic disease candidate for immunotherapy will be of outmost importance considering the rapid expansion of therapeutic indications also in early-stage solid tumors(146,147).

Administering anti-PD1/PD-L1 in earlier lines seemed to be associated with better PFS, OS and ORR at univariate analyses. Although the effect on PFS and OS might have been influenced by a potential lead time bias, it is also true that a less compromised immune system in untreated/less treated patients might favor the elicitation of more potent immune responses. At the same time, it is important to underline that treatment line lost its effect on all endpoints at multivariate analyses. Thus, this finding seems to suggest that treatment line should not be an eligibility criterion for ICI treatment.

Our initial analysis confirmed the capability of the LIPI score to successfully stratify patients with solid tumors treated with anti-PD1/PD-L1 in different prognostic subgroups, independently from all main clinicopathological characteristics (31,37,140,209), in a tumor agnostic fashion, both in terms of PFS and OS. Nevertheless, LIPI is only one within several prognostic scores that have been developed to improve ICI and/or phase I clinical trial candidates selection (38,49,59–63,117,188,189) so we decided to explore this field further and perform a second analysis.

We subdivided the study cohort into rapid progressors (RP) (PFS ≤ 4 months from ICI initiation) and non-rapid progressors (NRP) (PFS > 4 months from ICI initiation) and performed a direct comparison to explore which was the best prognostic predictor among LIPI, RMH, PMHI, NLR, dNLR, PIPO and GRIm scores in terms of PFS and OS. LIPI score was capable of satisfactorily detecting RP, differently from all other tested scores, as they performed worse than LIPI and many with an AUC close or equal to 0.5. Moreover, the association with rapid PD within 4 months was significant and independent from the cancer type only for baseline LIPI score. Its association with rapid PD was also noted independently of relevant clinical factors differentially distributed between RP and NRP. To note, when accounting for the same clinical factors along with baseline LIPI, LIPI at C2D1 showed a strong and independent prediction of rapid PD beyond the

baseline score, especially for what concerns the poor score category. In fact, patients in the poor group at C2D1, as compared to patients in the good and intermediate score groups showed an 831% and a 1122% increase in the odds of achieving rapid PD. Also, the multivariable model showed a higher goodness of fit when including either LIPI at both timepoints or only LIPI at C2D1, than the model including only LIPI at C1D1. Furthermore, when stratifying for relevant clinical factors and LIPI score at C1D1, the Cox model including C2D1 showed a higher goodness of fit than the stratified Cox model including only baseline LIPI, both in terms of PFS and OS prediction, suggesting a more refined prognostic accuracy, as also suggested by looking at the landmark analysis of 12- and 24-month PFS/OS. Therefore, LIPI score proved to be the best score identifying rapid progressions and showed a solid association with OS and PFS beyond tumor type and relevant clinical features.

In addition, we explored for the first time in this scenario the early dynamics of LIPI score, with the aim of assessing the ability to rapidly detect which patients might benefit from continuing immune check-point inhibition or may need a treatment change. Retaining a good prognostic score class between the C1D1 and C2D1 or experiencing a downstaging from poor to intermediate or good, or from intermediate to good was associated to significantly better OS and PFS. Our results suggest that a determination of LIPI score at C2D1 might help improving prognostic prediction, in terms of both PFS and OS beyond baseline score.

This score, assessed by detecting blood levels of LDH and dNLR (absolute neutrophil count/[white blood cell concentration − absolute neutrophil count])(59), is an elegantly simple biomarker accounting for peripheral pro-inflammatory status(119,199,200), which is a known poor prognostic factor in patients with cancer. Higher categories of LIPI score are the reflection of higher LDH and dNLR blood levels, suggesting more peripheral chronic inflammation. From a biological perspective, a downstaging in LIPI score category might be related to either a reduction in LDH and/or dNLR, which is associated to a lowering of neutrophils and/or an increase in lymphocytes levels. On one hand, LDH has been classically linked to tumor burden and cancer metabolism, but important immunosuppressive effects were also described in more recent years(203,204). Thus, its reduction might both directly reflect tumor response to treatment, as well as a reduction in immunosuppression, favoring response to ICI. On the other hand dNLR reduction due to lymphocytes increase suggests an effective ICI-promoted activation of adaptive immune response against the tumor(205). This combination of factors could thus explain not only the baseline prognostic role of the score, but also the association of its dynamics and levels at C2D1 with better long-term outcomes and less rapid

progressions. As far as we are concerned, this is the first study assessing LIPI score early dynamics and its association with survival. Additionally, in a pancancer context. Only another study assessed LIPI dynamics, but evaluated the association with side effects and was conducted in a cohort of patients with NSCLC(208). These results merit further validation, as LIPI dynamics might become a useful inexpensive on-treatment tool to identify patients either benefiting from continuing immune-checkpoint inhibition or candidates to change to alternative therapeutic strategies in the context of adequately designed clinical trials.

Our last objective was to explore the potential of tumor based biomarkers for ICI (PD1 mRNA levels, PD-L1 protein expression and Tumor Infiltrating Lymphocytes). In this study we did not perform any biopsy to our patients so we could only analyze archival samples if available. Besides, only 17.8% patients were able to achieve a CR or PR, PD-L1 status was mostly unknown as this information was only collected from medical notes if previously tested, so our analysis has some limitations. Although the number of cases with tumor tissue available for mRNA detection was too low for introducing the variable in the multivariate logistic regression models, we confirmed the capability of PD1 mRNA to identify patients more likely to achieve an objective response, CR above all, as our Dr Prat´s lab had previously demonstrated(87). Interestingly, while TILs seemed not to correlate with response and survival outcomes in a pan-cancer context, PD-L1% was positively associated with a slightly higher likelihood of achieving an objective response (OR: 1.03) and a 1% reduction in the risk of progression or death for each unitary increase. Additionally, a cut-off of 10% appeared to be optimal in discriminating between patients at higher likelihood of achieving an objective and durable response and at lower risk of progression or death, similarly to what observed for example, with pembrolizumab in metastatic triple negative breast cancer(157). Nevertheless, a larger casuistry is required to confirm the result independently from other variables and across cancer types, along with a uniform assessment of PD-L1 throughout cancer types.

Chapter 6. Conclusions

In conclusion, the analysis that are part of this thesis provided evidence to:

- Peripheral CD3+CD4+PD1+ lymphocytes subpopulations levels might be a tumor agnostic biomarker for resistance to ICI. Higher levels of peripheral CD3+CD4+PD1+ lymphocytes prior to start ICI o before the second cycle might be associated to worse outcomes discouraging ICI prescription.
- Peripheral CD3+CD4+ Highly Differentiated might have a role as prognosis biomarker. Higher levels seem correlated to shorter overall survival.
- Other peripheral cells like CD3+CD8+PD1+ or lymphocytes B merits further analysis but not conclusive evidence was found in our study.
- Patients who have not received ICI previously (immunotherapy-naïve status) have higher chances of benefit from ICIs. Other common clinicopathological factors seem not to be able to identify the best candidates for immunotherapy.
- LIPI score at baseline might be an effective tumor-agnostic prognosis biomarker able to identify patients more likely to benefit from ICI. Early LIPI dynamics between first and second ICI administration might improve LIPI prediction potential. LIPI score could be considered a stratification factor for clinical trials.
- PD1 mRNA might be tumor-agnostic predictive factor of response to ICI.
- PD-L1 protein levels, with a potential 10% cut-off, is a promising tumor-agnostic prognostic and predictive factor.

Bibliography

1. Robert C. A decade of immune-checkpoint inhibitors in cancer therapy. Vol. 11, Nature Communications. Nature Research; 2020.
2. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol [Internet]. 2020;20(11):651–68. Available from: http://dx.doi.org/10.1038/s41577-020-0306-5
3. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Vol. 14, Allergy, Asthma and Clinical Immunology. BioMed Central Ltd.; 2018.
4. Parry R V., Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi S V., et al. CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms. Mol Cell Biol. 2005 Nov 1;25(21):9543–53.
5. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. Vol. 20, Nature Reviews Immunology. Nature Research; 2020. p. 651–68.
6. Xu Editor J. Regulation of Cancer Immune Checkpoints [Internet]. Xu J, editor. Advances in Experimental Medicine and Biology. Singapore: Springer Singapore; 2020. (Advances in Experimental Medicine and Biology; vol. 1248). Available from: http://link.springer.com/10.1007/978-981-15-3266-5
7. Zuazo M, Arasanz H, Fernández-Hinojal G, García-Granda MJ, Gato M, Bocanegra A, et al. Functional systemic CD 4 immunity is required for clinical responses to PD -L1/ PD -1 blockade therapy . EMBO Mol Med. 2019 Jul;11(7).
8. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 1992 Nov;11(11):3887–95.
9. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the Pd-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. J Exp Med. 2000 Oct 2;192(7):1027–34.
10. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12(6):492–9.
11. Ando M, Ito M, Srirat T, Kondo T, Yoshimura A. Memory T cell, exhaustion, and tumor immunity. Immunol Med [Internet]. 2020;43(1):1–9. Available from: https://doi.org/10.1080/25785826.2019.1698261
12. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015;15(8):486–99.
13. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8 $^{+}$ T lymphocytes are prognostic factors of human ovarian cancer. Proceedings of the National Academy of Sciences. 2007 Feb 27;104(9):3360–5.
14. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 Is Associated with Poor Prognosis in Renal Cell Carcinoma Patients with Long-term Follow-up. Cancer Res. 2006 Apr 1;66(7):3381–5.

15. Flippot R, Teixeira M, Rey-Cardenas M, Carril-Ajuria L, Rainho L, Naoun N, et al. B cells and the coordination of immune checkpoint inhibitor response in patients with solid tumors. Vol. 12, Journal for ImmunoTherapy of Cancer. BMJ Publishing Group; 2024.
16. Chen DS, Mellman I. Oncology Meets Immunology : The Cancer Immunity Cycle. Immunity Review. 2013;39:1–10.
17. Munari E, Mariotti FR, Quatrini L, Bertoglio P, Tumino N, Vacca P, et al. PD-1/pd-l1 in cancer: Pathophysiological, diagnostic and therapeutic aspects. Vol. 22, International Journal of Molecular Sciences. MDPI AG; 2021.
18. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell. 2002 Aug;2(2):117–25.
19. Rodón J, Saura C, Dienstmann R, Vivancos A, Ramón y Cajal S, Baselga J, et al. Molecular prescreening to select patient population in early clinical trials. Nat Rev Clin Oncol. 2012 Jun;9(6):359–66.
20. Akkın S, Varan G, Bilensoy E. A review on cancer immunotherapy and applications of nanotechnology to chemoimmunotherapy of different cancers. Vol. 26, Molecules. MDPI AG; 2021.
21. Akkın S, Varan G, Bilensoy E. A Review on Cancer Immunotherapy and Applications of Nanotechnology to Chemoimmunotherapy of Different Cancers. Molecules. 2021 Jun 3;26(11).
22. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primers. 2020 May 7;6(1):38.
23. Schaft N, Dörrie J, Schuler G, Schuler-Thurner B, Sallam H, Klein S, et al. The future of affordable cancer immunotherapy. Vol. 14, Frontiers in Immunology. Frontiers Media SA; 2023.
24. Grossman JE, Vasudevan D, Joyce CE, Hildago M. Is PD-L1 a consistent biomarker for anti-PD-1 therapy? The model of balstilimab in a virally-driven tumor. Vol. 40, Oncogene. Springer Nature; 2021. p. 1393–5.
25. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. Mol Cancer Ther. 2017 Nov 1;16(11):2598–608.
26. Marabelle A, Le DT, Ascierto PA, Anna ;, Giacomo M Di, De Jesus-Acosta A, et al. Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/ Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. 2019; Available from: https://doi.
27. Marcus L, Fashoyin-Aje LA, Donoghue M, Yuan M, Rodriguez L, Gallagher PS, et al. FDA approval summary: Pembrolizumab for the treatment of tumor mutational burden-high solid tumors. Clinical Cancer Research. 2021 Sep 1;27(17):4685–9.
28. Pinato DJ, Howlett S, Ottaviani D, Urus H, Patel A, Mineo T, et al. Association of prior antibiotic treatment with survival and response to immune checkpoint inhibitor therapy in patients with cancer. JAMA Oncol. 2019 Dec 1;5(12):1774–8.

29. Cortellini A, Ricciuti B, Facchinetti F, Alessi JVM, Venkatraman D, Dall'Olio FG, et al. Antibiotic-exposed patients with non-small-cell lung cancer preserve efficacy outcomes following first-line chemo-immunotherapy. Annals of Oncology [Internet]. 2021;xxx(xxx):1–9. Available from: https://doi.org/10.1016/j.annonc.2021.08.1744
30. Arbour KC, Mezquita L, Long N, Rizvi H, Auclin E, Ni A, et al. Impact of Baseline Steroids on Efficacy of Programmed Cell Death-1 and Programmed Death-Ligand 1 Blockade in Patients With Non–Small-Cell Lung Cancer. Journal of Clinical Oncology [Internet]. 2018 Oct 1;36(28):2872–8. Available from: https://ascopubs.org/doi/10.1200/JCO.2018.79.0006
31. Drakaki A, Dhillon PK, Wakelee H, Chui SY, Shim J, Kent M, et al. Association of baseline systemic corticosteroid use with overall survival and time to next treatment in patients receiving immune checkpoint inhibitor therapy in real-world US oncology practice for advanced non-small cell lung cancer, melanoma, or urothelial carcinoma. Oncoimmunology. 2020 Jan 1;9(1).
32. De Giglio A, Mezquita L, Auclin E, Blanc-Durand F, Riudavets M, Caramella C, et al. Impact of intercurrent introduction of steroids on clinical outcomes in advanced non-small-cell lung cancer (Nsclc) patients under immune-checkpoint inhibitors (ici). Cancers (Basel). 2020 Oct 1;12(10):1–11.
33. Kartolo A, Deluce J, Holstead R, Hopman W, Lenehan J, Baetz T. Impact of Baseline Corticosteroids on Immunotherapy Efficacy in Patients With Advanced Melanoma. J Immunother. 2021 May 1;44(4):167–74.
34. Maslov D V., Tawagi K, Kc M, Simenson V, Yuan H, Parent C, et al. Timing of steroid initiation and response rates to immune checkpoint inhibitors in metastatic cancer. J Immunother Cancer. 2021 Jul 5;9(7).
35. Pan EY, Merl MY, Lin K. The impact of corticosteroid use during anti-PD1 treatment. Journal of Oncology Pharmacy Practice [Internet]. 2020 Jun 7;26(4):814–22. Available from: https://journals.sagepub.com/doi/10.1177/1078155219872786
36. Aldea M, Orillard E, Mansi L, Marabelle A, Scotte F, Lambotte O, et al. How to manage patients with corticosteroids in oncology in the era of immunotherapy? Vol. 141, European Journal of Cancer. Elsevier Ltd; 2020. p. 239–51.
37. Nelson BE, Greiner B, Hong A, Tipton S, Lee M, Dixon A, et al. Corticosteroid use and its impact on the efficacy of immunotherapy in multiple tumor types. Journal of Clinical Oncology [Internet]. 2021 May 20;39(15_suppl):e14583–e14583. Available from: https://ascopubs.org/doi/10.1200/JCO.2021.39.15_suppl.e14583
38. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: A systematic review and meta-analysis. J Natl Cancer Inst. 2014;106(6).
39. Ethier JL, Desautels D, Templeton A, Shah PS, Amir E. Prognostic role of neutrophil-to-lymphocyte ratio in breast cancer: A systematic review and meta-analysis. Breast Cancer Research [Internet]. 2017;19(1):1–13. Available from: http://dx.doi.org/10.1186/s13058-016-0794-1
40. Valero C, Lee M, Hoen D, Weiss K, Kelly DW, Adusumilli PS, et al. Pretreatment neutrophil-to-lymphocyte ratio and mutational burden as biomarkers of tumor

response to immune checkpoint inhibitors. Nat Commun [Internet]. 2021;12(1):1–9. Available from: http://dx.doi.org/10.1038/s41467-021-20935-9
41. Yu Y, Wang H, Yan A, Wang H, Li X, Liu J, et al. Pretreatment neutrophil to lymphocyte ratio in determining the prognosis of head and neck cancer: A meta-analysis. BMC Cancer. 2018;18(1):1–9.
42. Guo Y, Pan Y, Wan J, Gong B, Li Y, Kan X, et al. Prognosis stratification of cancer patients treated with immune checkpoint inhibitors through lung immune prognostic index: a meta-analysis and systematic review. Vol. 24, BMC Cancer. BioMed Central Ltd; 2024.
43. Bagley SJ, Kothari S, Aggarwal C, Bauml JM, Alley EW, Evans TL, et al. Pretreatment neutrophil-to-lymphocyte ratio as a marker of outcomes in nivolumab-treated patients with advanced non-small-cell lung cancer. Lung Cancer [Internet]. 2017 Apr;106:1–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28285682
44. Yann A, Stephane O, Marie A, Pauline T, Elaidi R, Thibault C, et al. Optimal cut-off for neutrophil-to-lymphocyte ratio : Fact or Fantasy ? A prospective cohort study in metastatic cancer patients. PLoS One. 2018;4(13):1–13.
45. Stephen J Bagley, Kothari S, Aggarwal C, Bauml JM, Alley EW, Evans TL, et al. Pretreatment neutrophil-to-lymphocyte ratio as a marker of outcomes in nivolumab-treated patients with advanced non-small-cell lung cancer. Lung Cancer. 2017;106:1–7.
46. Suh KJ, Kim SH, Kim YJ, Kim M, Keam B, Kim TM, et al. Post-treatment neutrophil-to-lymphocyte ratio at week 6 is prognostic in patients with advanced non-small cell lung cancers treated with anti-PD-1 antibody. Cancer Immunology, Immunotherapy [Internet]. 2018;67(3):459–70. Available from: https://doi.org/10.1007/s00262-017-2092-x
47. Riudavets M, Auclin E, Mezquita L. Host circulating biomarkers for immune-checkpoint inhibitors: single-agent and combinations. Future Oncol. 2020;16(23):1665–8.
48. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: A systematic review and meta-analysis. J Natl Cancer Inst. 2014;106(6).
49. Yang T, Hao L, Yang X, Luo C, Wang G, Lin Cai C, et al. Prognostic value of derived neutrophil-to-lymphocyte ratio (dNLR) in patients with non-small cell lung cancer receiving immune checkpoint inhibitors: A meta-analysis. BMJ Open. 2021;11(9).
50. Mezquita L, Preeshagul I, Auclin E, Saravia D, Hendriks L, Rizvi H, et al. Predicting immunotherapy outcomes under therapy in patients with advanced NSCLC using dNLR and its early dynamics. Eur J Cancer. 2021;151:211–20.
51. Duan J, Pan L, Yang M. Preoperative elevated neutrophil-to-lymphocyte ratio (NLR) and derived NLR are associated with poor prognosis in patients with breast cancer. Medicine. 2018 Dec;97(49):e13340.
52. Su S, Liu L, Li C, Zhang J, Li S. Prognostic role of pretreatment derived neutrophil to lymphocyte ratio in urological cancers: A systematic review and meta-analysis. International Journal of Surgery. 2019 Dec;72:146–53.

53. Seligmann JF, Hall P, Wilson H, Richman SD, Barrett J, Seymour M, et al. The Derived Neutrophil Lymphocyte Ratio (Dnlr) As a Biomarker in Advanced Colorectal Cancer (Acrc). Annals of Oncology. 2014 Sep;25:iv190.
54. Benitez JC, Recondo G, Rassy E, Mezquita L. The LIPI score and inflammatory biomarkers for selection of patients with solid tumors treated with checkpoint inhibitors. Vol. 64, Quarterly Journal of Nuclear Medicine and Molecular Imaging. Edizioni Minerva Medica; 2020. p. 162–74.
55. Vuagnat P, Auclin E, Mezquita L, Alfonso JA, Tocino MRV, Munoz FL, et al. Applicability of the LIPI score to metastatic microsatellite instability high cancer patients treated with immune checkpoint inhibitors. Annals of Oncology [Internet]. 2019 Dec;30:xi20. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0923753420344331
56. Aldea M, Benitez JC, Mezquita L. The lung immune prognostic index (LIPI) stratifies prognostic groups in advanced non-small cell lung cancer (NSCLC) patients. Vol. 9, Translational Lung Cancer Research. AME Publishing Company; 2020. p. 967–70.
57. Andrea Varga, Alice Bernard-Tessier, Edouard Auclin, Laura Mezquita, Capucine Baldini, David Planchard, et al. Applicability of the lung immune prognostic index (LIPI) in patients with metastatic solid tumors when treated with immune checkpoint inhibitors (ICI) in early clinical trials. In: TAT 2019. 2019.
58. Pauline P, Auclin E, Mezquita L, Chanza NM, Dumont C, Rodriguez-Vida A, et al. Association of the Lung Immune Prognostic Index with outcome in patients with metastatic urothelial cancer treated with immune checkpoint inhibitor. Journal of Clinical Oncology [Internet]. 2020 Feb 20;38(6_suppl):545–545. Available from: https://ascopubs.org/doi/10.1200/JCO.2020.38.6_suppl.545
59. Mezquita L, Auclin E, Ferrara R, Charrier M, Remon J, Planchard D, et al. Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. JAMA Oncol. 2018 Mar 1;4(3):351–7.
60. Bigot F, Castanon E, Baldini C, Hollebecque A, Carmona A, Postel-Vinay S, et al. Prospective validation of a prognostic score for patients in immunotherapy phase I trials: The Gustave Roussy Immune Score (GRIm-Score). Eur J Cancer. 2017;84:212–8.
61. Arkenau HT, Olmos D, Ang JE, De Bono J, Judson I, Kaye S. Clinical outcome and prognostic factors for patients treated within the context of a phase I study: The Royal Marsden Hospital experience. Br J Cancer. 2008 Mar 25;98(6):1029–33.
62. Chau NG, Florescu A, Chan KK, Wang L, Chen EX, Bedard P, et al. Early mortality and overall survival in oncology phase I trial participants: Can we improve patient selection? BMC Cancer. 2011 Oct 5;11.
63. Matos I, Villacampa G, Hierro C, Martin-Liberal J, Berché R, Pedrola A, et al. Phase I prognostic online (PIPO): A web tool to improve patient selection for oncology early phase clinical trials. Eur J Cancer [Internet]. 2021 Sep;155:168–78. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0959804921003889
64. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, Jansson M, Kulangara K, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1

of the “Blueprint PD-L1 IHC Assay Comparison Project.” Journal of Thoracic Oncology. 2016;12(2):208–22.
65. Grossman JE, Vasudevan D, Joyce CE, Hildago M. Is PD-L1 a consistent biomarker for anti-PD-1 therapy? The model of balstilimab in a virally-driven tumor. Vol. 40, Oncogene. Springer Nature; 2021. p. 1393–5.
66. Marabelle A, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord JP, et al. Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair–Deficient Cancer: Results From the Phase II KEYNOTE-158 Study. Journal of Clinical Oncology [Internet]. 2020 Jan 1;38(1):1–10. Available from: https://ascopubs.org/doi/10.1200/JCO.19.02105
67. Kavun A, Veselovsky E, Lebedeva A, Belova E, Kuznetsova O, Yakushina V, et al. Microsatellite Instability: A Review of Molecular Epidemiology and Implications for Immune Checkpoint Inhibitor Therapy. Vol. 15, Cancers. MDPI; 2023.
68. André T, Berton D, Curigliano G, Sabatier R, Tinker A V., Oaknin A, et al. Antitumor Activity and Safety of Dostarlimab Monotherapy in Patients With Mismatch Repair Deficient Solid Tumors. JAMA Netw Open. 2023 Nov 2;6(11):e2341165.
69. Kang YJ, O’Haire S, Franchini F, IJzerman M, Zalcberg J, Macrae F, et al. A scoping review and meta-analysis on the prevalence of pan-tumour biomarkers (dMMR, MSI, high TMB) in different solid tumours. Sci Rep. 2022 Dec 1;12(1).
70. Bonneville R, Krook MA, Kautto EA, Miya J, Wing MR, Chen HZ, et al. Landscape of Microsatellite Instability Across 39 Cancer Types. JCO Precis Oncol. 2017 Nov;(1):1–15.
71. Janku F, Tsimberidou A, Garrido-Laguna I et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. Mol Cancer Ther. 2011;7:558–65.
72. Druker BJ, Talpaz M, Resta DJ et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344(14):1031–7.
73. Demetri GD, von Mehren M, Blanke CD et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med. 2002;347(7):472–80.
74. Braiteh F, Kurzrock R. Uncommon tumors and exceptional therapies: paradox or paradigm? Mol Cancer Ther. 2007 Apr;6(4):1175–9.
75. Flaherty KT, Puzanov I, Kim KB et al. Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. J Mol Diagn. 2010;363(9):809–19.
76. Stewart DJ, Kurzrock R. Cancer: the road to Amiens. J Clin Oncol. 2009 Jan 20;27(3):328–33.
77. Seguí E, Paré L, Adamo B, Pineda E, Marín-Aguilera M, Reig Ó, et al. Immune gene expression, survivall, outcome and response to PD-1/PD-L1 blockade: a TCGA pan-cancer analysis. In: ASCO. Chicago; 2016.
78. Fields RC, Fleming MD, Gastman B, Gonzalez R, Johnson D, Joseph RW, et al. NCCN Clinical Practice Guidelines in Oncology. Melanoma. 2017;
79. Dummer R, Hauschild A, Lindenblatt N, Pentheroudakis G, Keilholz U. Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2015;

80. Aisner DL, Bauman J, Chirieac LR, D TA, DeCamp MM, Dilling TJ, et al. NCCN Clinical Practice Guidelines in Oncology. Non Samll Cell Lung Cancer. 2017;
81. Novello S, Barlesi F, Califano R, Cufer T, Ekman S, Levra MG, et al. Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2016;
82. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell [Internet]. 2002 Aug;2(2):117–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12204532
83. Drilon A, Oxnard GR, Tan DSW, Loong HHF, Johnson M, Gainor J, et al. Efficacy of Selpercatinib in *RET* Fusion–Positive Non–Small-Cell Lung Cancer. New England Journal of Medicine. 2020 Aug 27;383(9):813–24.
84. Wirth LJ, Sherman E, Robinson B, Solomon B, Kang H, Lorch J, et al. Efficacy of Selpercatinib in *RET* -Altered Thyroid Cancers. New England Journal of Medicine. 2020 Aug 27;383(9):825–35.
85. Yaeger R, Weiss J, Pelster MS, Spira AI, Barve M, Ou SHI, et al. Adagrasib with or without Cetuximab in Colorectal Cancer with Mutated *KRAS* G12C. New England Journal of Medicine. 2023 Jan 5;388(1):44–54.
86. Prat A, Pascual T, Juan M, Paz-Ares L, Felip E, Garralda E, et al. 1056P Efficacy of spartalizumab across multiple cancer types in patients with PD1-high mRNA expressing tumors (SOLTI-1904 ACROPOLI). Annals of Oncology. 2023 Oct;34:S639–40.
87. Paré L, Pascual T, Seguí E, Teixidó C, Gonzalez-Cao M, Galván P, et al. Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Annals of Oncology. 2018 Oct 1;29(10):2121–8.
88. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. Vol. 18, Cellular and Molecular Immunology. Springer Nature; 2021. p. 842–59.
89. Solis ES, Pascual T, Torres ES, Gonzalez-farre B, Brasó-Maristany F, Farre XG, et al. 2239P Gene expression profiling in tumors with and without tertiary lymphoid structures across cancer-types. Annals of Oncology. 2023 Oct;34:S1153–4.
90. Ladányi A. Prognostic and predictive significance of immune cells infiltrating cutaneous melanoma. Pigment Cell Melanoma Research. 2015;28:490–500.
91. Gooden, M.J.M., de Bock, G.H., Leffers, N., Daemen, T., and Nijman HW (2011). The prognostic influence of tumour-infiltrating lym- phocytes in cancer: a systematic review with meta-analysis. Br J Cancer. 2011;105:93–103.
92. Senovilla L, Vacchelli E, Galon J. Prognostic and predictive value of the immune infiltrate in cancer. OncoImmunol- ogy 1, 1323–1343. Oncoimmunology. 2012;1:1323–1343.
93. Goswami M, Toney NJ, Pitts SC, Celades C, Schlom J, Donahue RN. Peripheral immune biomarkers for immune checkpoint inhibition of solid tumours. Clin Transl Med. 2024 Aug;14(8).
94. Diehl A, Yarchoan M, Hopkins A, Jaffee E, Grossman SA. Relationships between lymphocyte counts and treatmentrelated toxicities and clinical responses in

patients with solid tumors treated with PD-1 checkpoint inhibitors. Oncotarget. 2017;8(69):114268–80.
95. Conroy MR, O'Sullivan H, Collins DC, Bambury RM, Power D, Grossman S, et al. Exploring the prognostic impact of absolute lymphocyte count in patients treated with immune-checkpoint inhibitors. BJC Reports. 2024 Apr 18;2(1).
96. Sun R, Champiat S, Dercle L, Aspeslagh S, Castanon E, Limkin EJ, et al. Baseline lymphopenia should not be used as exclusion criteria in early clinical trials investigating immune checkpoint blockers (PD-1/PD-L1 inhibitors). Eur J Cancer. 2017;84:202–11.
97. Bigot F, Castanon E, Baldini C, Hollebecque A, Carmona A, Postel-Vinay S, et al. Prospective validation of a prognostic score for patients in immunotherapy phase I trials: The Gustave Roussy Immune Score (GRIm-Score). Eur J Cancer. 2017;84:212–8.
98. Ho WJ, Yarchoan M, Hopkins A, Mehra R, Grossman S, Kang H. Association between pretreatment lymphocyte count and response to PD1 inhibitors in head and neck squamous cell carcinomas. J Immunother Cancer. 2018;6(1):1–8.
99. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat Med. 2016;22(4):433–8.
100. Zheng H, Liu X, Zhang J, Rice SJ, Wagman M, Kong Y, et al. Expression of PD-1 on CD4 + T cells in peripheral blood associates with poor clinical outcome in non-small cell lung cancer [Internet]. Vol. 7. Available from: www.impactjournals.com/oncotarget
101. Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. Proc Natl Acad Sci U S A. 2017;114(19):4993–8.
102. Kim KH, Cho J, Ku BM, Koh J, Sun JM, Lee SH, et al. The First-week Proliferative Response of Peripheral Blood PD-1+CD8+ T Cells Predicts the Response to Anti-PD-1 Therapy in Solid Tumors. Clinical Cancer Research. 2019 Apr 1;25(7):2144–54.
103. Liu J, Liu D, Hu G, Wang J, Chen D, Song C, et al. Circulating memory PD-1+CD8+ T cells and PD-1+CD8+T/PD-1+CD4+T cell ratio predict response and outcome to immunotherapy in advanced gastric cancer patients. Cancer Cell Int. 2023 Nov 16;23(1):274.
104. Han J, Duan J, Bai H, Wang Y, Wan R, Wang X, et al. TCR Repertoire Diversity of Peripheral PD-1+CD8+ T Cells Predicts Clinical Outcomes after Immunotherapy in Patients with Non–Small Cell Lung Cancer. Cancer Immunol Res. 2020 Jan 1;8(1):146–54.
105. Simon S, Voillet V, Vignard V, Wu Z, Dabrowski C, Jouand N, et al. PD-1 and TIGIT coexpression identifies a circulating CD8 T cell subset predictive of response to anti-PD-1 therapy. J Immunother Cancer. 2020 Nov 13;8(2):e001631.
106. Shen R, Postow MA, Adamow M, Arora A, Hannum M, Maher C, et al. LAG-3 expression on peripheral blood cells identifies patients with poorer outcomes after immune checkpoint blockade. Sci Transl Med. 2021 Aug 25;13(608).
107. Cirillo A, Zizzari IG, Botticelli A, Strigari L, Rahimi H, Scagnoli S, et al. Circulating CD137+ T Cell Levels Are Correlated with Response to Pembrolizumab

Treatment in Advanced Head and Neck Cancer Patients. Int J Mol Sci. 2023 Apr 12;24(8):7114.
108. Zuazo-Ibarra M, Arasanz H, Fernández-Hinojal G, María GC, Hernández-Marín B, Martínez-Aguillo M, et al. Highly differentiated CD4 T cells Unequivocally Identify Primary Resistance and Risk of Hyperprogression to PD-L1/PD-1 Immune Checkpoint Blockade in Lung Cancer [Internet]. 2018. Available from: http://biorxiv.org/lookup/doi/10.1101/320176
109. Saâda-Bouzid E, Defaucheux C, Karabajakian A, Coloma VP, Servois V, Paoletti X, et al. Hyperprogression during anti-PD-1/PD-L1 therapy in patients with recurrent and/or metastatic head and neck squamous cell carcinoma. Annals of Oncology. 2017 Jul;28(7):1605–11.
110. Arasanz H, Zuazo M, Bocanegra A, Gato M, Martínez-Aguillo M, Morilla I, et al. Early Detection of Hyperprogressive Disease in Non-Small Cell Lung Cancer by Monitoring of Systemic T Cell Dynamics. Cancers (Basel). 2020 Feb 4;12(2):344.
111. Duchemann B, Remon J, Naigeon M, Mezquita L, Ferrara R, Cassard L, et al. Integrating circulating biomarkers in the immune checkpoint inhibitor treatment in lung cancer. Vol. 12, Cancers. MDPI AG; 2020. p. 1–20.
112. Duchemann B, Remon J, Naigeon M, Cassard L, Jouniaux JM, Boselli L, et al. Current and future biomarkers for outcomes with immunotherapy in non-small cell lung cancer. Vol. 10, Translational Lung Cancer Research. AME Publishing Company; 2021. p. 2937–54.
113. García-Corbacho J, Indacochea A, González Navarro AE, Victoria I, Moreno D, Pesántez D, et al. Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study. Cancer Immunology, Immunotherapy. 2023 Jun 1;72(6):1709–23.
114. Arkenau HT, Barriuso J, Olmos D, Ang JE, De Bono J, Judson I, et al. Prospective validation of a prognostic score to improve patient selection for oncology phase I trials. Journal of Clinical Oncology. 2009;27(16):2692–6.
115. Chau NG, Florescu A, Chan KK, Wang L, Chen EX, Bedard P, et al. Early mortality and overall survival in oncology phase I trial participants: Can we improve patient selection? BMC Cancer. 2011;11:8–10.
116. Day D, Guo C, Kanjanapan Y, Tran B, Spreafico A, Joshua AM, et al. Survival in early phase immuno-oncology trials: Development and validation of a prognostic index. JNCI Cancer Spectr. 2019;3(4):1–8.
117. Riudavets M, Auclin E, Mezquita L. Host circulating biomarkers for immune-checkpoint inhibitors: single-agent and combinations. Future Oncol. 2020;16(23):1665–8.
118. Mezquita L, Auclin E, Ferrara R, Charrier M, Remon J, Planchard D, et al. Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. JAMA Oncol. 2018;4(3):351–7.
119. Paramanathan A, Saxena A, Morris DL. A systematic review and meta-analysis on the impact of pre-operative neutrophil lymphocyte ratio on long term outcomes after curative intent resection of solid tumours. Vol. 23, Surgical Oncology. Elsevier BV; 2014. p. 31–9.

120. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228–47.
121. Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol. 2017;18(3):e143–52.
122. Wen PY, Macdonald DR, Reardon DA, Cloughesy TF, Sorensen AG, Galanis E, et al. Updated response assessment criteria for high-grade gliomas: Response assessment in neuro-oncology working group. Journal of Clinical Oncology. 2010;28(11):1963–72.
123. Paré L, Pascual T, Seguí E, Teixidó C, Gonzalez-Cao M, Galván P, et al. Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Annals of Oncology. 2018 Oct 1;29(10):2121–8.
124. Garcia-Corbacho J, Indacochea A, González-Navarro AE, Victoria ID, Moreno D, Pesántez D, et al. Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study. Cancer Immunology, Immunotherapy. 2023;72(6).
125. Arrieta O, Montes-Servín E, Hernandez-Martinez JM, Cardona AF, Casas-Ruiz E, Crispín JC, et al. Expression of PD-1/PD-L1 and PD-L2 in peripheral T-cells from non-small cell lung cancer patients. Oncotarget. 2017 Nov 24;8(60):101994–2005.
126. Xia L, Guo L, Kang J, Yang Y, Yao Y, Xia W, et al. Predictable Roles of Peripheral IgM Memory B Cells for the Responses to Anti-PD-1 Monotherapy Against Advanced Non-Small Cell Lung Cancer. Front Immunol. 2021 Nov 24;12.
127. Carril-Ajuria L, Desnoyer A, Meylan M, Dalban C, Naigeon M, Cassard L, et al. Baseline circulating unswitched memory B cells and B-cell related soluble factors are associated with overall survival in patients with clear cell renal cell carcinoma treated with nivolumab within the NIVOREN GETUG-AFU 26 study. J Immunother Cancer. 2022 May 31;10(5).
128. Petitprez F, de Reyniès A, Keung EZ, Chen TWW, Sun CM, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. Nature. 2020 Jan;577(7791):556–60.
129. Pesántez D, Indacochea A, Angelats L, Sirico M, Victoria I, Sanfeliu E, et al. Unexpected Durable Complete Response With Anti–PD-L1 Blockade in Metastatic Undifferentiated Pleomorphic Sarcoma: A Case Report With Host and Tumor Biomarker Analysis. JCO Precis Oncol. 2023 Sep;(7).
130. Barth DA, Stanzer S, Spiegelberg JA, Bauernhofer T, Absenger G, Szkandera J, et al. Patterns of Peripheral Blood B-Cell Subtypes Are Associated With Treatment Response in Patients Treated With Immune Checkpoint Inhibitors: A Prospective Longitudinal Pan-Cancer Study. Front Immunol. 2022 Apr 1;13.
131. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228–47.
132. Wen PY, Van Den Bent M, Youssef G, Cloughesy TF, Ellingson BM, Weller M, et al. RANO 2.0: Update to the Response Assessment in Neuro-Oncology Criteria

for High- and Low-Grade Gliomas in Adults. Journal of Clinical Oncology. 2023 Nov 20;41(33):5187–99.
133. Schettini F, Conte B, Buono G, De Placido P, Parola S, Griguolo G, et al. T-DM1 versus pertuzumab, trastuzumab and a taxane as first-line therapy of early-relapsed HER2-positive metastatic breast cancer: an Italian multicenter observational study. ESMO Open. 2021 Apr 1;6(2).
134. Rothman KJ. Six persistent research misconceptions. Vol. 29, Journal of General Internal Medicine. Springer New York LLC; 2014. p. 1060–4.
135. Rothman KJ. No Adjustments Are Needed for Multiple Comparisons. Vol. 1. 1990.
136. Demaria S, Ng B, Devitt ML, Babb JS, Kawashima N, Liebes L, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys. 2004 Mar 1;58(3):862–70.
137. Seiwert TY, Kiess AP. Time to Debunk an Urban Myth? The “Abscopal Effect” With Radiation and Anti–PD-1. Journal of Clinical Oncology. 2021 Jan 1;39(1):1–3.
138. Lee NY, Ferris RL, Psyrri A, Haddad RI, Tahara M, Bourhis J, et al. Avelumab plus standard-of-care chemoradiotherapy versus chemoradiotherapy alone in patients with locally advanced squamous cell carcinoma of the head and neck: a randomised, double-blind, placebo-controlled, multicentre, phase 3 trial. Lancet Oncol. 2021 Apr;22(4):450–62.
139. Mcbride S, Sherman E, Tsai ; C Jillian, Baxi S, Aghalar J, Eng J, et al. Randomized Phase II Trial of Nivolumab With Stereotactic Body Radiotherapy Versus Nivolumab Alone in Metastatic Head and Neck Squamous Cell Carcinoma. J Clin Oncol [Internet]. 2020;39:30–7. Available from: https://doi.
140. Pinato DJ, Howlett S, Ottaviani D, Urus H, Patel A, Mineo T, et al. Association of prior antibiotic treatment with survival and response to immune checkpoint inhibitor therapy in patients with cancer. JAMA Oncol. 2019 Dec 1;5(12):1774–8.
141. Cortellini A, Ricciuti B, Facchinetti F, Alessi JVM, Venkatraman D, Dall’Olio FG, et al. Antibiotic-exposed patients with non-small-cell lung cancer preserve efficacy outcomes following first-line chemo-immunotherapy. Annals of Oncology. 2021 Nov 1;32(11):1391–9.
142. Maslov D V, Tawagi K, KC M, Simenson V, Yuan H, Parent C, et al. Timing of steroid initiation and response rates to immune checkpoint inhibitors in metastatic cancer. J Immunother Cancer. 2021 Jul 5;9(7):e002261.
143. Ruiz-Bañobre J, Areses-Manrique MC, Mosquera-Martínez J, Cortegoso A, Afonso-Afonso FJ, De Dios-Álvarez N, et al. Evaluation of the lung immune prognostic index in advanced nonsmall cell lung cancer patients under nivolumab monotherapy. Transl Lung Cancer Res. 2019 Dec 1;8(6):1078–85.
144. Meyers DE, Stukalin I, Vallerand IA, Lewinson RT, Suo A, Dean M, et al. The lung immune prognostic index discriminates survival outcomes in patients with solid tumors treated with immune checkpoint inhibitors. Cancers (Basel). 2019 Nov 1;11(11).
145. Inno A, Roviello G, Ghidini A, Luciani A, Catalano M, Gori S, et al. Rechallenge of immune checkpoint inhibitors: A systematic review and meta-analysis. Crit Rev Oncol Hematol. 2021 Sep;165:103434.

146. Schmid P, Salgado R, Park YH, Muñoz-Couselo E, Kim SB, Sohn J, et al. Pembrolizumab plus chemotherapy as neoadjuvant treatment of high-risk, early-stage triple-negative breast cancer: results from the phase 1b open-label, multicohort KEYNOTE-173 study. Annals of Oncology. 2020 May 1;31(5):569–81.
147. Keilson JM, Knochelmann HM, Paulos CM, Kudchadkar RR, Lowe MC. The evolving landscape of immunotherapy in solid tumors. J Surg Oncol. 2021 Mar 17;123(3):798–806.
148. Arpino G, Michelotti A, Truini M, Montemurro F, Russo S, Palumbo R, et al. Demographic, tumor and clinical features of clinical trials versus clinical practice patients with HER2-positive early breast cancer: results of a prospective study. J Cancer Res Clin Oncol. 2016 Mar 1;142(3):669–78.
149. De Placido S, Giuliano M, Schettini F, Von Arx C, Buono G, Riccardi F, et al. Human epidermal growth factor receptor 2 dual blockade with trastuzumab and pertuzumab in real life: Italian clinical practice versus the CLEOPATRA trial results. Breast. 2018 Apr;38:86–91.
150. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. Mol Cancer Ther. 2017 Nov 1;16(11):2598–608.
151. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. Vol. 18, Cellular and Molecular Immunology. Springer Nature; 2021. p. 842–59.
152. Hendry S, Salgado R, Gevaert T, Russell PA, John T, Thapa B, et al. Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method from the International Immuno-Oncology Biomarkers Working Group: Part 2: TILs in Melanoma, Gastrointestinal Tract Carcinomas, Non–Small Cell Lung Carcinoma and Mesothelioma, Endometrial and Ovarian Carcinomas, Squamous Cell Carcinoma of the Head and Neck, Genitourinary Carcinomas, and Primary Brain Tumors. Adv Anat Pathol. 2017 Nov;24(6):311–35.
153. Vigliar E, Malapelle U, Iaccarino A, Acanfora G, Pisapia P, Clery E, et al. PD-L1 expression on routine samples of non-small cell lung cancer: Results and critical issues from a 1-year experience of a centralised laboratory. J Clin Pathol. 2019 Jun 1;72(6):412–7.
154. Schettini F, Corona SP, Giudici F, Strina C, Sirico M, Bernocchi O, et al. Clinical, Radiometabolic and Immunologic Effects of Olaparib in Locally Advanced Triple Negative Breast Cancer: The OLTRE Window of Opportunity Trial. Front Oncol. 2021 Jun 28;11.
155. De Marchi P, Leal LF, Duval da Silva V, da Silva ECA, Cordeiro de Lima VC, Reis RM. PD-L1 expression by Tumor Proportion Score (TPS) and Combined Positive Score (CPS) are similar in non-small cell lung cancer (NSCLC). J Clin Pathol. 2021 Nov;74(11):735–40.
156. Hothorn T, Zeileis A. Generalized maximally selected statistics. Biometrics. 2008;64(4):1263–9.
157. Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im SA, Yusof MM, et al. Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative

breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. Lancet. 2020 Dec 5;396(10265):1817–28.
158. Choi JH, Ro JY. The 2020 WHO Classification of Tumors of Soft Tissue: Selected Changes and New Entities [Internet]. 2020. Available from: http://journals.lww.com/anatomicpathology
159. Bhalla AD, Landers SM, Singh AK, Landry JP, Yeagley MG, Myerson GSB, et al. Experimental models of undifferentiated pleomorphic sarcoma and malignant peripheral nerve sheath tumor. Laboratory Investigation. 2022 Jun 1;102(6):658–66.
160. Italiano A, Mathoulin-Pelissier S, Cesne A Le, Terrier P, Bonvalot S, Collin F, et al. Trends in survival for patients with metastatic soft-tissue sarcoma. Cancer. 2011 Mar 1;117(5):1049–54.
161. Cancer Genome Atlas Research Network. Electronic address: elizabeth.demicco@sinaihealthsystem.ca, Cancer Genome Atlas Research Network. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. Cell. 2017 Nov 2;171(4):950-965.e28.
162. Steele CD, Tarabichi M, Oukrif D, Webster AP, Ye H, Fittall M, et al. Undifferentiated Sarcomas Develop through Distinct Evolutionary Pathways. Cancer Cell. 2019 Mar 18;35(3):441-456.e8.
163. Blank CU, Haanen JB, Ribas A, Schumacher TN. CANCER IMMUNOLOGY. The "cancer immunogram". Science. 2016 May 6;352(6286):658–60.
164. Garcia-Corbacho J, Indacochea A, Gonzalez A, Victoria Ruiz I, Moreno Fernández D, Pesantez Coronel DS, et al. 125P Assessing the accuracy of multiple prognostic scores for immune checkpoint inhibitors (ICI) in patients with advanced solid tumors. Annals of Oncology [Internet]. 2022 Sep;33:S594–5. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0923753422020087
165. Schettini F, Brasó-Maristany F, Kuderer NM, Prat A. A perspective on the development and lack of interchangeability of the breast cancer intrinsic subtypes. NPJ Breast Cancer. 2022 Jul 19;8(1):85.
166. Fan C, Prat A, Parker JS, Liu Y, Carey LA, Troester MA, et al. Building prognostic models for breast cancer patients using clinical variables and hundreds of gene expression signatures. BMC Med Genomics. 2011 Jan 9;4:3.
167. Tawbi HA, Burgess M, Bolejack V, Van Tine BA, Schuetze SM, Hu J, et al. Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. Lancet Oncol. 2017 Nov;18(11):1493–501.
168. Toulmonde M, Penel N, Adam J, Chevreau C, Blay JY, Le Cesne A, et al. Use of PD-1 Targeting, Macrophage Infiltration, and IDO Pathway Activation in Sarcomas: A Phase 2 Clinical Trial. JAMA Oncol. 2018 Jan 1;4(1):93–7.
169. D'Angelo SP, Mahoney MR, Van Tine BA, Atkins J, Milhem MM, Jahagirdar BN, et al. Nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401): two open-label, non-comparative, randomised, phase 2 trials. Lancet Oncol. 2018 Mar;19(3):416–26.
170. Monga V, Skubitz KM, Maliske S, Mott SL, Dietz H, Hirbe AC, et al. A Retrospective Analysis of the Efficacy of Immunotherapy in Metastatic Soft-Tissue Sarcomas. Cancers (Basel). 2020 Jul 11;12(7).

171. Hutchinson L. Improving durable responses with TILs. Nature Publishing Group [Internet]. 2016;13(7):397. Available from: http://dx.doi.org/10.1038/nrclinonc.2016.95
172. Pollack SM, He Q, Yearley JH, Emerson R, Vignali M, Zhang Y, et al. T-cell infiltration and clonality correlate with programmed cell death protein 1 and programmed death-ligand 1 expression in patients with soft tissue sarcomas. Cancer. 2017 Sep 1;123(17):3291–304.
173. Sha D, Jin Z, Budczies J, Kluck K, Stenzinger A, Sinicrope FA. Tumor Mutational Burden as a Predictive Biomarker in Solid Tumors. Cancer Discov. 2020 Dec;10(12):1808–25.
174. Serrano C, Romagosa C, Hernández-Losa J, Simonetti S, Valverde C, Moliné T, et al. RAS/MAPK pathway hyperactivation determines poor prognosis in undifferentiated pleomorphic sarcomas. Cancer. 2016 Jan 1;122(1):99–107.
175. Napolitano A, Ostler AE, Jones RL, Huang PH. Fibroblast Growth Factor Receptor (FGFR) Signaling in GIST and Soft Tissue Sarcomas. Cells. 2021 Jun 17;10(6).
176. Patruni S, Khattab A, Abel S, Hasan S, Rizwan S, Finley GG, et al. A comparative analysis of survival in patients with non-small cell lung cancer with brain metastases receiving intracranial radiation with and without immunotherapy. Journal of Clinical Oncology. 2019 May 20;37(15_suppl):9025–9025.
177. Hendriks LEL, Henon C, Auclin E, Mezquita L, Ferrara R, Audigier-Valette C, et al. Outcome of Patients with Non-Small Cell Lung Cancer and Brain Metastases Treated with Checkpoint Inhibitors. J Thorac Oncol. 2019 Jul;14(7):1244–54.
178. Doroshow DB, Bhalla S, Beasley MB, Sholl LM, Kerr KM, Gnjatic S, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2021 Jun;18(6):345–62.
179. Paver EC, Cooper WA, Colebatch AJ, Ferguson PM, Hill SK, Lum T, et al. Programmed death ligand-1 (PD-L1) as a predictive marker for immunotherapy in solid tumours: a guide to immunohistochemistry implementation and interpretation. Pathology. 2021 Feb;53(2):141–56.
180. Roulleaux Dugage M, Nassif EF, Italiano A, Bahleda R. Improving Immunotherapy Efficacy in Soft-Tissue Sarcomas: A Biomarker Driven and Histotype Tailored Review. Front Immunol. 2021;12:775761.
181. Conte B, Brasó-Maristany F, Pascual T, Schettini F, Staaf J, Paré L, et al. 3MO Association of the research-based HER2DX signatures with survival in early-stage triple-negative breast cancer (eTNBC). Annals of Oncology. 2022 May;33:S124–5.
182. Schettini F, Paré L, Marín-Aguilera M, Puig S, Mezquita L, Baste N, et al. 14-gene immunoglobulin (IGG) and proliferation signatures and association with overall survival across cancer-types. Journal of Clinical Oncology. 2022 Jun 1;40(16_suppl):2636–2636.
183. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci. 2019 Jul 18;110(7):2080–9.
184. Italiano A, Bessede A, Pulido M, Bompas E, Piperno-Neumann S, Chevreau C, et al. Pembrolizumab in soft-tissue sarcomas with tertiary lymphoid structures: a phase 2 PEMBROSARC trial cohort. Nat Med. 2022 Jun;28(6):1199–206.
185. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors.

Nat Rev Dis Primers [Internet]. 2020 May 7;6(1):38. Available from: https://www.nature.com/articles/s41572-020-0160-6
186. Kohn C, Shcherbakova N|, Montero AJ. Cost Effectiveness and Financial Toxicity in New Cancer Therapies. In Springer Publishing Company; p. 231–40.
187. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primers [Internet]. 2020 May 7;6(1):38. Available from: https://www.nature.com/articles/s41572-020-0160-6
188. Arkenau HT, Barriuso J, Olmos D, Ang JE, De Bono J, Judson I, et al. Prospective validation of a prognostic score to improve patient selection for oncology phase I trials. Journal of Clinical Oncology. 2009 Jun 1;27(16):2692–6.
189. Day D, Guo C, Kanjanapan Y, Tran B, Spreafico A, Joshua AM, et al. Survival in early phase immuno-oncology trials: Development and validation of a prognostic index. JNCI Cancer Spectr. 2019 Dec 1;3(4).
190. Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol [Internet]. 2017;18(3):e143–52. Available from: http://dx.doi.org/10.1016/S1470-2045(17)30074-8
191. Akaike H. Information Theory and an Extension of the Maximum Likelihood Principle. In 1998. p. 199–213.
192. Tan S, Li D, Zhu X. Cancer immunotherapy: Pros, cons and beyond. Vol. 124, Biomedicine and Pharmacotherapy. Elsevier Masson SAS; 2020.
193. Sehgal K. Hyperprogression in Patients with Cancer Receiving Immune Checkpoint Inhibitors. Vol. 4, JAMA Network Open. American Medical Association; 2021.
194. Lawrence MS, Stojanov P, Polak P, Kryukov G V., Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499(7457):214–8.
195. Meléndez B, Van Campenhout C, Rorive S, Remmelink M, Salmon I, D'Haene N. Methods of measurement for tumor mutational burden in tumor tissue. Vol. 7, Translational Lung Cancer Research. AME Publishing Company; 2018. p. 661–7.
196. Sautès-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. Vol. 19, Nature Reviews Cancer. Nature Publishing Group; 2019. p. 307–25.
197. Chang TG, Cao Y, Sfreddo HJ, Dhruba SR, Lee SH, Valero C, et al. LORIS robustly predicts patient outcomes with immune checkpoint blockade therapy using common clinical, pathologic and genomic features. Nat Cancer. 2024 Jun 3;5(8):1158–75.
198. Hernando-Calvo A, Yang SYC, Vila-Casadesús M, Han M, Liu ZA, Berman AHK, et al. Combined Transcriptome and Circulating Tumor DNA Longitudinal Biomarker Analysis Associates With Clinical Outcomes in Advanced Solid Tumors Treated With Pembrolizumab. JCO Precis Oncol. 2024 Aug;(8).
199. Petrelli F, Cabiddu M, Coinu A, Borgonovo K, Ghilardi M, Lonati V, et al. Prognostic role of lactate dehydrogenase in solid tumors: A systematic review and meta-analysis of 76 studies. Vol. 54, Acta Oncologica. Informa Healthcare; 2015. p. 961–70.

200. McMillan DC. The systemic inflammation-based Glasgow Prognostic Score: A decade of experience in patients with cancer. Vol. 39, Cancer Treatment Reviews. 2013. p. 534–40.
201. McMillan DC. The systemic inflammation-based Glasgow Prognostic Score: A decade of experience in patients with cancer. Cancer Treat Rev. 2013 Aug;39(5):534–40.
202. Laird BJA, Fallon M, Hjermstad MJ, Tuck S, Kaasa S, Klepstad P, et al. Quality of Life in Patients With Advanced Cancer: Differential Association With Performance Status and Systemic Inflammatory Response. Journal of Clinical Oncology. 2016 Aug 10;34(23):2769–75.
203. Miholjcic TBS, Halse H, Bonvalet M, Bigorgne A, Rouanne M, Dercle L, et al. Rationale for LDH-targeted cancer immunotherapy. Vol. 181, European Journal of Cancer. Elsevier Ltd; 2023. p. 166–78.
204. Claps G, Faouzi S, Quidville V, Chehade F, Shen S, Vagner S, et al. The multiple roles of LDH in cancer. Vol. 19, Nature Reviews Clinical Oncology. Springer Nature; 2022. p. 749–62.
205. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Vol. 12, Nature Reviews Cancer. 2012. p. 252–64.
206. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic Diversity and Plasticity in Circulating Neutrophil Subpopulations in Cancer. Cell Rep. 2015 Feb;10(4):562–73.
207. Liu S, Wu W, Du Y, Yin H, Chen Q, Yu W, et al. The evolution and heterogeneity of neutrophils in cancers: origins, subsets, functions, orchestrations and clinical applications. Mol Cancer. 2023 Sep 7;22(1):148.
208. Nelli F, Fabbri A, Virtuoso A, Giannarelli D, Giron Berrios JR, Marrucci E, et al. Early Changes in LIPI Score Predict Immune-Related Adverse Events: A Propensity Score Matched Analysis in Advanced Non-Small Cell Lung Cancer Patients on Immune Checkpoint Blockade. Cancers (Basel). 2024 Jan 1;16(2).
209. Pinato DJ, Gramenitskaya D, Altmann DM, Boyton RJ, Mullish BH, Marchesi JR, et al. Antibiotic therapy and outcome from immune-checkpoint inhibitors. Vol. 7, Journal for ImmunoTherapy of Cancer. BioMed Central Ltd.; 2019.

APPENDIX

APPENDIX 1: First Publication (Cancer Inmunology, Immunotherapy 2023, Cuartile 1)

Cancer Immunology, Immunotherapy
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RESEARCH

Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study

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Abstract
Immune-checkpoint inhibitors (ICI) have revolutionized the therapeutic landscape of cancer. However, optimal patient selection is still an unmet need. One-hundred-forty-six patients with metastatic cancer candidates to ICI at the Hospital Clinic of Barcelona Clinical Trials Unit were prospectively recruited in this observational study. Blood samples were collected at different timepoints, baseline LIPI score calculated and pre-ICI archived tissues retrieved to evaluate PD-L1, tumor-infiltrating lymphocytes (TILs) and PD1 mRNA levels. Tumor assessments were centrally reviewed by RECIST 1.1 criteria. Associations with overall response rates (ORR), durable clinical benefit (DCB), progression-free survival (PFS) and overall survival (OS) were performed with univariable/multivariable logistic and Cox regressions, where appropriate. At a median follow-up of 26.9 months, median PFS and OS were 2.7 and 12.9 months. Response rates were 17.8% with duration of response (DOR) of 4.4 months. LIPI score was independently associated with PFS ($p=0.025$) and OS ($p<0.001$). Immunotherapy-naïve status was independently associated with better PFS ($p=0.005$). Time-to-best response (TTBR) and ORR ($p<0.001$ both) were associated with better OS at univariate analysis. PFS and DOR were moderately correlated with OS ($p<0.001$ both). A PD-L1 10% cut-off detected worse/best responders in terms of ORR (univariate p=0.011, multivariate $p=0.028$) and DCB (univariate $p=0.043$). PD1 mRNA levels were strikingly associated to complete responses ($p=0.021$). To resume, in our prospective observational pan-cancer study, baseline LIPI score, immunotherapy-naïve status, cancer type and RT before starting ICI were the most relevant clinical factors independently correlated with immunotherapy outcomes. Longer TTBR seemed to associate with better survival, while PD1 mRNA and PD-L1 protein levels might be tumor-agnostic predictive factors of response to ICI and should be furtherly explored.

Keywords Immunotherapy · Immune checkpoint inhibitors · PD-L1 · PD1 · Solid tumors

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Introduction

In the last decade, immunotherapy with immune-checkpoint inhibitors (ICI) has revolutionized the therapeutic landscape of many solid tumors. ICI-based therapeutic approach is based on the disruption of the activity of several immune system inhibitory mechanisms, so to unleash a potent immune response directed toward the tumor [1]. The majority of currently approved ICI act through the inhibition of the PD1/PD-L1 axis [2]. As of today, anti-PD1 (e.g., pembrolizumab, nivolumab) and anti-PD-L1 (e.g., atezolizumab, durvalumab) monoclonal antibodies (mAb) have become some of the most widely prescribed anticancer therapies and are recommended, in monotherapy or combination with other ICI or chemotherapy (CT), in a broad spectrum of cancer types [1]. However, the degree of benefit is different according to the cancer type and within each tumor type, and only a limited proportion of patients seem to benefit [3].

The only predictive biomarkers of response that can be used in clinical practice are the assessment of PD-L1 levels by immunohistochemistry (IHC), micro-satellite instability (MSI) and tumor mutational burden (TMB), though the latter only in the USA [4–7]. However, they have been variably successful in predicting responders according to different cancers and their use is limited to specific contexts [4–6]. The outcome of ICI therapy has also been linked to the quality and magnitude of tumor-infiltrating lymphocytes (TILs)' responses within the tumor microenvironment, though without current clinical applicability [8]. Additionally, the optimal metastatic therapeutic setting (earlier or further lines), the efficacy in immune-pretreated patients, the effects of exposure to immediately previous or concurrent radiotherapy (RT), and the optimal duration of treatment remain questions unanswered. To note, the impact of systemic corticosteroids and exposure to antibiotic (ATB) therapy on response to ICI are another major concern, with only few and/or conflicting data being published so far [9–18]. Finally, easy-to-detect and relatively low cost prognostic predictors able to stratify patients for either ICI clinical trial inclusion or better tailoring of the treatment strategy are urgently needed and the LIPI score, based on a relative neutrophil count and LDH is a promising one, which merits further validation in a pan-cancer setting [19, 20].

The Bioimmunoblood project is a prospective observational study which is currently ongoing at the Clinical Trials Unit of the Hospital Clinic of Barcelona (HCB) Medical Oncology Department. Within this project we aim at characterizing the patterns of response to anti-PD1 and anti-PD-L1 ICI in metastatic solid tumors and exploring patients' clinicopathological, molecular and blood features that can be useful to improve the selection of candidates for this relatively novel therapeutic approach. Here we report the main clinical results, while extensive molecular characterization and blood biomarker study are currently ongoing.

Materials and methods

Study design and participants

To enter the Bioimmunoblood study, eligible patients had to be diagnosed of metastatic solid tumor and about to start a treatment with an ICI in a clinical trial. Full inclusion/exclusion criteria are reported in Fig. 1.

We considered evaluable for this analysis all participants treated with an anti-PD1 or anti-PD-L1 ICI with radiological data available for an independent assessment of tumor

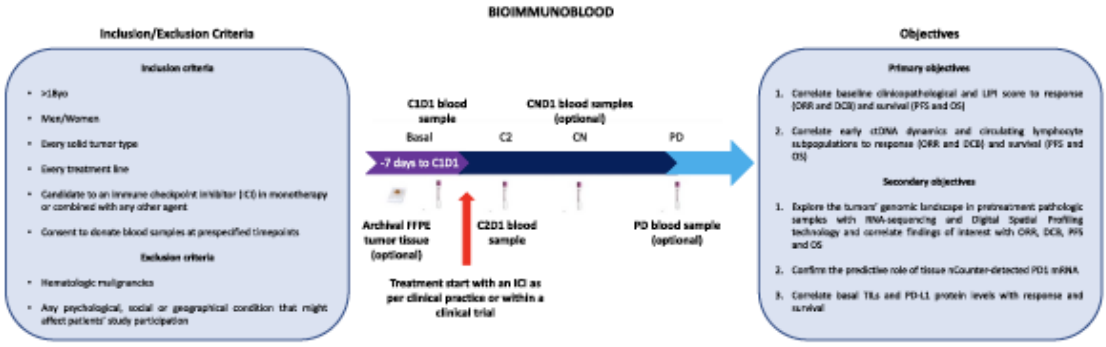

Fig. 1 Bioimmunoblood study design. *C* cycle, *D* day, *FFPE* fresh-frozen paraffin-embedded, *ICI* immune-checkpoint inhibitors, *ORR* overall response rate, *DCB* durable clinical benefit, *PFS* progression-free survival, *OS* overall survival, *TILs* tumor-infiltrating lymphocytes, *ctDNA* circulating tumor DNA, *PD* progressive disease, *yo* years old

responses according to RECIST 1.1 criteria [21]. Patients with available baseline imaging experiencing a rapid progression leading to death, hence with no available radiologic reassessment, were also included.

Procedures

A blood sample was collected from each patient at the first day of cycle 1 (C1D1) and 2 (C2D1) prior to receive the therapy and at each radiological evaluation of response until progression. For this analysis only basal samples were considered. Blood chemistry tests were carried out, including the evaluation of albumin, hemoglobin (Hb), LDH and standard leukocyte populations. The lung immune prognostic index (LIPI) score was also calculated [22]. Treatments and follow-up procedures were decided outside of this study according to study protocol, since patients received ICI in interventional clinical trials. All data were retrieved from electronic patient charts. In case of availability and explicit patient consent, archived tumor sections from the primary or the latest available metastatic biopsy before starting ICI were collected. An expert pathologist from the HCB (ES) carried out an assessment of TILs according to the methodology proposed by the International Immuno-Oncology Biomarkers Working Group [23]. PD1 mRNA expression was evaluated using the Nanostring nCounter® platform as we elsewhere described [24]. PD-L1 was assessed according to the HCB clinical practice and using the anti-PD-L1 mouse monoclonal antibody 22C3 (Dako), following manufacturer's recommendation [25, 26] (Supplementary materials).

Study endpoints and outcomes

There was no prespecified sample size because of the exploratory nature of this study. The accrual was terminated after 4 years, and the clinical data cut-off was established when a minimum follow-up including at least one reassessment of the disease for every included patient was reached.

This first analysis was intended to correlate baseline clinicopathological factors to response, in terms of overall response rate (ORR) and durable clinical benefit (DCB), and survival, in terms of progression-free survival (PFS) and overall survival (OS) (Primary Objective 1, Fig. 1). The primary features of interest were treatment line at which an anti-PD1 or PD-L1 ICI is delivered (1st vs. subsequent lines), patients' immune-naïve status (yes vs. no), the regimen type (ICI monotherapy vs. ICI-based combination), the ICI target (anti-PD1 vs. anti-PD-L1), having received RT, systemic ATB or corticosteroids (> 10 mg prednisone equivalent dose) within 30 days before, or during ICI treatment, as well as cancer type according to the following groups: NSCLC, genitourinary (GU) tumors, gastrointestinal (GI) tumors, breast cancer/gynecological tumors, other rarer tumors. The effect on OS for the time-to-best response (TTBR) and duration of response (DOR) in patients achieving at least a stable disease (SD), was investigated, as well. The prognostic value of the LIPI score in terms of PFS and OS in a pan-cancer context was also assessed.

Further objectives of this first report were to explore TILs, PD-L1 protein and PD1 mRNA impact on ORR, DCB, PFS and OS in patients treated with anti-PD1/PD-L1 ICI (Secondary Objectives 2–3, Fig. 1).

The evaluation of response for the purpose of this study were performed in accordance to RECIST 1.1 criteria [21]. Best responses (BR) were classified as SD, progressive disease (PD), complete (CR) or partial response (PR) independently by the same expert (JGC) from the Clinical Trials Unit of the HCB [21]. For the ORR assessment we considered all patients achieving CR + PR as BR, while for DCB we included all patients achieving CR + PR + SD retained at 6 months as BR.

Statistical analysis

Multiple χ^2 tests and one-way ANOVA were used to calculate differences among poor, best and non-responders with respect to categorical and continuous variables of interest, respectively. For the purpose of this study, we considered as poor responders all patients that achieved SD as their BR, while best responders were those achieving PR or CR as their BR and non-responders were represented by patients with PD as BR. Correlations between continuous variables were evaluated with Pearson's r. Univariate and multivariable logistic regression analyses were performed to investigate the association between PD1 mRNA abundance with tumor response. Odds ratios (OR) with 95% confidence intervals (CI) were used as measure of association with ORR and DCB. The maximally selected rank statistics (MSRS) method was adopted to identify an exploratory optimal cut-off for PD1 mRNA, TILs and PD-L1 protein, considering PFS as the time-dependent endpoint [27]. Survival curves were estimated by the Kaplan–Meier method and differences between curves were evaluated by the log-rank test. Cox regression models were applied to estimate univariate and multivariate hazard ratios (HR) with their 95% CI to explore the association among clinicopathological/biological variables, TTBR, DOR, PFS and OS. For the primary endpoint of PFS, the proportional hazard assumption for the univariate and multivariate Cox regression models was previously tested using correlation coefficients between transformed survival times and scaled Schoenfeld residuals and further checked with the smoothed plots of Schoenfeld residuals [28]. The clinical data cut-off date for this analysis was 25 August 2021. Patients alive were censored at the date of the last follow-up.

A two-sided alfa error of 0.5 was considered for statistical significance. Considering the observational and exploratory nature of the study, we decided not to take into account the multiplicity issue [29, 30]. All statistical analyses were carried out using R Studio vers.1.0.153 (PBC, Boston, MA) and SPSS vers 24.0 (IBM SPSS Statistics, Armonk, NY: IBM Corp) for MacOSX. Full methods are reported in Supplementary materials.

Results

Between May 2017 and June 2021, 156 patients entered the study and 146 received an anti-PD1/anti-PD-L1-based treatment. The selection process for the purpose of this analysis is resumed in Fig. 2.

The median follow-up at the data cut-off (31/08/2021) was 26.9 months (95% CI: 13.1–31.7). All patients and tumors characteristics are detailed in Table 1.

A summary of activity and efficacy outcomes is reported in Table 2.

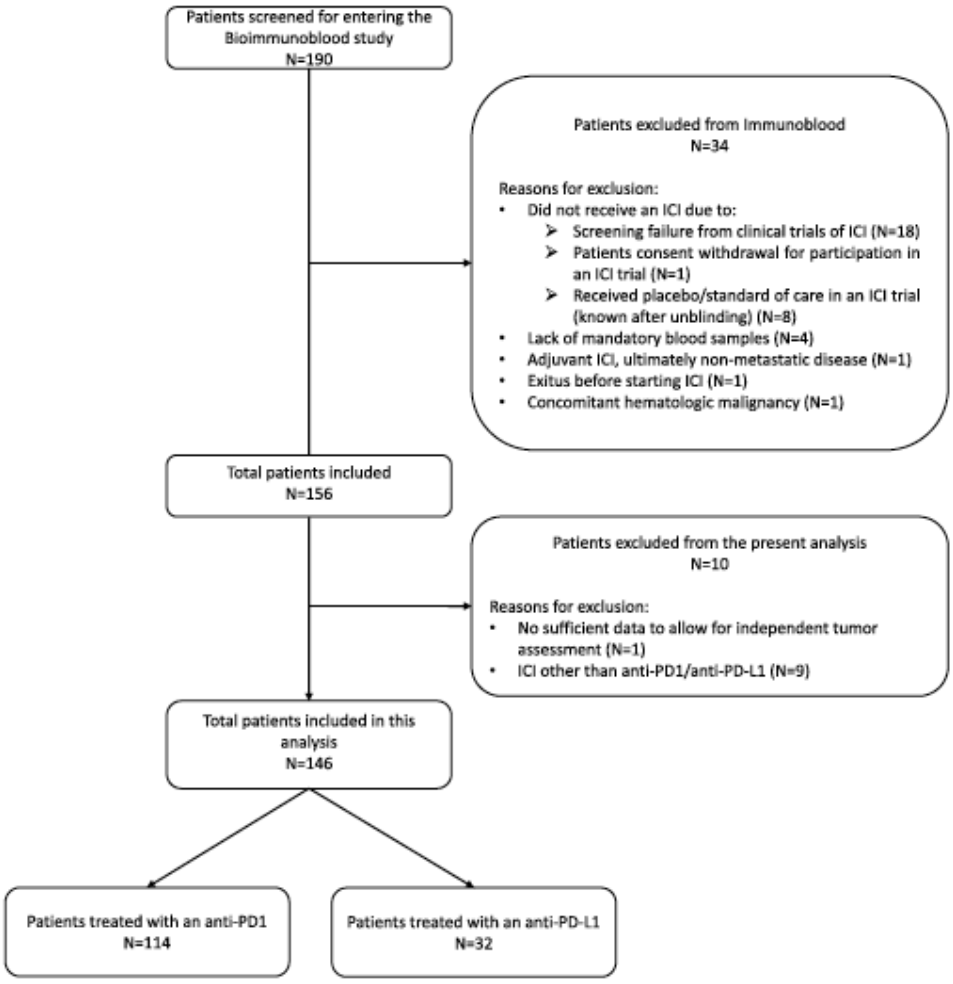

Fig. 2 STROBE flowchart. *ICI* immune-checkpoint inhibitors

Table 1 Population characteristics

Characteristics	Non-responders		Poor responders		Best responders		Overall		P*
	N	%	*N*	%	*N*	%	*N*	%	
	71	48.6	49	33.6	26	17.8	146	100.0	
Age									
Mean	63.3	-	62.6	-	65.0	-	63.3	-	0.69
SD	±12.3	-	±13.1	-	±8.2	-	±11.9	-	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Sex									
Female	27	38.0	15	30.6	7	26.9	49	33.6	0.51
Male	44	62.0	34	69.4	19	73.1	97	66.4	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
ECOG									
0-1	58	87.9	40	93.0	22	88.0	120	89.6	0.67
2-3	8	12.1	3	7.0	3	12.0	14	10.4	
Overall	66	93.0	43	87.8	25	96.2	134	91.8	
Cancer type									
NSCLC	19	26.8	8	11.3	14	28.6	41	28.1	**0.03**
GI cancers	22	31.0	12	16.9	5	10.2	39	26.7	
GU cancers	7	9.9	11	15.5	3	6.1	21	14.4	
CNS, H&N, melanoma and rare cancers	13	18.3	13	18.3	3	6.1	29	19.9	
Breast+gyneco	10	14.1	5	7.0	1	2.0	16	11.0	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic at diagnosis									
Yes	40	56.3	26	53.1	17	65.4	83	56.8	0.59
No	31	43.7	23	46.9	9	34.6	63	43.2	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic treatment line									
1st	16	22.5	12	24.5	12	46.2	40	27.4	0.14
2nd	20	28.2	17	34.7	7	26.9	44	30.1	
≥3rd	35	49.3	20	40.8	7	26.9	62	42.5	
Min-Max	1st - 10th	-	1st - 6th	-	1st - 4th	-	1st - 10th	-	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Immunotherapy-naïve									
Yes	58	81.7	47	95.9	25	96.2	130	89.0	**0.02**
No	13	18.3	2	4.1	1	3.8	16	11.0	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Type of regimen									
Monotherapy	35	49.3	18	36.7	14	53.8	67	45.9	0.20
Immunotherapy combination	20	28.2	11	22.4	4	15.4	35	24.0	
Immunotherapy + other	16	22.5	20	40.8	8	30.8	44	30.1	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	

Table 1 (continued)

Immunotherapy target									
PD1	60	84.5	33	67.3	21	80.8	114	78.1	0.08
PD-L1	11	15.5	16	32.7	5	19.2	32	21.9	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Clinical Trial									
Yes	47	66.2	39	79.6	14	53.8	100	68.5	0.06
No	24	33.8	10	20.4	12	46.2	46	31.5	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Number of metastatic sites									
<3	12	16.9	11	22.4	6	23.1	29	19.9	0.68
≥3	59	83.1	38	77.6	20	76.9	117	80.1	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Metastatic sites									
Visceral	55	77.5	37	75.5	21	80.8	113	77.4	0.87
Non-visceral	16	22.5	12	24.5	5	19.2	33	22.6	
Bone	15	21.1	13	26.5	4	15.4	32	21.9	
CNS*	5	7.0	2	4.1	1	3.8	8	5.5	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
TILs (%)									
Mean	7	-	5	-	7	-	6	-	0.44
SD	±8.9	-	±7.4	-	±8.4	-	±8.4	-	
Overall	54	76.1	29	59.2	19	73.1	102	69.9	
PD-L1									
Positive	15	65.2	9	75.0	11	100.0	35	76.1	0.08
Negative	8	34.8	3	25.0	0	0.0	11	23.9	
Overall	23	32.4	12	24.5	11	42.3	46	31.5	
LIPI Score									
Good	29	44.6	22	45.8	14	63.6	65	48.1	0.46
Intermediate	26	40.0	21	43.8	7	31.8	54	40.0	
Poor	10	15.4	5	10.4	1	4.5	16	11.9	
Overall	65	91.5	48	98.0	22	84.6	135	92.5	
PD1 mRNA									
Mean	-6.34	-	-7.13	-	-6.15	-	-6.5	-	0.14
SD	±1.45	-	±1.76	-	±1.50	-	±1.57	-	
Overall	36	50.7	18	36.7	14	53.8	68	46.6	
RT									
Yes in the 30 days before ICI	6	8.7	2	4.1	1	4.0	9	6.3	0.52
Not in the 30 days before ICI	63	91.3	47	95.9	24	96.0	134	93.7	
Overall	69	97.2	49	100.0	25	96.2	143	97.9	
Yes during ICI	21	30.4	9	18.4	6	24.0	36	25.2	0.33
No during ICI	48	69.6	40	81.6	19	76.0	107	74.8	
Overall	69	97.2	49	100.0	25	96.2	143	97.9	

Table 1 (continued)

Corticosteroids									
Yes in the 30 days before ICI	8	11.3	11	22.4	3	12.0	22	15.2	0.22
Not in the 30 days before ICI	63	88.7	38	77.6	22	88.0	123	84.8	
Overall	71	100.0	49	100.0	25	96.2	145	99.3	
Yes during ICI	15	21.1	24	49.0	14	53.8	53	36.3	**<0.01**
No during ICI	56	78.9	25	51.0	12	46.2	93	63.7	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
sATB									
Yes in the 30 days before ICI	5	7.0	1	2.0	1	3.8	7	4.8	0.44
Not in the 30 days before ICI	66	93.0	48	98.0	25	96.2	139	95.2	
Overall	71	100.0	49	100.0	26	100.0	146	100.0	
Yes during ICI	12	17.1	18	37.5	11	42.3	41	28.5	**0.01**
No during ICI	58	82.9	30	62.5	15	57.7	103	71.5	
Overall	70	98.6	48	98.0	26	100.0	144	98.6	

Non-responders progressive disease as best response, *Poor responders* stable disease as best response, *Best responders* complete response or partial response as best response, *SD* standard deviation, *CNS* central nervous system, *ICI* immune-checkpoint inhibitors, *TILs* tumor-infiltrating lymphocytes, *sATB* systemic antibiotics, *RT* radiotherapy, *GI* gastrointestinal, including colorectal, gastric, esophageal, pancreatic cancer and cholangiocarcinoma, *GU* genitourinary, including kidney, bladder urothelial and prostate cancer, *Gyneco* gynecological, including ovarian and cervix cancer, CNS tumors includes only glioblastoma, *H&N* head and neck tumors; rare tumors include sarcomas, thymic and suprarenal carcinomas, *NSCLC* non-small cell lung cancer, *χ^2 test for differences in proportions and unpaired Student's t test for differences in means, # primary CNS tumors excluded

Progression-free survival

At the time of data cut-off, 120 PFS events had occurred and median PFS was 2.7 months (95% CI 2.0–3.8) (Supplementary Fig. 1 and Table 2).

Cancer site showed a significant association with PFS at the univariate analysis ($p=0.007$) (Fig. 3), with NSCLC patients treated with ICI being significantly favored over patients with GI tumors ($p=0.011$), breast cancer and other gynecological malignancies ($p=0.012$), melanoma, H&N tumors and other rare malignancies ($p=0.003$) but not genitourinary cancers ($p=0.628$). Patients treated in first-line showed better PFS than patients treated in later lines ($p=0.037$) (Fig. 3), and the later the line, the worse the outcome ($p=0.001$). LIPI score was significantly associated with PFS ($p=0.008$) (Fig. 3), with intermediate ($p=0.035$) and poor scores ($p=0.005$) associated with worse PFS than good scores. Immuno-naïve status, systemic ATB and corticosteroids during ICI were also associated with significant PFS improvement ($p=0.001$, $p=0.004$ and $p=0.004$, respectively) (Fig. 3). No other clinical or hematological factors were associated with PFS (full results in Supplementary Table 1).

At the multivariate analysis, only immunotherapy-naïve status ($p=0.005$) and LIPI score ($p=0.025$) were associated with PFS independently from each other, cancer site, treatment line, ATB, corticosteroids and previous RT (Table 3).

PFS showed a positive moderate correlation with OS: $r=0.75$, $p<0.001$.

Activity

The median TTBR was 2.5 months (95%CI 2.0–2.7) (Supplementary Fig. 1), with an ORR of 17.8% (95%CI 12.0–25.0%) (Table 2). Excluding patients who experienced a PD as best response, the median DOR was 4.4 months (95%CI 3.3–10.5) (Supplementary Fig. 1), with 17.8% (95%CI 11.6–24.0%) patients experiencing a CR, PR or SD lasting ≥ 6 months (Table 2). The DOR showed a positive moderate correlation with OS ($r=0.60$, $p<0.001$).

Cancer site appeared to be correlated with the achievement of ORR ($p=0.044$), with NSCLC and GU tumors being associated with better ORR, compared to other cancers ($p=0.011$) (Fig. 4, Supplementary Fig. 2).

First-line ICI appeared to be associated with stronger responses, compared to later lines ($p=0.021$) (Supplementary Fig. 2). Systemic ATB during ICI were associated with increased DCB ($p=0.001$) but not ORR ($p=0.089$). Notably, systemic corticosteroids administered during ICI were associated with significantly better ORR ($p=0.044$) and

Table 2 Overall ICI activity and efficacy

ACTIVITY AND EFFICACY	POPULATION	
	N (146)	% (100.0)
TTBR (months)		
Median (95% CI)	2.5 (2.0 - 2.7)	-
Response		
CR (95% CI)	7	4.8 (2.0 - 9.6)
PR (95% CI)	19	13.1 (8.0 - 19.6)
SD (95% CI)	49	33.6 (26.0 - 41.8)
PD (95% CI)	71	48.6 (40.3 - 57.0)
ORR (95% CI)	26	17.8 (12.0 - 25.0)
DCB (95% CI)	26	17.8 (11.6 - 24.0)
Evaluable patients	146	100.0
DOR (months)		
Median (95% CI)	4.4 (3.3 - 10.5)	-
PFS (months)		
Median (95% CI)	2.7 (2.0 - 3.8)	-
6-month PFS	44 patients at risk	31.5 (24.7 - 40.0)
12-month PFS	20 patients at risk	20.6 (14.8 - 28.6)
OS (months)		
Median (95% CI)	12.9 (9.9 - 17.4)	-
6-month OS	99 patients at risk	72.1 (65.2 - 79.9)
12-month OS	54 patients at risk	50.8 (42.9 - 60.1)

TTBR time-to-best response, *DOR* duration of response, *PFS* progression-free survival, *OS* overall survival, *CI* confidence interval, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *ORR* overall response rate, *DCB* durable clinical benefit

DCB ($p=0.015$). There were no other significant associations with ORR and DCB (Supplementary Table 2).

Overall results were not significant at the multivariate analysis for ORR (Table 3). Conversely, sATB during ICI were independently associated with more favorable DCB ($p=0.004$) and a trend for better DCB was observed for NSCLC and GU tumors versus all others ($p=0.079$) (Table 3).

Overall survival

At the time of data cut-off, 91 deaths had occurred, and median OS was of 12.9 months (95%CI 9.9–17.4) (Supplementary Fig. 1 and Table 2). Similarly to PFS, tumor site, number of treatment lines and LIPI score were significantly associated with OS ($p=0.021$, $p=0.037$ and $p<0.001$, respectively) (Fig. 5). When RT was administered within 30 days before ICI treatment start, a significantly worse OS was observed ($p=0.009$). Patients achieving an objective response were also prognostically favored over patients

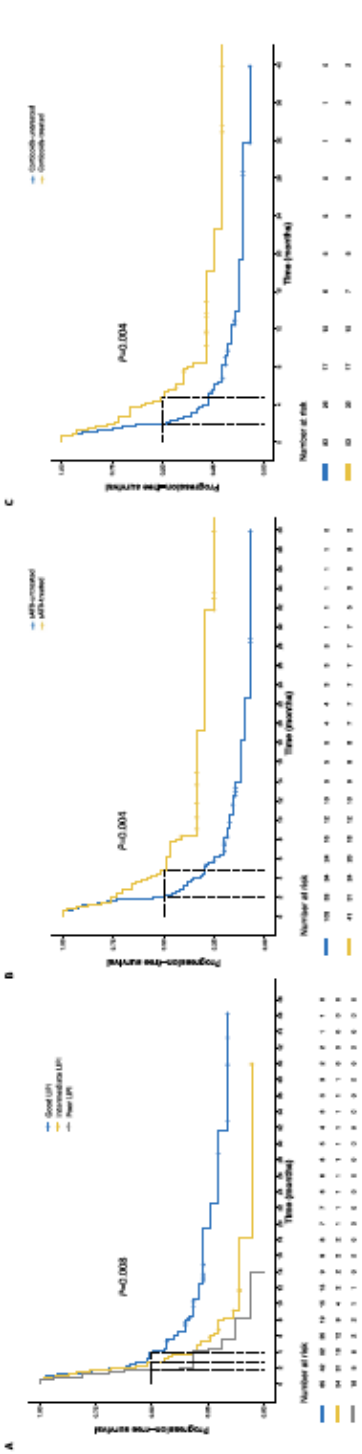

Fig. 3 Progression-free survival curves according to significant population characteristics. *A* PFS according to LIPI score, *B* PFS according to sATB administration during ICI treatment, *C* PFS according to systemic corticosteroids administration during ICI treatment, *PFS* progression-free survival, *sATB* systemic antibiotics, *ICI* immune-checkpoint inhibitors

Table 3 Multivariate survival analyses

Variables	PFS				OS			
	HR	Inf 95%CI	Sup 95%CI	*P*	HR	Inf 95%CI	Sup 95%CI	*P*
Cancer site (All others vs. NSCLC+GU)	1.51	0.95	2.39	0.084	1.93	1.09	3.43	**0.025**
ICI treatment line (1st vs. ≥2nd)	0.87	0.53	1.41	0.561	0.78	0.43	1.43	0.422
Immunotherapy-naïve status (Yes vs. No)	0.42	0.23	0.78	**0.005**	0.61	0.32	1.18	0.144
Basal LIPI Score				**0.025**				**<0.001**
Intermediate vs. Good	1.32	0.86	2.03	0.211	1.67	1.01	2.77	**0.045**
Intermediate vs. Poor	0.59	0.32	1.07	0.081	0.40	0.21	0.77	**0.006**
Poor vs. Good	2.24	1.24	4.03	**0.007**	4.22	2.16	8.23	**<0.001**
sATB during ICI (Yes vs. No)	0.76	0.47	1.23	0.270	0.93	0.54	1.60	0.789
Corticosteroids during ICI (Yes vs. No)	0.71	0.45	1.11	0.136	0.90	0.55	1.50	0.695
Previous RT (Yes vs. No)	1.35	0.52	3.49	0.535	3.10	1.05	9.15	**0.041**
Variables	**ORR**				**DCB**			
	OR	Inf 95%CI	Sup 95%CI	*P*	OR	Inf 95%CI	Sup 95%CI	*P*
Cancer site (NSCLC+GU vs. all others)	2.19	0.85	5.64	0.105	2.39	0.91	6.29	0.079
ICI treatment line (1^{st} vs. $\geq 2^{nd}$)	1.98	0.78	5.05	0.154	0.89	0.32	2.46	0.823
sATB during ICI (Yes vs. No)	1.58	0.62	4.04	0.341	3.89	1.54	9.85	**0.004**
Corticosteroids during ICI (Yes vs. No)	1.82	0.73	4.54	0.198	2.07	0.81	5.27	0.127

HR hazard ratio, *OR* odds ratio, *Inf* inferior, *Sup* superior, *PFS* progression-free survival, *OS* overall survival, *ORR* overall response rate, *DCB* durable clinical benefit, *ICI* immune-checkpoint inhibitor, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NSCLC* non-small cell lung cancer, *GU* genitourinary, *sATB* systemic antibiotics, *RT* radiotherapy

Significant *p* values are reported in bold

achieving SD or PD as their best response ($p < 0.001$) (Fig. 5), with better prognosis for longer TTBR ($p < 0.001$). No other clinical or hematological factors were associated with OS (Supplementary Table 1).

At the multivariate analysis, the independent prognostic value of the LIPI score ($p < 0.001$) was confirmed, along with a detrimental effect for RT received within 30 days before ICI was confirmed ($p = 0.041$), as well. Also, compared to NSCLC and GU tumors, all other cancers showed significantly worse OS ($p = 0.025$) (Table 3).

Tissue biomarkers exploratory analysis

PD-L1 protein expression, TILs levels and PD1 mRNA levels could be assessed for 46 (31.5%), 102 (69.9%) and 68 (46.6%) patients, respectively.

Increasing protein levels of PD-L1 were found to be associated with slightly better PFS (HR: 0.987, 95%CI 0.978–0.995, p = 0.003). The MSRS method was then applied to detect a potential cut-off of PD-L1 expression to identify patients at better/worse prognosis in terms of PFS. An optimal cut-off of 10% could identify patients with significantly different PFS (≤ 10% vs. > 10% HR: 3.12, 95%CI 1.53–6.36, $p = 0.002$), also when adjusting for cancer site ($p = 0.030$) (Fig. 4, Supplementary Table 1). Additionally, higher levels of PD-L1 were associated with significantly better ORR (OR: 1.03, 95%CI 1.01–1.05, $p = 0.007$) and DCB (OR: 1.03, 95%CI 1.00–1.05, $p = 0.028$). The previously established 10% cut-off was able to distinguish between best/worst responders in terms of ORR ($p = 0.011$) and DCB ($p = 0.043$) at univariate analysis, as well (Supplementary Table 2). When adjusting for cancer site, the cut-off retained its significance in terms of ORR (OR: 11.67, 95%CI 1.30–104.82, $p = 0.028$). Finally, the PD-L1 cut-off was also able to distinguish between patients with worse/better OS at univariate analysis (HR: 2.83, 95%CI 1.22–6.57, $p = 0.016$) and when adjusting for cancer site ($p = 0.024$) (Fig. 4, Supplementary Table 1).

Both TILs and PD1 mRNA levels were not significantly associated to PFS ($p = 0.730$ and $p = 0.682$, respectively), ORR ($p = 0.742$ and $p = 0.331$, respectively), DCB ($p = 0.870$ and $p = 0.352$, respectively) and OS ($p = 0.509$ and $p = 0.208$, respectively) (Supplementary Tables 1 and 2). However, PD1 mRNA levels were strikingly associated to the achievement of CR (Fig. 4), compared to all other responses (OR: 2.35, 95%CI 1.14–4.87, $p = 0.021$) and achieving an objective response was associated to better

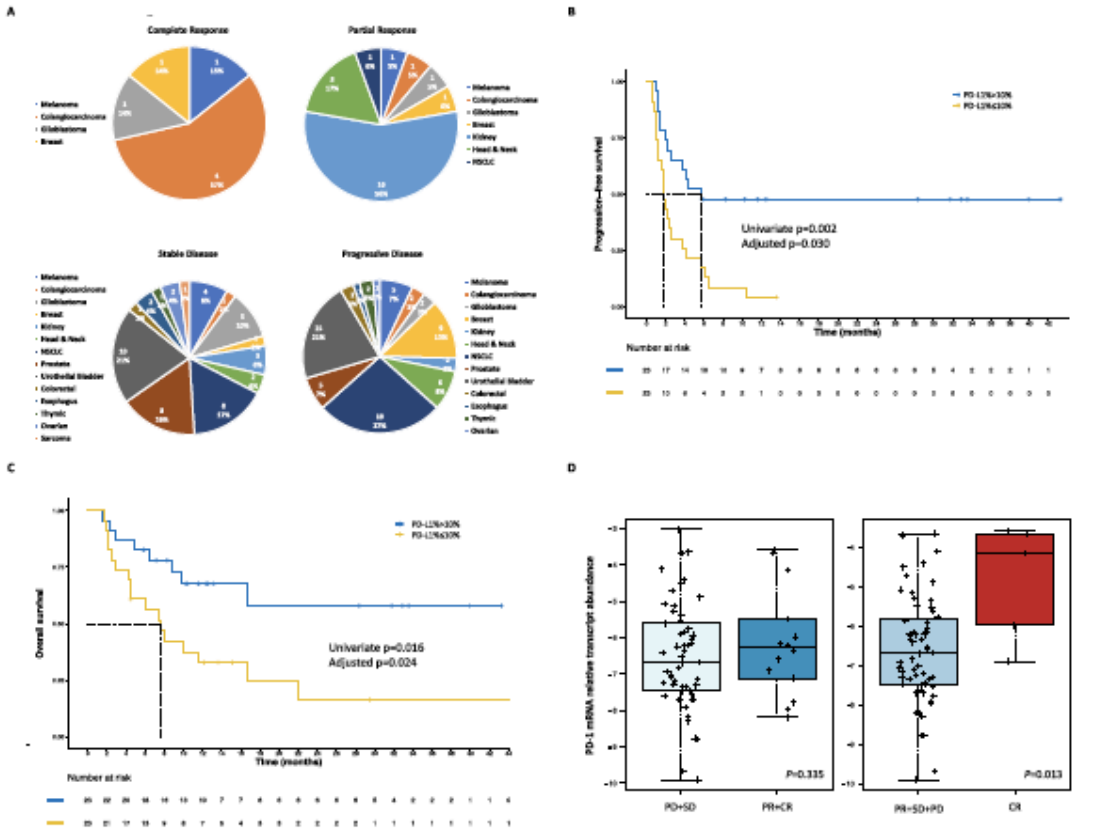

Fig. 4 PD-L1 protein and PD1 mRNA levels' main associations with outcomes and best responses according to tumor site. *A* Best response according to tumor site, *B* Progression-free survival KM curves according to a PD-L1 cut-off selected with the Maximally Selected Rank Statistics method, *C* Overall survival KM curves according to the selected PD-L1 cut-off, *D* PD1 mRNA levels in patients achieving an objective response versus patient not achieving an objective response in the left box plot and PD1 mRNA levels in patients achieving a complete response vs. patients not achieving a complete response in the right box plot, *PFS* Progression-free survival, *OS* Overall survival, *KM* Kaplan–Meier, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *p* values in box plots are referred to Student's t tests for differences in mean PD1 mRNA levels

OS, as previously reported (HR: 0.12, 95%CI 0.05–0.30, $p < 0.001$).

Discussion

Here we assessed the correlation among many clinicopathological and biological factors with activity and efficacy endpoint of ICI treatment, so to identify an easily detectable profile of the patients that might gain the most benefit out of anti-PD1/PD-L1 immunotherapy. Overall, baseline LIPI score, immunotherapy-naïve status, cancer type and RT before starting ICI were the most relevant clinical factors independently correlated with immunotherapy outcomes. Longer TTBR seem to associate with better survival, suggesting the need for not interrupting ICI therapy unless required for tumor progression, tolerability issues or patient's preference. We also observed that PD1 mRNA and PD-L1 protein levels might be tumor-agnostic predictive factors of response to ICI.

We confirmed that roughly 18% of patients treated with anti-PD1/PD-L1 ICI experienced a durable clinical response of at least 6 months, including SD. In patients achieving disease control, the DOR moderately correlated with OS and the longer the DOR, the better the OS. Importantly, the TTBR also seemed to be positively correlated with OS. Considering that no specific factors are currently able to prospectively predict the best response the patient

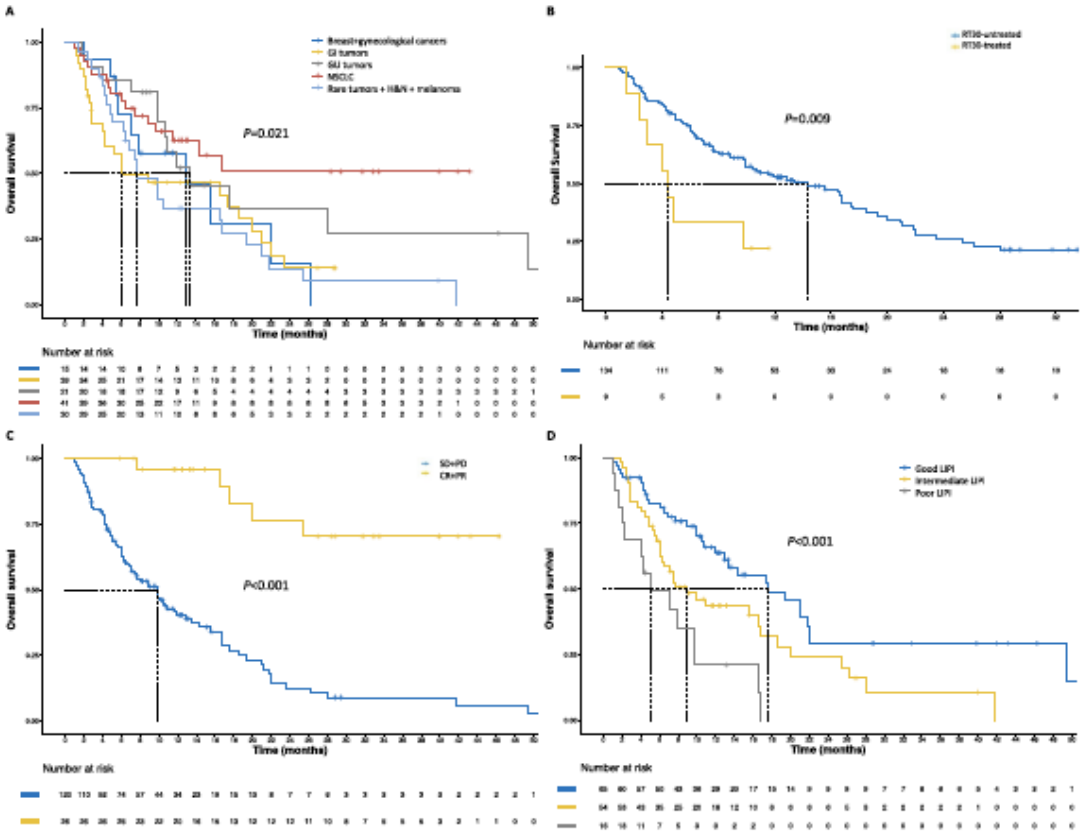

Fig. 5 Overall survival curves according to significant population characteristics. *A* OS according to cancer site, *B* OS according to treatment line, *C* OS according to best responses, *D* OS according to LIPI score, *OS* overall survival, *NSCLC* non-small cell lung cancer, *H&N* head and neck tumors, *GI* gastrointestinal, *GU* genitourinary, *SD* stable disease, *PD* progressive disease, *CR* complete responses; *PR* partial responses, *RT30* radiotherapy received within 30 days from ICI start

will achieve, nor for how long it will last, these results suggest that anti-PD1/PD-L1 ICI might be preferably discontinued at tumor progression or unacceptable toxicity, justifying maintenance/durable treatment strategies.

Unfortunately, only 17.8% patients were able to achieve an objective response (CR or PR), and the type of response was associated with OS, with patients achieving CR or PR as best response experiencing an 88% reduction in the risk of death, compared to patients not achieving an objective response. In this perspective, although the number of cases with tumor tissue available for mRNA detection was too low for introducing the variable in the multivariate logistic regression models, we confirmed the capability of PD1 mRNA to identify patients more likely to achieve an objective response, CR above all (Fig. 4), as our group previously demonstrated [24]. Interestingly, while TILs seemed not to correlate with response and survival outcomes in a pan-cancer context, PD-L1% was positively associated with a slightly higher likelihood of achieving an objective response (OR: 1.03) and a 1% reduction in the risk of progression or death for each unitary increase. Additionally, a cut-off of 10% appeared to be optimal in discriminating between patients at higher likelihood of achieving an objective and durable response and at lower risk of progression or death, similarly to what observed for example, with pembrolizumab in metastatic triple negative breast cancer [31]. Nevertheless, a larger casuistry is required to confirm the result independently from other variables and across cancer types, along with a uniform assessment of PD-L1 throughout cancer types.

We investigated in our study the role of palliative RT administered right before or during anti-PD1/PD-L1 ICI

therapy. It has been considered that RT might potentially contribute to determine a stronger systemic immune response (i.e., the abscopal effect) via immunogenic cell death and antigen release, thus enhancing the efficacy of ICI [32, 33]. However, in our cohort, RT administered during ICI was not associated to PFS, OS or tumor responses. Surprisingly, RT administered within 30 days from ICI treatment start was associated with worse OS, independently from all other clinicopathological factors considered. We have no current explanation for this observation and only 9 patients had received palliative RT immediately before ICI start, making this finding difficult to generalize. Conversely, in line with other findings [34, 35], we did not observe any abscopal effect, providing more evidence to debunk a widely postulated, yet scarcely objectivized phenomena [33].

Recently, Pinato et al. showed that systemic ATB administered prior to, but not during ICI monotherapy, are associated with a worse treatment response and OS in solid tumors [9], while ATB treatment in general seems not to impact on chemo-immunotherapy outcomes [10]. In our cohort, only ATB during, but not previous to anti-PD1/PD-L1 treatment, were associated with better PFS (univariate analysis) and DCB (univariate and multivariate analysis). To note, considering the very low number of patients (n = 7) that received ATB prior to ICI, we cannot completely exclude that an ATB-induced gut microbial dysbiosis might impair ICI efficacy. At the same time, we had no sign of detrimental effect during ICI-based therapy in a wider number of patients (n = 41), in line with recent evidences [9, 10], with a significant and independent association to DCB which merits further investigation.

Whether systemic corticosteroids, due to their immunosuppressive effect, might impair or not ICI when administered right before or during treatment is another matter of debate. Several studies led to the conclusion that avoiding or delaying the use of corticosteroids may result in maximizing the potential treatment benefits of immunotherapy [12–16]. However, other evidences highlight that corticosteroids have no detrimental effect on immunotherapy and high doses of steroids might reflect poorer basal conditions (e.g., active brain metastases, concurrent diseases, larger tumor volume), ultimately responsible for the more scarce outcomes observed with ICI [17, 18]. In our study, systemic administration of corticosteroids during ICI was associated with better PFS, ORR and DCB at the univariate analysis but lost any significant effect when adjusting for other clinicopathological factors. Corticosteroids prior to ICI did not show any significant effect on outcomes. We did not observe any difference when dividing steroid-receiving patients according to dose (above or below an equivalent of 30 mg of prednisone; not shown), as well. To note, in 48 out of 61 (78%) cases, systemic corticosteroids were administered to treat immune-related adverse events and in 5 (8%) further cases were administered as premedication to CT scan contrast medium. Thus, in our study corticosteroid use did not reflect a baseline unfavorable condition beyond tumor type and there was no hint that successfully treating ICI immune-mediated toxicities with corticosteroids might ultimately impair anti-PD1/PD-L1 efficacy.

Multiple evidences have highlighted so far the capability of the simple LIPI score, based on the derived neutrophile-to-lymphocyte ratio (dNLR) and LDH, to successfully predict the prognosis of patients with NSCLC treated with immunotherapy [36, 37]. LIPI score prognostic ability has been also evaluated in patients with various tumor types treated with ICI, like melanoma, bladder cancer or solid tumors harboring MSI [19, 22, 36, 38–40]. Our study confirms the capability of the LIPI score to successfully stratify patients with solid tumors treated with anti-PD1/PD-L1 in different prognostic subgroups, independently from all main clinicopathological characteristics, in a tumor-agnostic fashion, both in terms of PFS and OS. Patients with poor basal LIPI had a poor benefit from ICI, hence the evaluation of LIPI may identify a subset of patients with no or reduced benefit to anti-PD1/PD-L1 therapy. Considering the evidence available on this score, we strongly encourage its use at least for the selection of patients for clinical trials with ICI or as a stratification factor within such trials.

Noteworthy, an immunotherapy-naïve status was associated to a significantly better PFS, independently from other characteristics. Concordant recommendations regarding the opportunity to retreat patients already treated with immunotherapy do not exist. Furthermore, these patients are usually excluded from clinical trials that evaluate new ICI drugs or combinations so the evidence of activity in this setting is limited. A recent meta-analysis pooling 49 available studies showed that in patients who had previously discontinued ICI because of PD, ORR and median PFS were inferior to those of patients who had previously discontinued ICI because of toxicity (15.2% and 2.9 months vs. 44% and 13.2 months, respectively) [41]. Our findings, taken together with current literature, seems to confirm that rechallenges with ICI, at least with anti-PD1/PD-L1, should not be encouraged broadly, although in specific cases this strategy could be considered. Understanding the clinical impact of neo/adjuvant ICI in patients with relapsing metastatic disease candidate for immunotherapy will be of outmost importance considering the rapid expansion of therapeutic indications also in early-stage solid tumors [42, 43].

Importantly, administering anti-PD1/PD-L1 in earlier lines seemed to be associated with better PFS, OS and ORR at univariate analyses. Although the effect on PFS and OS might have been influenced by a potential lead time bias, it is also true that a less compromised immune system in untreated/less treated patients might favor the elicitation

of more potent immune responses. At the same time, it is important to underline that treatment line lost its effect on all endpoints at multivariate analyses. Thus, this finding seems to suggest that treatment line should not be an eligibility criterion for ICI treatment.

Finally, we observed that NSCLC and GU tumors were associated with better survival and activity outcomes compared to the rest of solid malignancies included in our study. This result, for which a specific explanation cannot be provided in the context of this analysis, is somewhat confirmatory of the good sensitivity to immune-checkpoint inhibition observed in the clinical practice scenario. In fact, most ICI are currently approved for NSCLC, prostate, kidney and bladder urothelial cancer [44].

Our study presents several limitations worth noting. First, its observational nature limited any possibility of control with respect to the administered treatment or for a more homogeneous tumor site distribution or treatment line. Second, being a non-interventional trial, we could not realize any tumor biopsy for patients lacking tumor tissues. This prevented us from testing for PD-L1 protein levels and PD1 mRNA in all patients' tumors. Additionally, there was no control arm. Finally, patients were treated in clinical trials, which means that some agents are not currently approved for the same clinical scenario. At the same time, this potential bias highlights the added value of a Clinical Trials Unit in an Oncology Department, which gives patients real therapeutic possibilities not otherwise or readily available in a pure clinical practice scenario. Despite limitations, our study comprehensively assessed all main clinicopathological characteristics considered in clinical practice. Data were prospectively collected and there was no specific selection bias related to excessively strict inclusion criteria, which is the typical Achilles' heel when generalizing clinical trial results to the "real-life" population [45, 46]. Furthermore, the sample size was in line with most phase II single arm trials.

To resume, only < 20% of patients with solid tumors obtain an objective and durable response with anti-PD1/PD-L1 ICI, with the magnitude and duration of response being directly associated with outcomes. The appropriate selection for patients more likely to achieve a durable response to ICI should be a priority. In this perspective, common clinicopathological factors seem not to be able to identify the best candidates for immunotherapy, except for immunotherapy-naïve status. Systemic corticosteroid administration for treating ICI-related adverse events is a feasible therapeutic strategy which seem not to negatively affect ICI efficacy, as well as systemic ATB administered during treatment. Importantly, none of our RT-treated patients experienced a beneficial abscopal effect, while RT detrimental effect when administered before starting ICI should be further elucidated in wider casuistries. Importantly, our study provides additional evidence to support the use of basal LIPI score and PD1 mRNA in tumor tissue at least to select patients for clinical trials with anti-PD1/PD-L1 ICI and/or as stratification factors, while PD-L1%, with a potential 10% cut-off, is a promising tumor-agnostic prognostic and predictive factor. However, it should be further validated in appropriately powered prospective studies and with the same detecting methodology, preferably CPS, potentially more generalizable than TPS (Supplementary materials).

To conclude, the selection of the best candidate to anti-PD1/PD-L1 therapy remains an unmet need. A better molecular characterization of responders and non-responders is key to identify currently elusive factors that prevent us from efficiently select patients for this therapeutic strategy. The ongoing evaluation of blood and tissue biomarkers from our Bioimmunoblood study will hopefully provide a much-needed contribution to this field.

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Authors' contributions JGC, FS and AP conceived the study. JGC, AI, IV, DP, LA, AM, LM, NV, MN, BA, NB and TS participated in patient recruitment. JGC, AI, IV, LA, DM and PG collected data. AGN, PB, OC, PG, ES, JM processed and analyzed blood/tissue samples. FS performed the statistical analyses. JGC, FS and AP interpreted study results and wrote the first manuscript draft. All authors revised and approved the final submitted manuscript.

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Availability of data and material The datasets generated during and/or analyzed during the current study are available from the Corresponding Authors (AP and FS) upon reasonable request.

Declarations

Conflict of interest NB participated in advisory boards for Nanobiotix, Merck Serono, MSD, BioNtech, Roche, and BMS. LM declared Sponsored Research funds from Bristol-Myers Squibb, Boehringer Ingelheim, Inivata, Stilla, Amgen; consulting, advisory role fees from Roche, Takeda; personal fees and funding for lectures and educational activities from Bristol-Myers Squibb, AstraZeneca, Takeda, Roche; travel, accommodations, expenses from Bristol-Myers Squibb, Roche, AstraZeneca and Takeda. AP declared no competing non-financial interests, but reported advisory and consulting fees from Roche, Pfizer, Novartis, Amgen, BMS, Puma, Oncolytics Biotech, MSD, Guardant Health, Peptomyc and Lilly, lecture fees from Roche, Pfizer, Novartis, Amgen, BMS, Nanostring Technologies and Daiichi

Sankyo, institutional financial interests from Boehringer, Novartis, Roche, Nanostring, Sysmex Europe GmbH, Medica Scientia inno. Research, SL, Celgene, Astellas and Pfizer; and shares ownership and a leadership role in Reveal Genomics, SL. FS declared personal fees for educational activities from Novartis. All other authors declared no conflict of interest.

Ethics approval and consent to participate The study protocol was approved by the Ethic Committee of the HCB (IRB n. HCB/2017/0371) and was conducted according to the Declaration of Helsinki, good clinical practice guidelines and in comply with applicable national and local laws. All patients signed an informed consent before entering the study.

Consent for publication All patients gave their informed consent to publish study results based on their anonymized data.

References

1. Robert C (2020) A decade of immune-checkpoint inhibitors in cancer therapy. Nat Commun 11:3801. https://doi.org/10.1038/s41467-020-17670-y
2. Munari E, Mariotti FR, Quatrini L et al (2021) PD-1/PD-L1 in cancer: pathophysiological, diagnostic and therapeutic aspects. Int J Mol Sci 22:5123. https://doi.org/10.3390/ijms22105123
3. Akkın S, Varan G, Bilensoy E (2021) A review on cancer immunotherapy and applications of nanotechnology to chemoimmunotherapy of different cancers. Molecules 26:3382. https://doi.org/10.3390/molecules26113382
4. Grossman JE, Vasudevan D, Joyce CE, Hildago M (2021) Is PD-L1 a consistent biomarker for anti-PD-1 therapy? The model of balstilimab in a virally-driven tumor. Oncogene 40:1393–1395. https://doi.org/10.1038/s41388-020-01611-6
5. Goodman AM, Kato S, Bazhenova L et al (2017) Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. Mol Cancer Ther 16:2598–2608. https://doi.org/10.1158/1535-7163.MCT-17-0386
6. Marabelle A, Le DT, Ascierto PA et al (2020) Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 38:1–10. https://doi.org/10.1200/JCO.19.02105
7. Marcus L, Fashoyin-Aje LA, Donoghue M et al (2021) FDA approval summary: pembrolizumab for the treatment of tumor mutational burden-high solid tumors. Clin Cancer Res 27:4685–4689. https://doi.org/10.1158/1078-0432.CCR-21-0327
8. Paijens ST, Vledder A, de Bruyn M, Nijman HW (2021) Tumor-infiltrating lymphocytes in the immunotherapy era. Cell Mol Immunol 18:842–859. https://doi.org/10.1038/s41423-020-00565-9
9. Pinato DJ, Howlett S, Ottaviani D et al (2019) Association of prior antibiotic treatment with survival and response to immune checkpoint inhibitor therapy in patients with cancer. JAMA Oncol 5:1774–1778. https://doi.org/10.1001/jamaoncol.2019.2785
10. Cortellini A, Ricciuti B, Facchinetti F et al (2021) Antibiotic-exposed patients with non-small-cell lung cancer preserve efficacy outcomes following first-line chemo-immunotherapy. Ann Oncol 32:1391–1399. https://doi.org/10.1016/j.annonc.2021.08.1744
11. Arbour KC, Mezquita L, Long N et al (2018) Impact of baseline steroids on efficacy of programmed cell death-1 and programmed death-ligand 1 blockade in patients with non-small-cell lung cancer. J Clin Oncol 36:2872–2878. https://doi.org/10.1200/JCO.2018.79.0006
12. Drakaki A, Dhillon PK, Wakelee H et al (2020) Association of baseline systemic corticosteroid use with overall survival and time to next treatment in patients receiving immune checkpoint inhibitor therapy in real-world US oncology practice for advanced non-small cell lung cancer, melanoma, or urothelial carcinoma. Oncoimmunology 9:1824645. https://doi.org/10.1080/2162402X.2020.1824645
13. De Giglio A, Mezquita L, Auclin E et al (2020) Impact of intercurrent introduction of steroids on clinical outcomes in advanced non-small-cell lung cancer (NSCLC) patients under immune-checkpoint inhibitors (ICI). Cancers (Basel) 12:2827. https://doi.org/10.3390/cancers12102827
14. Kartolo A, Deluce J, Holstead R et al (2021) Impact of baseline corticosteroids on immunotherapy efficacy in patients with advanced melanoma. J Immunother 44:167–174. https://doi.org/10.1097/CJI.0000000000000360
15. Maslov DV, Tawagi K, Kc M et al (2021) Timing of steroid initiation and response rates to immune checkpoint inhibitors in metastatic cancer. J Immunother Cancer 9:e002261. https://doi.org/10.1136/jitc-2020-002261
16. Pan EY, Merl MY, Lin K (2020) The impact of corticosteroid use during anti-PD1 treatment. J Oncol Pharm Pract 26:814–822. https://doi.org/10.1177/1078155219872786
17. Aldea M, Orillard E, Mansi L et al (2020) How to manage patients with corticosteroids in oncology in the era of immunotherapy? Eur J Cancer 141:239–251. https://doi.org/10.1016/j.ejca.2020.09.032
18. Nelson BE, Greiner B, Hong A et al (2021) Corticosteroid use and its impact on the efficacy of immunotherapy in multiple tumor types. JCO 39:e14583–e14583. https://doi.org/10.1200/JCO.2021.39.15_suppl.e14583
19. Benitez JC, Recondo G, Rassy E, Mezquita L (2020) The LIPI score and inflammatory biomarkers for selection of patients with solid tumors treated with checkpoint inhibitors. Q J Nucl Med Mol Imaging 64:162–174. https://doi.org/10.23736/S1824-4785.20.03250-1
20. Varga A, Bernard-Tessier A, Auclin E et al (2019) Applicability of the lung immune prognostic index (LIPI) in patients with metastatic solid tumors when treated with immune checkpoint inhibitors (ICI) in early clinical trials. Ann Oncol 30:i2. https://doi.org/10.1093/annonc/mdz027.001
21. Eisenhauer EA, Therasse P, Bogaerts J et al (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45:228–247. https://doi.org/10.1016/j.ejca.2008.10.026
22. Aldea M, Benitez JC, Mezquita L (2020) The lung immune prognostic Index (LIPI) stratifies prognostic groups in advanced non-small cell lung cancer (NSCLC) patients. Transl Lung Cancer Res 9:967–970. https://doi.org/10.21037/tlcr.2020.04.14
23. Hendry S, Salgado R, Gevaert T et al (2017) Assessing tumor infiltrating lymphocytes in solid tumors: a practical review for pathologists and proposal for a standardized method from the international immuno-oncology biomarkers working group. Adv

Anat Pathol 24:235–251. https://doi.org/10.1097/PAP.0000000000000162
24. Paré L, Pascual T, Seguí E et al (2018) Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Ann Oncol 29:2121–2128. https://doi.org/10.1093/annonc/mdy335
25. Vigliar E, Malapelle U, Iaccarino A et al (2019) PD-L1 expression on routine samples of non-small cell lung cancer: results and critical issues from a 1-year experience of a centralised laboratory. J Clin Pathol 72:412–417. https://doi.org/10.1136/jclinpath-2019-205732
26. Schettini F, Corona SP, Giudici F et al (2021) Clinical, radiometabolic and immunologic effects of olaparib in locally advanced triple negative breast cancer: the OLTRE window of opportunity trial. Front Oncol 11:2496. https://doi.org/10.3389/fonc.2021.686776
27. Hothorn T, Zeileis A (2008) Generalized maximally selected statistics. Biometrics 64:1263–1269. https://doi.org/10.1111/j.1541-0420.2008.00995.x
28. Schettini F, Conte B, Buono G et al (2021) T-DM1 versus pertuzumab, trastuzumab and a taxane as first-line therapy of early-relapsed HER2-positive metastatic breast cancer: an Italian multicenter observational study. ESMO Open 6:100099. https://doi.org/10.1016/j.esmoop.2021.100099
29. Rothman KJ (2014) Six Persistent research misconceptions. J Gen Intern Med 29:1060–1064. https://doi.org/10.1007/s11606-013-2755-z
30. Rothman KJ (1990) No adjustments are needed for multiple comparisons. Epidemiology 1:43–46
31. Cortes J, Cescon DW, Rugo HS et al (2020) Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): a randomised, placebo-controlled, double-blind, phase 3 clinical trial. The Lancet 396:1817–1828. https://doi.org/10.1016/S0140-6736(20)32531-9
32. Demaria S, Ng B, Devitt ML et al (2004) Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys 58:862–870. https://doi.org/10.1016/j.ijrobp.2003.09.012
33. Seiwert TY, Kiess AP (2021) Time to debunk an urban myth? The "abscopal effect" with radiation and anti-PD-1. J Clin Oncol 39:1–3. https://doi.org/10.1200/JCO.20.02046
34. Lee NY, Ferris RL, Psyrri A et al (2021) Avelumab plus standard-of-care chemoradiotherapy versus chemoradiotherapy alone in patients with locally advanced squamous cell carcinoma of the head and neck: a randomised, double-blind, placebo-controlled, multicentre, phase 3 trial. Lancet Oncol 22:450–462. https://doi.org/10.1016/S1470-2045(20)30737-3
35. McBride S, Sherman E, Tsai CJ et al (2021) Randomized phase II trial of nivolumab with stereotactic body radiotherapy versus nivolumab alone in metastatic head and neck squamous cell carcinoma. J Clin Oncol 39:30–37. https://doi.org/10.1200/JCO.20.00290
36. Mezquita L, Auclin E, Ferrara R et al (2018) Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. JAMA Oncol 4:351–357. https://doi.org/10.1001/jamaoncol.2017.4771
37. Ruiz-Bañobre J, Areses-Manrique MC, Mosquera-Martínez J et al (2019) Evaluation of the lung immune prognostic index in advanced non-small cell lung cancer patients under nivolumab monotherapy. Transl Lung Cancer Res 8:1078–1085. https://doi.org/10.21037/tlcr.2019.11.07
38. Vuagnat P, Auclin E, Mezquita L et al (2019) Applicability of the LIPI score to metastatic microsatellite instability high cancer patients treated with immune checkpoint inhibitors. Ann Oncol 30:xi20. https://doi.org/10.1093/annonc/mdz449.008
39. Pauline P, Auclin E, Mezquita L et al (2020) Association of the Lung Immune Prognostic Index with outcome in patients with metastatic urothelial cancer treated with immune checkpoint inhibitor. JCO 38:545–545. https://doi.org/10.1200/JCO.2020.38.6_suppl.545
40. Meyers DE, Stukalin I, Vallerand IA et al (2019) The lung immune prognostic index discriminates survival outcomes in patients with solid tumors treated with immune checkpoint inhibitors. Cancers (Basel) 11:E1713. https://doi.org/10.3390/cancers11111713
41. Inno A, Roviello G, Ghidini A et al (2021) Rechallenge of immune checkpoint inhibitors: A systematic review and meta-analysis. Crit Rev Oncol Hematol 165:103434. https://doi.org/10.1016/j.critrevonc.2021.103434
42. Schmid P, Salgado R, Park YH et al (2020) Pembrolizumab plus chemotherapy as neoadjuvant treatment for high-risk, early-stage triple-negative breast cancer: results from the phase 1b open-label, multicohort KEYNOTE-173 study. Ann Oncol. https://doi.org/10.1016/j.annonc.2020.01.072
43. Keilson JM, Knochelmann HM, Paulos CM et al (2021) The evolving landscape of immunotherapy in solid tumors. J Surg Oncol 123:798–806. https://doi.org/10.1002/jso.26416
44. FDA Approval Timeline of Active Immunotherapies. In: Cancer Research Institute. https://www.cancerresearch.org/fda-approval-timeline-of-active-immunotherapies. Accessed 13 Dec 2022
45. Arpino G, Michelotti A, Truini M et al (2016) Demographic, tumor and clinical features of clinical trials versus clinical practice patients with HER2-positive early breast cancer: results of a prospective study. J Cancer Res Clin Oncol 142:669–678. https://doi.org/10.1007/s00432-015-2033-z
46. De Placido S, Giuliano M, Schettini F et al (2018) Human epidermal growth factor receptor 2 dual blockade with trastuzumab and pertuzumab in real life: Italian clinical practice versus the CLEOPATRA trial results. Breast 38:86–91. https://doi.org/10.1016/j.breast.2017.12.012

APPENDIX 2: Second Publication (JCO Precision Oncology, Cuartile 1)

Unexpected Durable Complete Response With Anti–PD-L1 Blockade in Metastatic Undifferentiated Pleomorphic Sarcoma: A Case Report With Host and Tumor Biomarker Analysis

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Background

Soft-tissue sarcomas (STSs) are a heterogeneous group of tumors, representing approximately 1% of adult malignancies.[1] Within STSs, undifferentiated pleomorphic sarcomas (UPSs) are one of the most frequent subgroups (5%-15%).[2] Treatment options of advanced UPS remain limited, and the prognosis of patients with metastatic disease is poor, with a median survival of approximately 12 months.[3] UPS is characterized by a high level of genomic instability, as indicated by its complex karyotype with low tumor mutational burden (TMB) but high copy number alterations.[4,5] This feature can be theoretically associated with higher immunogenicity because of a potential increase in neoantigen formation.[6] For this reason, there is potential role for immunotherapy with immune checkpoint inhibitors (ICIs) in this subset of patients.[7]

Here, we report the case of a patient with metastatic UPS of the chest wall successfully treated with an anti–PD-L1 ICI at the Clinical Trial Unit of the Hospital Clinic of Barcelona (HCB), who experienced an exceptionally prolonged complete response (CR). Because of the lack of biomarkers for the correct identification of patients with UPS benefiting the most from ICIs, an extensive clinicopathological and molecular profiling was performed to explain this uncommon response.

Case Presentation

A 69-year-old man without a relevant medical history was diagnosed in June 2017 at the HCB with a stage IV UPS of the chest wall with one pulmonary metastasis. The patient received standard first-line chemotherapy with doxorubicin + ifosfamide, obtaining stable disease (SD) as best response. A tumorectomy with pulmonary metastasectomy was performed afterward. However, after 2 months, the patient was admitted to our hospital because of seizures. A magnetic resonance imaging (MRI) was performed and showed brain metastasis (Figs 1A and 1B). The lack of previous symptoms and brain imaging prevents to know if it was already present. A new CT scan showed bilateral lung metastases, as well (Fig 1C). The patient was treated with whole brain radiotherapy (WBRT); then, after approximately 1 month, second-line treatment was started with an experimental probody directed against PD-L1. After 8 months of anti–PD-L1 treatment, the patient experienced a CR in extracranial target lesions according to RECIST 1.1 criteria[8] (Fig 1D) and minimal residual changes in brain MRI. After 52 months, the patient discontinued the treatment because of the lack of production of the study drug. In the last reassessment in June 2023, after 71 months, the patient was still with no evidence of disease progression. The clinical case is resumed in Figure 2.

Consent for Publication

The patient provided informed consent to publish the study results on the basis of anonymized data.

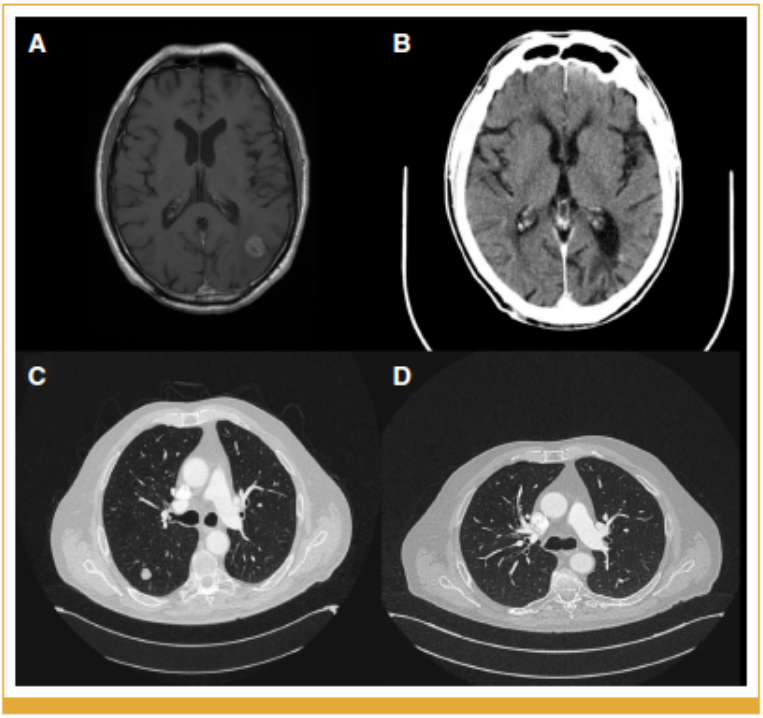

FIG 1. Representative imaging from baseline and at CR in target lesions. (A) CNS metastasis at baseline MRI; (B) CT CNS images at the moment of obtaining a CR (MRI no longer used after baseline); (C) lung metastases at baseline CT scan; the largest lung lesion measured 16 mm in its maximum diameter, with several additional satellite lesions; (D) lung CT scan at the moment of obtaining a CR. CR, complete response; CT, computed tomography; MRI, magnetic resonance imaging.

Molecular Assessments

Host Biomarkers

Previously to start the anti–PD-L1 treatment, we calculated the derived neutrophil-to-lymphocyte ratio (dNLR) and the lung immune prognostic index (LIPI), which we proved to be a highly performing tumor-agnostic prognostic score for ICI-treated patients.[9] The dNLR was 1.51, and the LIPI score was 0, both suggesting a good prognosis.[10,11] Such scores did not substantially change after the first cycle of ICI.

In addition, we performed an exploratory study of the T-cell population in peripheral blood. Blood samples were collected at baseline, at cycle 2, and at each radiologic evaluation. Flow cytometry analyses were performed using the lineage and differentiation markers CD25, CD3, FOXP3, CD40L, HLA-DR, CD4, CD62L, CD69, CD8, CTLA4, CD19, CD16/56, CD28, PDL1, PD1, CD45RO/RA, and CCR7.

Before cycle 1 of anti–PD-L1 treatment, the patient had high blood levels of effector memory T cells with low naïve T cells. At the time of CR, the naïve T cell increased from 9.3% to 21.5% with a decrease of 15% in effector memory T cells. When the patient did not receive the anti–PD-L1 treatment at cycle 10, the naïve T-cell population was 9.9%, similar to baseline (Fig 3A). However, T subpopulations levels in blood did not show a clear pattern in relation to treatment response and maintenance. NK lymphocytes and Tregs showed undulatory noninformative patterns (Fig 3B). At the same time, B-cell levels in blood showed a substantial increase through time (Fig 3C).

Tumor Biomarkers

To interrogate genomic alterations of well-known genes altered in cancer that might both potentially explain the unexpected therapeutic response, as well as representing potential future targets, we performed a molecular testing in pretreatment formalin-fixed paraffin-embedded (FFPE) tumor tissue through the Oncomine Focus assay (ThermoFisher Scientific, Waltham, MA; Table 1). This next-generation sequencing (NGS)–based assay detected the following pathogenetic hotspot mutations: *BRAF* G469V, *FGFR4* W460Ter, *NRAS* G12S, and *PIK3CA* H1047R.

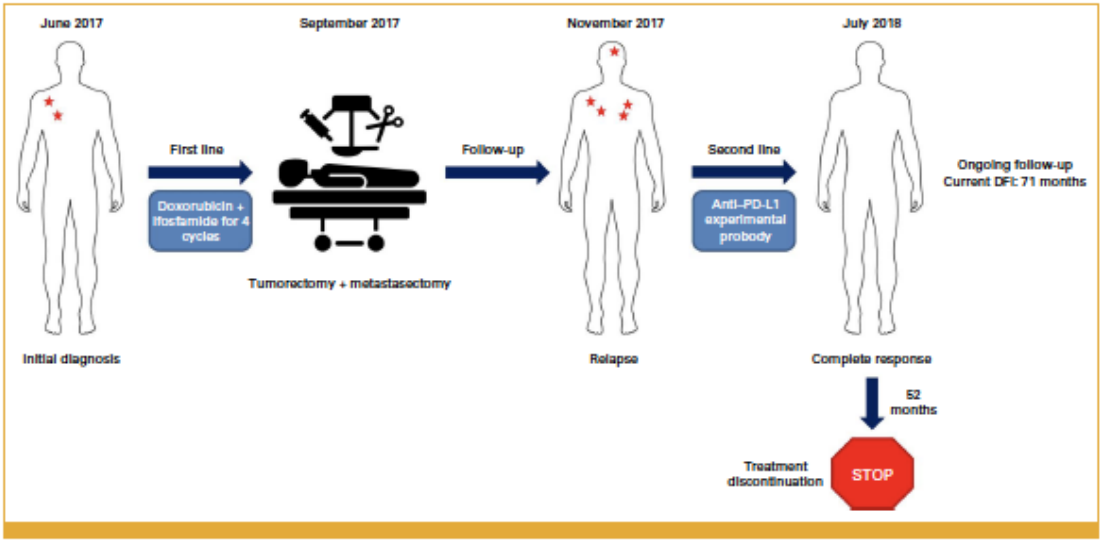

FIG 2. Case report timeline. The first-line scheme consisted in doxorubicin 50 mg/m^2 in continuous infusion at day 1 plus ifosfamide 2,000 mg/m^2 with MESNA uroprotection for 3 consecutive days, administered once every 3 weeks. Whole-brain radiotherapy was administered to reach a total of 30 Gy to stabilize the brain lesion. The experimental probody directed against PD-L1 was administered once every 2 weeks, in cycles of 8 weeks. DFI, disease-free interval.

FFPE tumor tissue was used to perform a gene expression-based assay[12] with a Nanostring nCounter platform (Nanostring Technologies, Seattle, MA) at our laboratory. The assay included cancer- and immune-related genes, including *PDCD1* (PD1) expression, which we considered worthy assessing in this context (Table 2). The PD1 mRNA level detected was -3.657 (relative transcript abundance), meaning high levels of expression according to the cutoff from Paré et al[14] predicting benefit with anti-PD1 ICI. With the same assay, we compared the levels of expression of multiple immune genes associated with B cells, T cells, innate immunity cells, and cytokines, as well as the established immunoglobulin G (IGG) signature, originally identified in breast tumors (Table 2).[13] The mean mRNA levels for IGG-related genes and of all immune genes taken together were higher than mean mRNA levels of all the 192 genes included in the research-based PAM50 codeset (ANOVA $P = .029$; Fig 3D). The relative transcript abundance of the IGG signature and of all immune genes together corresponded to the 72nd and 58th percentile of the entire codeset, respectively.

PD-L1 was evaluated using immunohistochemistry (IHC) 22C3 pharmDx (Agilent, Santa Clara, CA). PD-L1 was positive with a combined positive score of 70%. We also checked for the presence of high microsatellite instability (MSI-H) at IHC, but no MSI-H was observed at baseline.[15] Additionally, we explored the tumor microenvironment in the primary tumor through IHC and found a high CD4$^+$ and CD8$^+$ T-cell infiltration with low CD20$^+$ (B cells) and FOXP3+ Tregs infiltration (Fig 4). We also assessed in hematoxylin and eosin slides from FFPE samples the presence of tertiary lymphoid structures (TLSs), which are ectopic lymphoid tissues identified as highly organized lymphoid nonencapsulated aggregates resembling secondary lymphoid organs.[16] No TLS were found.

Discussion

UPS is a rare and difficult-to-treat solid tumor with limited therapeutic options.[3] Recently, several phase II studies investigated the outcomes of anti-PD1/PD-L1 ICIs in patients with advanced sarcoma.[3,17,18] Responses were observed only for some histological subtypes, including UPS, with a 40% objective response rate to anti-PD1 in the SARC028 trial.[17] Other studies showed similar results, suggesting that a subset of patients with UPS may respond to immune checkpoint blockade.[19,20] On the basis of this evidence and considering the poor performance of chemotherapy beyond first-line, immunotherapy with ICIs is recommended by the National Comprehensive Cancer Network guidelines for refractory UPS.[21] Nevertheless, the PEMBROSARC phase II trial demonstrated that in an unselected population, the clinical benefit of ICI was extremely limited, with a 6-month nonprogressing rate of 4.9% (95% CI, 0.6 to 16.5) and an overall response rate of 2.4% (95% CI, 0.1 to 12.9).[18] However, results were impressive when intratumor TLSs were observed.[18] These evidences suggest that ICIs can be beneficial to patients with sarcoma, including UPS, but correct biomarker identification is essential.[22] In our case, no TLS was observed at baseline, despite impressive response to ICI. Interestingly, the patient had good prognosis according to

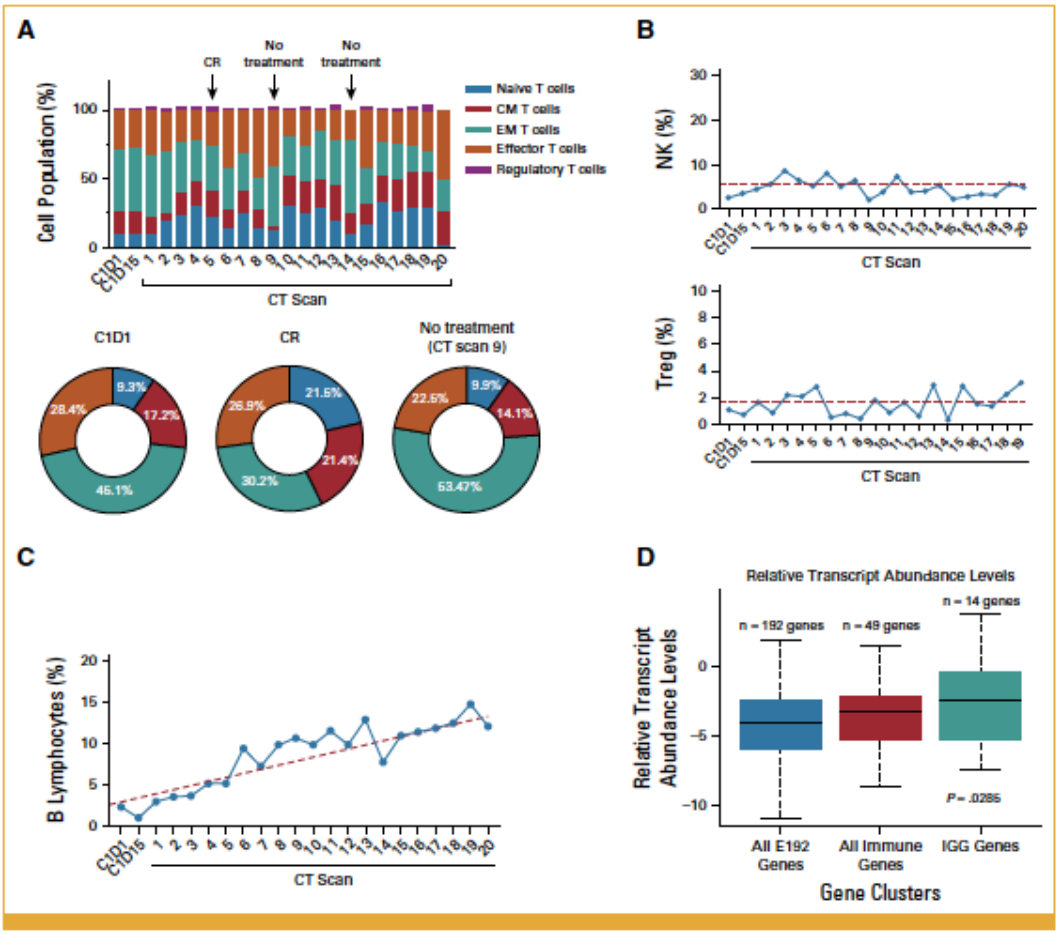

FIG 3. Lymphocyte subpopulations levels at different time points and immune genes relative transcript abundance. (A) Circulating T lymphocyte subpopulations levels at different time points; (B) circulating NK and Tregs lymphocyte subpopulations levels trends through time; (C) circulating B lymphocyte subpopulations levels trends through time; (D) boxplots of mRNA levels of different gene clusters. The numbers 1-20 (A-C) are referred to the number of TAC evaluation. Red lines (B and C) are representative of the different lymphocytes levels trends. C1, first cycle; CM, central memory; CR, complete response; CT, computed tomography; D1, cycle day 1; D15, cycle day 15; EM, effector memory; IGG, immunoglobulin G; NK, natural killer; Tregs, regulatory T lymphocytes.

both basal LIPI score and dNLR, confirming that these scores provide valuable prognostic information in patients with solid tumors treated with immunotherapy.[9,11,23]

It has been reported that UPSs present with a high expression of genes related to both antigen presentation and T-cell–mediated immunity and is among the most mutated STS subtypes, suggesting that it may be well suited to treatment with ICI.[24] Unfortunately, we could not measure the TMB of our patient's tumor, which is an established biomarker of response to ICI with anti-PD1 pembrolizumab.[25] However, not many genomic mutations were found, nor were observed alterations clearly associated with immunotherapy benefit. Conversely, the presence of *BRAF* G469V and *NRAS* G12S mutations suggested a possible hyperactivation of the RAS/MAPK pathway, which usually confers poor prognosis in UPS.[26] An *FGFR4* mutation was observed, as well (ie, *FGFR4* W460Ter), which is a driver gene for rhabdomyosarcomas.[27] Despite these potentially unfavorable mutations, our patients showed an impressive response to ICI with a durable CR that translated into a disease-free interval of almost 6 years, which is uncommon.

Interestingly, our patient received WBRT 1 month before starting anti–PD-L1 treatment. This approach could have increased the permeability of the blood-brain barrier and

TABLE 1. List of Genes Included in the Oncomine Focus Gene Panel

Oncomine Gene Panel					
Hotspot Mutation Target Gene		CNV Target Gene		Pathogenetic Fusions Involved Gene	
AKT1	*IDH1*	*AKT1*	*MYCN*	*ABL1*	*NTRK3*
ALK	*IDH2*	*ALK*	*PDGFRA*	*AKT3*	*PDGFRA*
AR	*JAK1*	*AR*	*PIK3CA*	*ALK*	*PPARG*
BRAF	*JAK2*	*BRAF*	—	*AXL*	*RAF1*
CDK4	*KIT*	*CCND1*	—	*BRAF*	*RET*
CTNNB1	*KRAS*	*CDK4*	—	*EGFR*	*ROS1*
DDR2	*MAP2K1*	*CDK6*	—	*ERBB2*	—
EGFR	*MAP2K2*	*EGFR*	—	*ERG*	—
ERBB2	*MET*	*ERBB2*	—	*ETV1*	—
ERBB3	*MTOR*	*FGFR1*	—	*ETV4*	—
ERBB4	*NRAS*	*FGFR2*	—	*ETV5*	—
ESR1	*PDGFRA*	*FGFR3*	—	*FGFR1*	—
FGFR2	*PIK3CA*	*FGFR4*	—	*FGFR2*	—
FGFR3	*RAF1*	*KIT*	—	*FGFR3*	—
GNA11	*RET*	*KRAS*	—	*MET*	—
GNAQ	*ROS1*	*MET*	—	*NTRK1*	—
HRAS	*SMO*	*MYC*	—	*NTRK2*	—

Abbreviations: CNV, copy number variation.

TABLE 2. List of Genes Included in the Custom 192-Gene Panel

PAM50 E192 Gene Panel					
ABCC11	*CD7*	*EOMES*	*IL18R1*	*MKI67*	*RRM2*
ACTG2	*CD79A*[a]	*ERBB2*	*IL23A*	*MLPH*	*S100A9*
ACTR3B	*CD84*	*ERBB3*	*IL2RG*[a]	*MMP1*	*SERPINB5*
AFF3	*CD86*	*ERBB4*	*IL34*	*MMP11*	*SFRP1*
AGR2	*CD8A*	*ESR1*	*IRF1*	*MND1*	*SH2D1A*
AGR3	*CDC20*	*ETFA*	*IRF4*	*MPHOSPH6*	*SIAH2*
ANLN	*CDC6*	*EXO1*	*IRF8*	*MRAS*	*SLAMF1*
AR	*CDCA1*	*F12*	*ISG20*	*MSLN*	*SLC39A6*
ASPM	*CDCA5*	*FA2H*	*ITK*	*MUCL1*	*SPDEF*
AURKA	*CDCA8*	*FGFR1*	*KCTD9*	*MYBL2*	*STARD3*
BAG1	*CDH3*	*FGFR2*	*KIF23*	*MYC*	*STAT1*
BCL2	*CDKN3*	*FGFR4*	*KIF2C*	*NAT1*	*STAT4*
BIRC5	*CENPA*	*FHOD1*	*KLK5*	*NDRG2*	*TCAP*
BLVRA	*CENPF*	*FOXA1*	*KLRB1*	*NECTIN4*	*TFCP2L1*
BOC	*CEP55*	*FOXC1*	*KLRD1*	*NEK2*	*THSD4*
BRCA1	*CLUAP1*	*GABRP*	*KNTC2*	*NFIB*	*TMEM45B*
BRCA2	*CNTNAP2*	*GAPD*	*KRT14*	*NQO1*	*TNFRSF17*[a]
BUB1	*CREB3L4*	*GARS*	*KRT17*	*NTN3*[a]	*TOP2A*
C2orf54	*CRYAB*	*GATA3*	*KRT18*	*ORC6L*	*TROP2*
CCNB1	*CTLA4*	*GNLY*	*KRT5*	*ORMDL3*	*TRPV6*
CCNB2	*CX3CL1*	*GPNMB*	*KRT6B*	*PDCD1*	*TSPAN13*
CCND1	*CXCL13*	*GPR160*	*KYNU*	*PGR*	*TTK*
CCNE1	*CXCL8*[a]	*GRB7*	*LAX1*[a]	*PHGDH*	*TYMS*
CD19	*CXCL9*	*GSDMB*	*LGALS9*	*PIM2*[a]	*UBE2C*
CD2	*CXCR6*	*GZMA*	*LY9*	*PNMT*	*UBE2T*
CD27[a]	*CXXC5*	*GZMB*	*MAGED2*	*POU2AF1*[a]	*XBP1*
CD274	*DGKD*	*HLA-C*[a]	*MAPT*	*PSMD3*	*ZNF552*
CD3D	*DNAJC12*	*ID4*	*MDM2*	*PTTG1*	*ACTB*
CD3G	*DNAJ1*	*IGJ*[a]	*MELK*	*PUM1*	*MRPL19*
CD4	*E2F1*	*IGKC*[a]	*MFSD2A*	*RAD51*	*PSMC4*
CD40	*EAF2*	*IGL*[a]	*MIA*	*RBT*	*RPLP0*
CD68	*EGFR*	*IGLV3-25*[a]	*MIDT*	*RRAGA*	*SF3A1*

[a]Identifies the 14 genes integrating the immunoglobulin G signature. These genes are implicated in the maturation of T and B lymphocytes progenitors (*IL2RG*), CD4+ and B lymphocytes activation and survival (*CD27, TNFRSF17, PIM2*), B lymphocytes differentiation in germinal centers (*POU2AF1*), immunoglobulin production (*CD79a, IGJ, IGKC, IGL, IGLV3-25*), chemotaxis (*CXCL8, NTN3*), and regulation of B, T, and NK lymphocytes activity (*LAX1, HLA-C*).[13]

improved the brain metastasis response, as suggested from studies conducted in other tumor types. This combined approach might thus merit further evaluation in wider cohorts also in the context of UPS.[28-30]

Importantly, high PD-L1 protein levels were observed at baseline. This biomarker has been associated with response to ICI directed against the PD1/PD-L1 axis in multiple trials,[31-33] a predictive potential that seems to find confirmation in our case. However, PD-L1 is a suboptimal biomarker since different and not interchangeable assays and methodologies for assessment are available, with different indications depending on the tumor and leading to different ICI prescriptions.[31,34] Moreover, several meta-analyses led to opposite conclusions.[22] Noteworthy, PD1 mRNA levels were also considered high, if taking into account the cutoff for prediction of anti-PD1 ICI benefit recently established in a pan-cancer context.[14,33] This biomarker has the advantage over PD-L1 to be detectable with a standardized and high reproducible methodology and might be applied potentially in all solid tumors. In our case, it successfully predicted anti–PD-L1 benefit. Hence, we believe that further confirmation of its predictive potential should be pursued, also in the context of patients treated with anti–PD-L1 ICI. In this perspective, the ongoing trial SOLTI-1904 ACROPOLI (ClinicalTrials.gov identifier: NCT04802876) will likely provide more solid evidence on this promising biomarker.

Another common biomarker of response to anti-PD1 ICI is the presence of MSI-H, a condition usually associated with DNA mismatch repair deficiency (dMMR).[15] In fact, two prospective trials have demonstrated, so far, that solid malignancies with MSI-H/dMMR experience clinically meaningful response rates with durable effect over time when treated with anti-PD1 pembrolizumab or dostarlimab,[15,35] leading to a tumor histology-agnostic approval by the US Food and Drug Administration for both ICIs. However, in our case, there was no MSI-H at baseline, suggesting other potential mechanisms underlying the response obtained to immunotherapy.

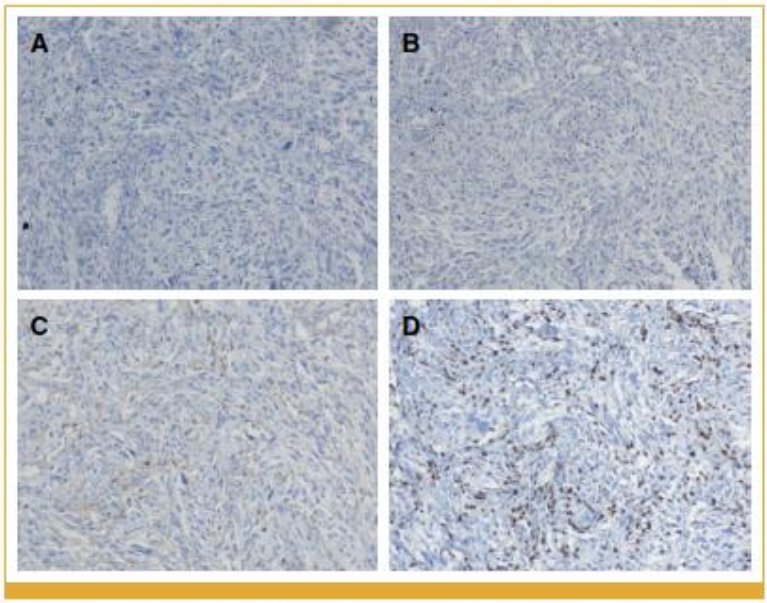

FIG 4. Representative images of immune infiltrate in the patients' tumor. (A) $CD4^+$ T-helper lymphocytes; (B) $CD8^+$ cytotoxic T lymphocytes; (C) $CD20^+$ B lymphocytes; (D) FOXP3+ regulatory T lymphocytes (Tregs). All pathology images are magnified at 40×. +, positive; Tregs, regulatory T lymphocytes.

Interesting from a biologic perspective is the finding that peripheric B lymphocytes levels increased during the treatment and basal levels of the IGG immune signature were higher than overall mean gene expression. This signature seems to reflect adaptive immune response activation mostly associated with B-cell response and immunoglobulin production and was associated with more favorable outcomes in the aggressive triple-negative breast cancer subtype.[36] Interestingly, we recently observed in a publicly available data set from The Cancer Genome Atlas that the IGG signature was associated with better overall survival in STS (hazard ratio, 0.78 [95% CI, 0.62 to 0.97]; $P = .029$).[37] Another study showed that TLSs enriched in B cells in sarcoma's microenvironment are associated with better prognosis and response to immunotherapy,[38] though in our case there were no TLSs in baseline tumor tissue. Overall our case, along with these findings, suggest that anti–PD-L1 ICIs are an effective treatment option in UPS and that B-cell immunity is likely responsible for the antitumoral effect of this therapeutic approach in this disease. Moreover, B cells can contribute to the upregulation of T-cell responses. In our patient's tumor microenvironment, high cytotoxic T-cell infiltration was observed, with reduced Tregs infiltrates, usually negative regulators of antitumoral immune responses,[39] consistent with the recent report from a subcohort of the PEMBROSARC trial.[40] Whether this might be a proxy for tumor immune sensitivity should be further clarified.

Finally, although B-cell infiltrates were not extensive at baseline, circulating B lymphocytes progressively increased throughout the treatment, raising the question of whether B-cell levels might represent a good tool to monitor therapeutic response. Unfortunately, we had no available posterior biopsy to evaluate potential treatment-induced modifications in the tumoral immune infiltrate and correlate B lymphocyte levels through time and TLSs in the tumor microenvironment, which have been elsewhere associated with response to ICIs in sarcoma.[38]

Conclusions

Despite being a poor prognostic disease, metastatic UPS can be successfully treated with immunotherapy interfering with the PD1/PD-L1 axis. The correct selection of optimal candidates for such a therapeutic approach is imperative, considering the high costs and potential life-threatening toxicities associated with immune checkpoint blockade.[41,42] In this perspective, PD-L1 levels or PD1 mRNA at baseline might be useful to identify candidates. In addition, the role of WBRT to increase the therapeutic response to ICIs in UPS with brain metastasis

should be assessed. Considering the prognostic role and the potential association between response to ICI and B-cell immunity, the role of baseline IGG signature merits further exploration to define its role as predictor of response to anti-PD1/PD-L1 inhibitors. Similarly, the role of peripheric B lymphocyte levels as a tool to monitor antitumor response also merits further evaluation in prospective wider cohorts.

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DISCLAIMER

Any views, opinions, findings, conclusions, or recommendations expressed in this material are those solely of the author(s) and do not necessarily reflect those of Funding Entities.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Choi JH, Ro JY: The 2020 WHO classification of tumors of soft tissue: Selected changes and new entities. Adv Anat Pathol 28:44-58, 2021
2. Bhalla AD, Landers SM, Singh AK, et al: Experimental models of undifferentiated pleomorphic sarcoma and malignant peripheral nerve sheath tumor. Lab Invest 102:658-666, 2022
3. Italiano A, Mathoulin-Pelissier S, Cesne AL, et al: Trends in survival for patients with metastatic soft-tissue sarcoma. Cancer 117:1049-1054, 2011
4. Cancer Genome Atlas Research Network: Comprehensive and integrated genomic characterization of adult soft tissue sarcomas. Cell 171:950-965.e28, 2017
5. Steele CD, Tarabichi M, Oukrif D, et al: Undifferentiated sarcomas develop through distinct evolutionary pathways. Cancer Cell 35:441-456.e8, 2019
6. Blank CU, Haanen JB, Ribas A, et al: The "cancer immunogram." Science 352:658-660, 2016
7. Robert C: A decade of immune-checkpoint inhibitors in cancer therapy. Nat Commun 11:3801, 2020
8. Eisenhauer EA, Therasse P, Bogaerts J, et al: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). Eur J Cancer 45:228-247, 2009
9. Garcia-Corbacho J, Indacochea A, Gonzalez A, et al: 125P Assessing the accuracy of multiple prognostic scores for immune checkpoint inhibitors (ICI) in patients with advanced solid tumors. Ann Oncol 33:S594-S595, 2022
10. Mezquita L, Auclin E, Ferrara R, et al: Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. JAMA Oncol 4:351-357, 2018
11. Yang T, Hao L, Yang X, et al: Prognostic value of derived neutrophil-to-lymphocyte ratio (dNLR) in patients with non-small cell lung cancer receiving immune checkpoint inhibitors: A meta-analysis. BMJ Open 11:e049123, 2021
12. Schettini F, Brasó-Maristany F, Kuderer NM, et al: A perspective on the development and lack of interchangeability of the breast cancer intrinsic subtypes. NPJ Breast Cancer 8:85, 2022
13. Fan C, Prat A, Parker JS, et al: Building prognostic models for breast cancer patients using clinical variables and hundreds of gene expression signatures. BMC Med Genomics 4:3, 2011
14. Paré L, Pascual T, Seguí E, et al: Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Ann Oncol 29:2121-2128, 2018
15. Marabelle A, Le DT, Ascierto PA, et al: Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: Results from the phase II KEYNOTE-158 study. J Clin Oncol 38:1-10, 2020
16. Sautès-Fridman C, Petitprez F, Calderaro J, et al: Tertiary lymphoid structures in the era of cancer immunotherapy. Nat Rev Cancer 19:307-325, 2019
17. Tawbi HA, Burgess M, Bolejack V, et al: Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): A multicentre, two-cohort, single-arm, open-label, phase 2 trial. Lancet Oncol 18:1493-1501, 2017
18. Toulmonde M, Penel N, Adam J, et al: Use of PD-1 targeting, macrophage infiltration, and IDO pathway activation in sarcomas: A phase 2 clinical trial. JAMA Oncol 4:93-97, 2018
19. D'Angelo SP, Mahoney MR, Van Tine BA, et al: Nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401): Two open-label, non-comparative, randomised, phase 2 trials. Lancet Oncol 19:416-426, 2018
20. Monga V, Skubitz KM, Maliske S, et al: A retrospective analysis of the efficacy of immunotherapy in metastatic soft-tissue sarcomas. Cancers (Basel) 12:1873, 2020
21. National Comprehensive Cancer Network (NCCN): NCCN Clinical Practice Guidelines for Soft Tissue Sarcoma. Vers.2.2023—April 25, 2023, 2023. https://www.nccn.org/professionals/physician_gls/pdf/sarcoma.pdf
22. Roulleaux Dugage M, Nassif EF, Italiano A, et al: Improving immunotherapy efficacy in soft-tissue sarcomas: A biomarker driven and histotype tailored review. Front Immunol 12:775761, 2021
23. Benitez JC, Recondo G, Rassy E, et al: The LIPI score and inflammatory biomarkers for selection of patients with solid tumors treated with checkpoint inhibitors. Q J Nucl Med Mol Imaging 64:162-174, 2020
24. Pollack SM, He Q, Yearley JH, et al: T-cell infiltration and clonality correlate with programmed cell death protein 1 and programmed death-ligand 1 expression in patients with soft tissue sarcomas. Cancer 123:3291-3304, 2017
25. Sha D, Jin Z, Budczies J, et al: Tumor mutational burden as a predictive biomarker in solid tumors. Cancer Discov 10:1808-1825, 2020
26. Serrano C, Romagosa C, Hernández-Losa J, et al: RAS/MAPK pathway hyperactivation determines poor prognosis in undifferentiated pleomorphic sarcomas. Cancer 122:99-107, 2016
27. Napolitano A, Ostler AE, Jones RL, et al: Fibroblast growth factor receptor (FGFR) signaling in GIST and soft tissue sarcomas. Cells 10:1533, 2021
28. Patruni S, Khattab A, Abel S, et al: A comparative analysis of survival in patients with non-small cell lung cancer with brain metastases receiving intracranial radiation with and without immunotherapy. J Clin Oncol 37, 2019 (suppl 15; abstr 9025)
29. Hendriks LEL, Henon C, Auclin E, et al: Outcome of patients with non-small cell lung cancer and brain metastases treated with checkpoint inhibitors. J Thorac Oncol 14:1244-1254, 2019
30. Suwinski R: Combination of immunotherapy and radiotherapy in the treatment of brain metastases from non-small cell lung cancer. J Thorac Dis 13:3315-3322, 2021
31. Doroshow DB, Bhalla S, Beasley MB, et al: PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol 18:345-362, 2021
32. Paver EC, Cooper WA, Colebatch AJ, et al: Programmed death ligand-1 (PD-L1) as a predictive marker for immunotherapy in solid tumours: A guide to immunohistochemistry implementation and interpretation. Pathology 53:141-156, 2021
33. Garcia-Corbacho J, Indacochea A, González Navarro A, et al: Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: A longitudinal prospective biomarker-based study. Cancer Immunol Immunother 72:1709-1723, 2023
34. Schettini F, Corona SP, Giudici F, et al: Clinical, radiometabolic and immunologic effects of olaparib in locally advanced triple negative breast cancer: The OLTRE window of opportunity trial. Front Oncol 11:2496, 2021
35. Berton D, Banerjee SN, Curigliano G, et al: Antitumor activity of dostarlimab in patients with mismatch repair-deficient/microsatellite instability–high tumors: A combined analysis of two cohorts in the GARNET study. J Clin Oncol 39:2564, 2021
36. Conte B, Brasó Maristany F, Pascual T, et al: 3MO Association of the research-based HER2DX signatures with survival in early-stage triple-negative breast cancer (eTNBC). Ann Oncol 33:S124-S125, 2022
37. Schettini F, Paré L, Marín-Aguilera M, et al: 14-gene immunoglobulin (IGG) and proliferation signatures and association with overall survival across cancer-types. J Clin Oncol 40, 2022 (suppl 16; abstr 2636)
38. Petitprez F, de Reyniès A, Keung EZ, et al: B cells are associated with survival and immunotherapy response in sarcoma. Nature 577:556-560, 2020
39. Ohue Y, Nishikawa H: Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci 110:2080-2089, 2019
40. Italiano A, Bessede A, Pulido M, et al: Pembrolizumab in soft-tissue sarcomas with tertiary lymphoid structures: A phase 2 PEMBROSARC trial cohort. Nat Med 28:1199-1206, 2022
41. Ramos-Casals M, Brahmer JR, Callahan MK, et al: Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primers 6:38, 2020
42. Kohn C, Shcherbakova N, Montero AJ: Cost effectiveness and financial toxicity in new cancer therapies, in Ernstoff MS, Igor P, Robert Caroline, et al (eds): SITC's Guide to Managing Immunotherapy Toxicity. New York, NY, Springer Publishing Company, pp 231-240, 2019

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RESEARCH

Blood-based prognostic scores and early dynamics under immunotherapy to select patients with metastatic solid tumors for continuing immune check-point inhibition: a prospective longitudinal study

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Abstract

Introduction Immune check-point inhibitors (ICI) were a major breakthrough in cancer care, but optimal patient selection remains elusive in most tumors.

Methods Overall 173 adult patients with metastatic solid tumors candidates to ICI in clinical trials at our Institution were prospectively recruited. Blood samples were collected at cycle 1 (C1D1) and 2 (C2D1) and until the occurrence of progressive disease (PD). C1D1 LIPI, RMH, PMHI, NLR, dNLR, PIPO and GRIm prognostic scores were calculated. The primary endpoint was identifying the best score to predict rapid PD (≤ 4 months) with ICI using logistic regressions accounting for tumor type, and receiving operators characteristics (ROC) with area under curve (AUC), accompanied by an extensive comparison of the score performances in the prediction of overall survival (OS), progression-free survival (PFS), overall response rates (ORR) and durable clinical benefit (DCB). Secondary objectives included describing study cohort outcomes and studying the association between the selected score at C1D1, C2D1 and its dynamics with OS and PFS.

Results C1D1 LIPI was the best predictor of rapid PD, OS and PFS, regardless of cancer type, compared to other scores. No score was associated to ORR and only RMH to DCB. Baseline LIPI detected three categories of patients with significantly different OS ($p < 0.001$) and PFS ($p = 0.013$). The same was observed at C2D1 for OS and PFS (both $p = 0.020$). Significant LIPI class shifts were observed in the overall population ($p < 0.001$), rapid progressors ($p = 0.029$) and non-rapid progressors ($p = 0.009$). Retaining a good LIPI or experiencing a shift towards a better prognostic class was associated to improved OS ($p = 0.009$) and PFS ($p = 0.006$). C2D1 LIPI, but not C1D1, remained significantly associated to rapid PD in multivariable analysis.

Conclusions LIPI may improve patient selection for ICI and guide treatment adjustments according to on-treatment dynamics in a pancancer context.

Keywords LIPI score · Immune check-point inhibitors · Immunotherapy · Metastatic · Cancer

Introduction

Immunotherapy with immune check-point inhibitors (ICI) has represented a major breakthrough for the treatment of solid malignancies in the last decade. This therapeutic approach unleashes a potent immune response against the tumor by interfering with the activity of key molecules implied in the negative regulation of immune response [1].

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However, ICI treatment efficacy is extremely heterogeneous and unpredictable, not only across different cancer histologies but also within a particular cancer type [2, 3]. Furthermore, ICIs may lead to harmful and potentially lethal immune-mediated side effects. Therefore, the identification of proper biomarkers of response is crucial to improve therapeutic outcomes, avoid unnecessary toxicities and optimize resources, since ICIs are considered an expensive treatment that can yearly cost more than $100,000 per patient [4]. Unfortunately, few biomarkers have proved to be effective for a proper patient selection and with several cancer-specific and/or technical-related limitations [5–7].

With the aim of characterizing clinical features, tissue-based and blood-based biomarkers that could predict prognosis and response to ICI in a pancancer context, we carried out the Bioimmunoblood prospective observational study at the Clinical Trials Unit (CTU) of the Hospital Clinic of Barcelona (HCB) [8]. In this report, we assessed clinical outcomes in the entire study cohort, compared the most relevant baseline prognostic scores developed for selecting or stratifying candidates to ICI and/or phase I trial across different tumor types [9–18] to pick the best one in predicting rapid progression to ICI, and further assess the contribution of the most successful score's early dynamics to detect rapid progressors and improve patient selection for continuing immune check-point inhibition.

Methods

Study design and participants

To participate in the Bioimmunoblood study, eligible patients had an advanced solid cancer and a scheduled initiation of an ICI-based treatment in a clinical trial. Full inclusion/exclusion criteria were previously reported [8]. Patients were recruited between November 2016 and March 2022 and followed-up until December 2023. We considered evaluable for this analysis all participants treated with an ICI with radiological data available for an independent assessment of tumor responses according to the tumor type.

Procedures

A blood sample was collected from each patient at the first day of cycle 1 (C1D1) and 2 (C2D1) prior to receive the treatment and, subsequently, at each radiological evaluation until progressive disease (PD) was determined [8]. For the purpose of the present analysis only blood samples at C1D1 and C2D1 were interrogated. Blood chemistry tests were carried out, including the evaluation of albumin, hemoglobin (Hb), lactate dehydrogenase (LDH) and standard leukocyte populations. The use of antibiotics (ATB) and/or corticoids during ICI was assessed. C1D1 samples were used for the election of the best prognostic predictor among the most commonly used, namely the Lung Immune Prognostic Index (LIPI), Royal Marsden Hospital (RMH), Princess Margaret Hospital Index (PMHI), neutrophil-to-lymphocyte ratio (NLR), derived NLR (dNLR), Phase I Prognostic Online (PIPO) and Gustave Roussy Immune (GRIm) prognostic scores [9–18]. C2D1 samples were also analyzed to assess the best score's dynamics between C1D1 and C2D1. Treatment decisions were made outside of this study according to trial protocol and investigators criteria as ICI-based treatments were given as part of the clinical trials conducted at the CTU of the HCB. All data were retrieved from electronic patient charts.

Study endpoints and outcomes

There was no prespecified sample size because of the exploratory nature of this study. The clinical data cut-off was established when a minimum follow-up including at least one reassessment of the disease for every included patient was reached.

The primary objective of this analysis was to provide an extensive comparison of the prognostic scores in terms of prediction of overall survival (OS), progression-free survival (PFS), overall response rates (ORR) and durable clinical benefit (DCB) with ICI, and identify the best score predicting rapid PD to ICI. The key secondary objective was a more refined assessment of the association between the selected best score at C1D1 with OS, PFS, ORR and DBC by accounting for relevant confounding factors. Other secondary objectives were the assessment of the association between the selected score at C2D1 with PFS and at C2D1 with OS, the evaluation of the best score's category changes from C1D1 to C2D1 as well as the evaluation of the association of the best score's dynamics with PFS and OS.

PFS was defined as the time from C1D1 to PD or death from any cause, whichever occurred first. OS was defined as the time from C1D1 to death from any cause. Rapid PD was defined as PFS $\leq$ 4 months from ICI initiation. This cut-off was determined by the study authors as the minimum clinically acceptable benefit achievable with ICIs, taking into account the balance between the potential treatment toxicity, costs, potential need to detect pseudo-progressions and the prognosis of advanced solid tumors [19]. The evaluation of response was performed in accordance to RECIST 1.1 criteria [20], or RANO criteria in the case of glioblastomas (GB) [21]. Best overall responses (BOR) were classified as PD, stable disease (SD), complete (CR) or partial response (PR) by the same expert (Dr. García-Corbacho). ORR included all patients achieving CR or PR as BOR. DCB was defined as absence of PD at 6 months [8]. The score selection process and main study analyses are resumed in Fig. 1.

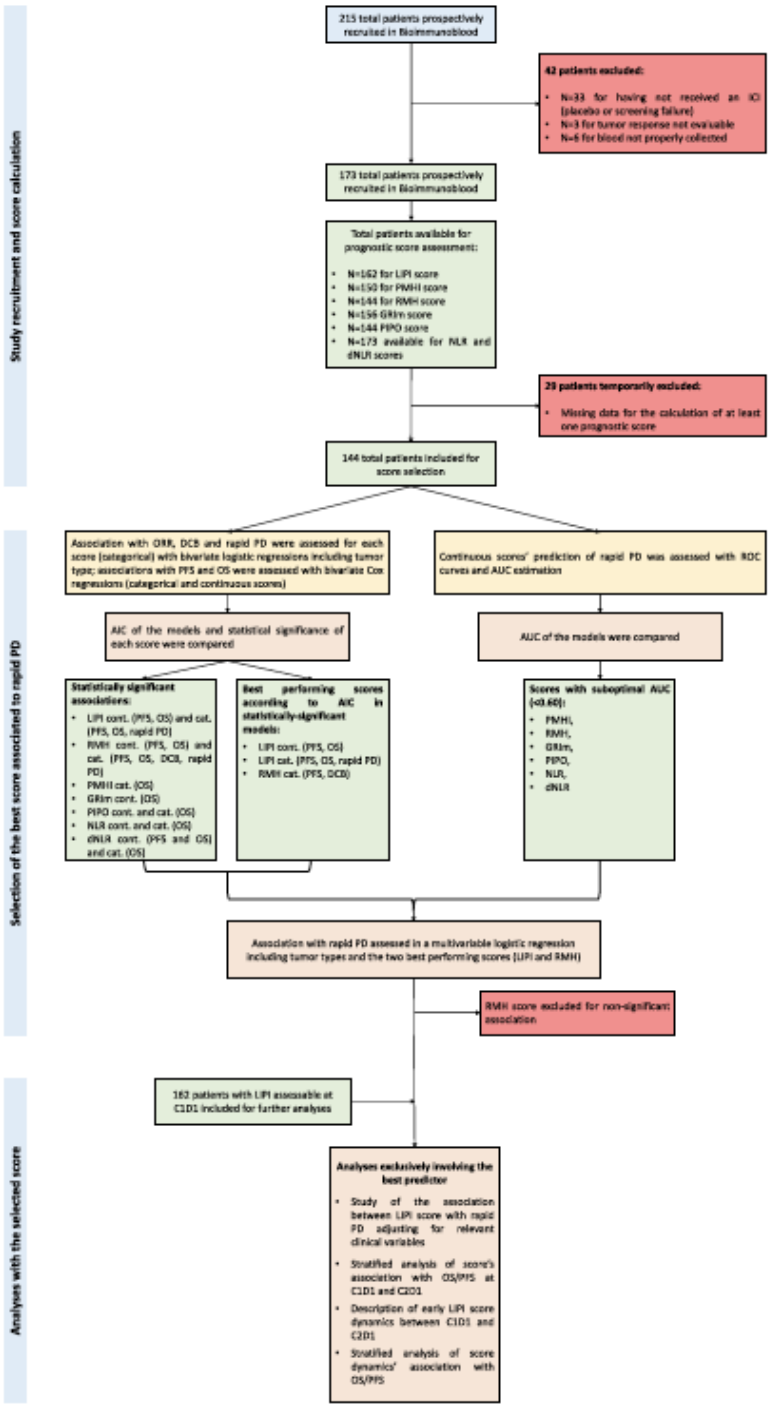

Fig. 1 Study description. AIC: Akaike information criterion; AUC: ROC's area under curve; C: treatment cycle; D: day; dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score; N: number of patients; NLR: neutrophil-to-lymphocyte ratio; LIPI: Lung Immune Prognostic Index; OS: overall survival; PD: progression of the disease; PFS: progression-free survival; PMHI: Princess Margaret Hospital Index; PIPO: Phase I Prognostic Online; RMH: Royal Marsden Hospital; ROC: receiving operator characteristics

Statistical analysis

When appropriate, χ^2, Kruskal–Wallis and Wilcoxon rank-sum tests, for unpaired variables, and McNemar and Wilcoxon signed-rank tests, for paired variables, were used to calculate differences between patients with different prognostic score classes and rapid vs. non-rapid PD. Bivariate logistic regression analyses were performed to estimate the odds ratios (OR) with their 95% confidence intervals (CI) to investigate the association of the prognostic scores at C1D1 with rapid PD, ORR and DCB. Bivariate Cox proportional hazard models including tumor type and each score at C1D1 were used to estimate hazard ratios (HR) with their 95% CI to explore associations with PFS and OS. Patients alive were censored at the date of the last follow-up. Akaike information criterion (AIC) was used to compare the goodness of fit among multivariable regression models [22]. A difference of more than 2 points between models was considered significant, and the model with the lowest AIC was considered to be the best [23]. Receiving operator characteristics (ROC) curves and their area under curve (AUC) were then used for each score to assess the capability of predicting rapid PD. AUCs were then compared with the DeLong test. After identifying the score with the best performance on all endpoints and the best capability of predicting rapid PD, Cox proportional hazard models stratified for selected confounders were then used to explore the association of the best score at C1D1, C2D1 and score dynamics between C1D1-C2D1 with PFS and OS (Supplementary methods). Multivariable logistic regressions accounting for the same confounders were carried out to assess the best score at C1D1, C2D1 and C1D1-C2D1 dynamics with rapid PD. The proportional hazard assumption was properly checked [24] for both OS and PFS (Supplementary Fig. 1). Survival curves were estimated by the Kaplan–Meier method and differences between curves were evaluated by the log-rank test. Landmark analyses to assess 12-month and 24-month OS and PFS according to the best score classes were conducted, as well. No imputation was done for missing data. A two-sided alfa error of 0.05 was considered for statistical significance.

All statistical analyses were carried out using R Studio vers.1.0.153 (PBC, Boston, MA) and SPSS vers. 24.0 (IBM SPSS Statistics, Armonk, NY: IBM Corp) for MacOSX.

Results

Population characteristics, risk stratification and outcomes

A total of 173 patients were included. Population demographics at baseline are reported in Table 1. The baseline prognostic stratification provided by the selected

Table 1 Bioimmunoblood population characteristics

Demographics	Overall	
	N	%
	173	100.0
Age		
Median	64	–
IQR	56.8—71.5	–
Sex		
Male	108	62.4
Female	65	37.6
Overall	*173*	*100.0*
ECOG		
0–1	153	88.4
≥2	20	11.6
Overall	*173*	*100.0*
Tumor type		
Breast cancer	15	8.7
Colorectal adenocarcinoma	33	19.1
NSCLC	47	27.2
Head and neck	10	5.8
Gynecologic tumors (Cervix, endometrium, ovary)	9	5.2
Pancreas and biliary tract tumors	6	3.5
Esophageal and gastric carcinoma	9	5.2
Melanoma	9	5.2
Prostate adenocarcinoma	8	4.6
Renal cell carcinoma	7	4.0
Urothelial bladder cancer	6	3.5
Glioblastoma	8	4.6
Other*	6	3.5
Overall	*173*	*100.0*
Number of metastatic sites		
<3	34	19.7
≥3	139	80.3
Overall	*173*	*100.0*
Metastatic involvement		
Visceral	139	80.3
CNS§	10	5.8
Overall	*173*	*100.0*
RT ≤ 30 days from ICI start		
Yes	10	5.8
No	162	94.2
Overall	*172*	*99.4*
Systemic ATB ≤ 30 days from ICI start		
Yes	9	5.2
No	164	94.8
Overall	*173*	*100.0*
Systemic ATB during ICI		
Yes	53	30.6
No	120	69.4
Overall	*173*	*100.0*

Table 1 (continued)

Demographics	Overall	
	N	%
Systemic corticosteroids ≤ 30 days from ICI start		
Yes	23	13.3
No	150	86.7
Overall	*173*	*100.0*
Systemic corticosteroids during ICI		
Yes	67	38.7
No	106	61.3
Overall	*173*	*100.0*
ICI treatment line		
1st	46	26.6
2nd	54	31.2
≥ 3rd	73	42.2
Overall	*173*	*100.0*
ICI type		
Anti-PD1	131	75.7
Anti-PD-L1	32	18.5
Other	10	5.8
Overall	*173*	*100.0*
Regimen type		
ICI monotherapy	92	53.2
ICI combination	36	20.8
ICI + other agent	45	26.0
Overall	*173*	*100.0*
Previous immunotherapy in every setting		
Yes	139	80.3
No	34	19.7
Overall	*173*	*100.0*

PS: performance status; IQR: interquartile range; NSCLC: non-small cell lung cancer; ATB: antibiotics; RT: radiotherapy; CNS: central nervous system; ICI: immune-checkpoint inhibitor. *: thymic carcinoma, Merkel cell carcinoma, carcinomas of unknown primary site, soft tissue sarcomas, adrenal gland adenocarcinoma, hepatocarcinoma; #: 2 patients received ICI in 1st or 2nd line, but the precise information was not reported in our records; §: excluding glioblastomas

prognostic scores is reported in Fig. 2A. The proportion of patients pertaining to the same prognostic category according to the different predictors varied significantly ($p < 0.001$).

At a median follow-up of 38.3 months (95%CI 36.1–58.6), median PFS (mPFS) was 2.5 months (95%CI 2.0–3.7) and median OS (mOS) was 13.3 months (95%CI 9.9–17.4), with rapid PD or death ≤ 4 months from ICI initiation observed in 60.7% (95%CI 53.0–68.0%) cases. Objective responses were scarce, with an ORR of 16.8% (95%CI 11.5–23.2%). Main outcomes are detailed in Table 2.

Identification of the best score predicting PFS, OS, ORR, DCB and rapid PD

We subdivided the study cohort into rapid progressors (RP) (PFS ≤ 4 months from ICI initiation) and non-rapid progressors (NRP) (PFS > 4 months from ICI initiation). The groups differed significantly in several baseline clinicopathological factors, as reported in Table 3. We explored which was the best prognostic predictor among LIPI, RMH, PMHI, NLR, dNLR, PIPO and GRIm scores in terms of PFS and OS. First, we removed from this analysis all patient with at least one of the scores not assessable, in order to avoid a biased comparison between scores due to their different populations. Following this selection we reached a total population of 144 patients. We run multiple bivariate Cox regression models each one including one of the scores as continuous or categorical variable and tumor types. Only LIPI ($p = 0.001$), dNLR ($p = 0.024$) and RMH ($p = 0.006$) continuous scores, and LIPI ($p = 0.002$) and RMH ($p = 0.001$) categorical scores were significantly associated to PFS, regardless of tumor type (Supplementary Table 1). The best AIC was observed for the model including continuous LIPI score (AIC: 1085.70). Similar AIC was observed for the models including categorical LIPI (AIC: 962.59) and RMH (AIC: 968.62). LIPI ($p < 0.001$), NLR ($p = 0.025$), dNLR ($p < 0.001$), RMH ($p = 0.005$), GRIm ($p = 0.006$) and PIPO ($p = 0.030$) continuous scores were significantly associated to OS independently of tumor type. Similarly, LIPI ($p < 0.001$), NLR ($p = 0.023$), dNLR ($p < 0.001$), RMH ($p < 0.001$), PMHI ($p = 0.046$) and PIPO ($p = 0.033$) categorical scores were significantly associated to OS (Supplementary Table 1). The bivariate model including LIPI showed the best AIC both for the continuous (AIC: 961.12) and categorical (AIC: 962.59) score. No score was significantly associated to ORR (not shown) and only RMH was associated to DCB (adjusted OR [aOR] for high score vs. low: 0.34, 95%CI: 0.14-0.83, $p = 0.017$).

We then assessed the association of all categorical scores with rapid PD in bivariate logistic regression models including tumor types. A significant and independent association was observed with LIPI categorical score ($p = 0.003$); specifically, the poor vs. good (aOR: 6.00, 95%CI 1.47–24.53, $p = 0.013$) and the intermediate vs. the good categories (aOR: 3.36, 95%CI 1.48–7.62, $p = 0.004$), without significant differences between the poor and intermediate score categories ($p = 0.422$). RMH categorical score was also associated to rapid PD (aOR: 2.88, 95%CI 1.29–6.46, $p = 0.010$), but the best AIC was observed for the LIPI score-containing model (AIC: 192.69 and 189.31, respectively). All other scores were not significantly associated to rapid PD (not shown). All statistically significant models' AIC for each endpoint are reported in Supplementary results.

The majority of the scores were unable to predict rapid PD at the ROC curve analysis, with an AUC ranging

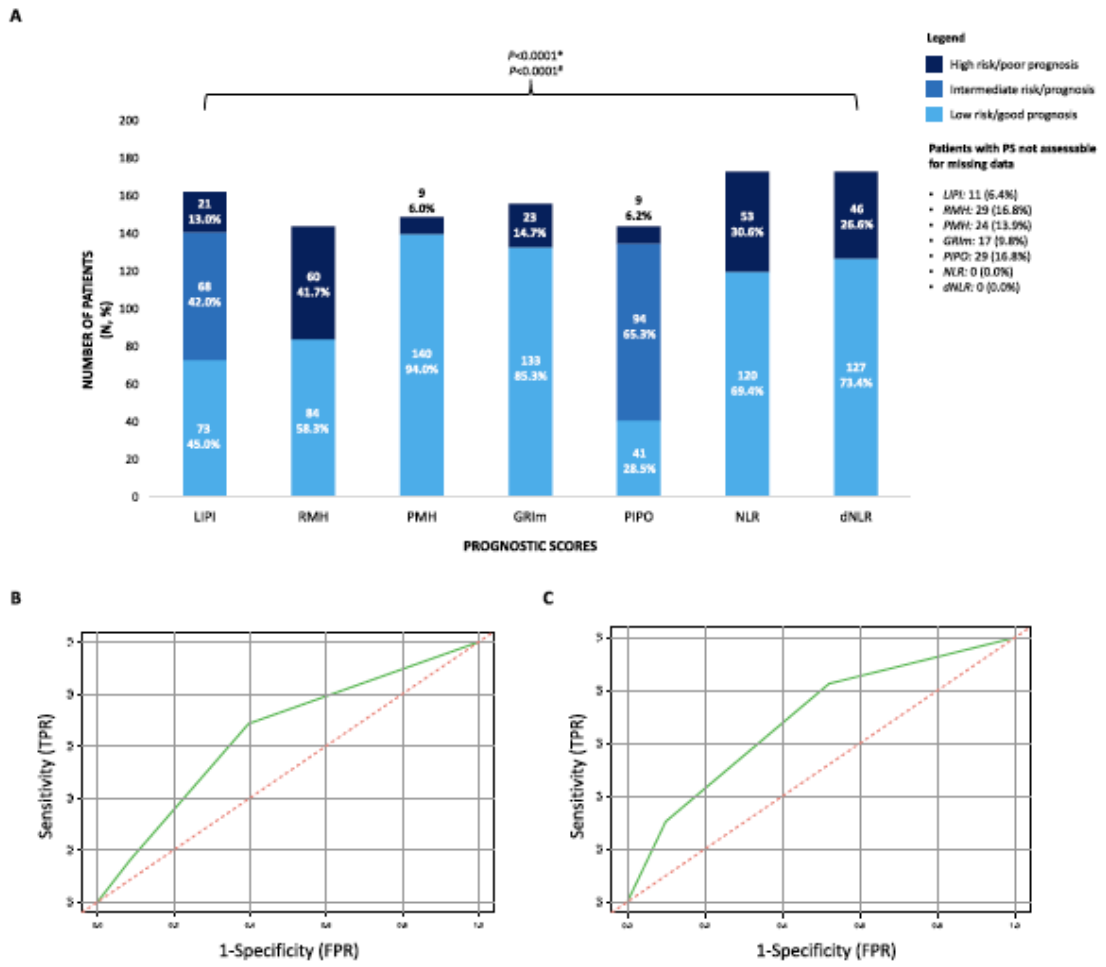

Fig. 2 Prognostic stratification of Bioimmunoblood patients according to different prognostic scores and ROC curves of LIPI score as a predictor of rapid PD. **A** Prognostic stratification of study population according to different prognostic scores at baseline; **B** ROC curve of the LIPI score as a predictor of rapid PD; **C** ROC curve of the LIPI score as a predictor of rapid death, excluding disease progression without survival events. dNLR: derived NLR; GRIm: Gustave Roussy Immune prognostic score LIPI: Lung Immune Prognostic Index; NLR: neutrophil-to-lymphocyte ratio; PD: progression of the disease; PIPO: Phase I Prognostic Online; PMHI: Princess Margaret Hospital Index; RMH: Royal Marsden Hospital; ROC: receiving operator characteristics. % were calculated according to the total number of patients with available parameters to assess each prognostic score. Missing values for each score are reported in the in-figure legend. *: for this analysis the intermediate prognostic group of the LIPI and PIPO score was jointed with the respective poor prognostic group. #: for this analysis the intermediate prognostic group of the LIPI and PIPO score was jointed with the respective good prognostic group

0.51–0.60. LIPI score was the only one significantly able to predict rapid PD, with satisfactory efficacy (AUC: 0.65, 95%CI 0.56–0.74, DeLong $p<0.001$) (Fig. 2B). The sensitivity and specificity were 68.6% and 60.3%, respectively, for a score class intermediate or poor to be able to detect RP, meaning that out of 100 true RP, 69 could be correctly identified and out of 100 NRP, 60 were correctly detected. Notably, the performance of LIPI score improved when early deaths were the only events considered (AUC: 0.69, 95%CI 0.56–0.82) (Fig. 2C).

Finally, we built a multivariable model to predict rapid PD including LIPI and RMH categorical scores and tumor types. Only LIPI score retained a significant association with rapid PD (poor/intermediate vs. good score category aOR: 3.10, 95%CI 1.12–8.60 p=0.029).

Table 2 Activity and efficacy of ICI in the overall study population

Study population outcomes		
PD timing	*N*	*%*
≤4 months from ICI start	105	60.7
>4 months from ICI start	68	39.3
Best response	*N*	*%*
Complete response	7	4.0
Partial response	22	12.7
Stable disease	59	34.1
Progressive disease	85	49.1
ORR	*%*	*95%CI*
CR + PR	16.8	11.5%—23.2%
DCB	*%*	*95%CI*
CR + PR + SD ≥ 6 months	30.1	23.2% – 36.9%
Median PFS (months)	*N*	*95%CI*
	2.5	2.0—3.7
Median OS (months)	*N*	*95%CI*
	13.3	9.9—17.4
12-month PFS	*N (%)*	*95%CI*
	32 (18.5)	13.5%—25.3%
12-month OS	*N (%)*	*95%CI*
	88 (50.9)	45.0%—60.0%

CI: confidence interval; CR: complete response; DCB: durable clinical benefit; ICI: immune-checkpoint inhibitor; N: number; ORR: overall response rate; PD: disease progression; PR: partial response; PFS: progression-free survival; SD: stable disease; OS: overall survival

Considering the globally superior performance of LIPI score in the previous assessments, it was ultimately selected for further analyses of its dynamics and more refined study of the associations with survival outcomes. To note, LIPI score at C1D1 was available for 162 (93.6%) patients. Baseline clinicopathological features according to LIPI score class are reported in Supplementary Table 2.

Association between LIPI at C1D1 and OS/PFS

C1D1 LIPI score detected three categories of patients with significantly different OS ($p < 0.001$), where patients with a poor (stratified HR [HR_{st}]: 2.74, 95%CI 1.56–4.80) and intermediate score (HR_{st}: 1.63, 95%CI 1.09–2.44) showed significantly worse OS than patients with a good score (Fig. 3A) (stratified model's AIC: 719.41). Similarly, LIPI score was also prognostic in terms of PFS ($p = 0.013$). At C1D1, the poor/intermediate scores (HR_{st}: 1.55, 95%CI 1.10–2.18) score were associated with worse PFS than the good score (Fig. 3A) (stratified model's AIC: 807.88). Landmark analyses of 12-month and 24-month OS and PFS provided consistent results (Supplementary Table 3).

Association between LIPI at C2D1 and OS/PFS

LIPI at C2D1 was assessable for 114 (65.9%) patients. When considering LIPI score at C2D1 and stratifying also for LIPI at C1D1, a global significant difference in OS between the three score classes was observed ($p = 0.020$) (stratified model's AIC: 60.57). The intermediate (HR_{st}: 8.34, 95%CI 1.03–67.42) and poor (HR_{st}: 40.75, 95%CI 1.59–1045.04) scores performed worse than the good (Fig. 3B). In terms of PFS, the intermediate (HR_{st}: 1.64, 95%CI 1.01–2.67) and poor (HR_{st}: 2.61, 95%CI 1.24–5.51) score classes showed a significantly worse outcome than the good score class ($p = 0.020$), stratifying also for baseline LIPI (Fig. 3B) (stratified model's AIC: 453.49). Landmark analyses of 12-month and 24-month OS and PFS provided consistent results (Supplementary Table 3).

LIPI score dynamics and association with rapid PD, OS and PFS

Paired LIPI scores at C1D1 and C2D1 were assessable for 113 (65.3%) patients. We explored potential LIPI score changes from C1D1 to C2D1. A significant number of patients changed their LIPI class from C1D1 to C2D1. It happened in the overall population ($p < 0.001$), RP ($p = 0.029$) and NRP ($p = 0.009$). Despite those changes, the proportion of good, intermediate and poor classes between the two timepoints remained similar (Fig. 4A). When separating the study population into RP vs. NRP, by taking into account all patients (not only those with paired C1D1-C2D1 samples), we observed that NRP had 38 (60.3%) good, 19 (30.2%) intermediate and 6 (9.5%) poor LIPI score class cases, as compared to 35 (35.4%) good, 49 (49.5%) intermediate and 15 (15.1%) poor score cases in RP at C1D1 ($p = 0.008$). Conversely, no significant difference was observed at C2D1 ($p = 0.305$). Similarly, when restricting the comparison only to NRP and RP patients with paired LIPI score at C1D1 and C2D1, a significantly higher proportion of LIPI good cases for NRP vs. RP was still observed, with more intermediate/poor cases in the RP group ($p = 0.026$) and a similar proportion of LIPI score classes at C2D1 ($p = 0.258$) (Fig. 4A). Similarly to what previously done for LIPI at C1D1, we built a bivariate logistic regression model adjusting for tumor type, so to test the association of LIPI at C2D1 with rapid PD. A significant association was observed, as well ($p = 0.049$), especially for the poor vs. good score (aOR: 9.91, 95%CI 1.58–62.04) and the poor vs. intermediate (aOR: 6.83, 95%CI 1.06–43.87). Other comparisons did not reach statistical significance. A shift towards poorer score classes or stability of the intermediate and poor classes was not formally associated to rapid PD (OR: 2.28, $p = 0.096$).

When assessing the association of rapid PD with either LIPI at C1D1 or LIPI at C2D1 in a multivariable model

Table 3 Baseline clinicopathological features according to rapid tumor progression status

Demographics	Rapid progressors		Non-rapid progressors		*P*
	N	%	N	%	
	105	60.7	68	39.3	
Age					
Median	62.6	–	67.1	–	*0.030*
IQR	54.2—69.0	–	60.5—72.6	–	
Sex					
Male	61	58.1	47	69.1	0.144
Female	44	41.9	21	30.9	
Overall	105	100.0	68	100.0	
ECOG					
0–1	94	89.5	59	86.8	0.579
≥ 2	11	10.5	9	13.2	
Overall	105	100.0	68	100.0	
Tumor type					
Breast cancer	12	11.4	3	4.4	*0.031*
Colorectal adenocarcinoma	23	21.9	10	14.7	
NSCLC	22	21.0	25	36.8	
Head and neck	7	6.7	3	4.4	
Gynecologic tumors (Cervix, endometrium, ovary)	5	4.8	4	5.9	
Pancreas and biliary tract tumors	4	3.8	2	2.9	
Esophageal and gastric carcinoma	6	5.7	3	4.4	
Melanoma	7	6.7	2	2.9	
Prostate adenocarcinoma	0	0.0	8	11.8	
Renal cell carcinoma	4	3.8	2	2.9	
Urothelial bladder cancer	5	4.8	2	2.9	
Glioblastoma	6	5.7	2	2.9	
Other*	4	3.8	2	2.9	
Overall	105	100.0	68	100.0	
Number of metastatic sites					
< 3	21	20.0	13	19.1	0.887
≥ 3	84	80.0	55	80.9	
Overall	105	100.0	68	100.0	
Metastatic involvement					
Visceral	86	81.9	53	77.9	0.522
Non-visceral	19	18.1	15	22.1	
Overall	105	100.0	68		
CNS metastases	6	6.1	4	6.1	1.000
No CNS metastases	93	93.9	62	93.9	
Overall§	99	94.3	66	97.1	
RT ≤ 30 days from ICI start					
Yes	6	5.8	4	5.9	0.975
No	98	94.2	64	94.1	
Overall	104	99.0	68	100.0	
Systemic ATB ≤ 30 days from ICI start					
Yes	5	4.8	4	5.9	0.746
No	100	95.2	64	94.1	
Overall	105	100.0	68	100.0	

Table 3 (continued)

Demographics	Rapid progressors		Non-rapid progressors		*P*
	N	%	N	%	
	105	60.7	68	39.3	
Systemic ATB during ICI					
Yes	24	22.9	29	42.6	*0.006*
No	81	77.1	39	57.4	
Overall	105	100.0	68	100.0	
Systemic corticosteroids ≤ 30 days from ICI start					
Yes	13	12.4	10	14.7	0.660
No	92	87.6	58	85.3	
Overall	105	100.0	68	100.0	
Systemic corticosteroids during ICI					
Yes	28	26.7	39	57.4	*< 0.001*
No	77	73.3	29	42.6	
Overall	105	100.0	68	100.0	
ICI treatment line					
1st	21	20.0	25	36.8	*0.044*[#]
2nd	33	31.4	20	29.4	
≥ 3rd	51	48.6	23	33.8	
Overall	105	100.0	68	100.0	
ICI type					
Anti-PD1	78	74.3	54	79.4	*0.044*
Anti-PD-L1	18	17.1	14	20.6	
Other	9	8.6	0	0.0	
Overall	105	100.0	68	100.0	
Regimen type					
ICI monotherapy	54	51.4	38	55.9	*0.043*
ICI combination	28	26.7	8	11.8	
ICI + other agent	23	21.9	22	32.4	
Overall	105	100.0	68	100.0	
Previous immunotherapy					
Yes	81	77.1	58	85.3	0.188
No	24	22.9	10	14.7	
Overall	105	100.0	68	100.0	

ICI: immune-checkpoint inhibitors; ATB: antibiotics; NSCLC: non-small cell lung cancer; CNS: central nervous system; IQR: interquartile range. *thymic carcinoma, Merkel cell carcinoma, COD, STS, adrenal gland adenocarcinoma, hepatocarcinoma; §: excluding glioblastomas; #: chi-square for variable dichotomized in 1st and ≥ 2nd line

accounting for all clinicopathological features differently distributed between RP and NRP (Table 3), as well as ECOG performance status (PS), LIPI at C1D1 was no longer associated ($p = 0.593$) whereas LIPI at C2D1 was. More specifically, the poor score vs. the good (OR_{adj}: 6.95, 95%CI 1.19–40.51, $p = 0.031$) or intermediate (OR_{adj}: 9.63, 95%CI 1.47–63.22, $p = 0.018$) scores retained a strong significant association with rapid PD, independently of baseline LIPI, age, tumor type, treatment line at which the ICI was delivered, ICI regimen type, the ICI target and the use of systemic ATB or corticosteroids during ICI treatment and ECOG. In addition, the multivariable model including both LIPI at C1D1 and C2D1 presented an AIC of 135.16, similar to the AIC of 133.07 exhibited by the model including only LIPI at C2D1 (not shown), while the model including only LIPI at C1D1 (not shown) showed an AIC of 191.65, supporting the value of assessing early LIPI dynamics to predict rapid PD.

In terms of prognosis, retaining a good prognostic score class between the C1D1 and C2D1 or experiencing a downstaging from poor to intermediate or good, or from intermediate to good was associated to significantly better OS (HR_{st}: 1.90, 95%CI 1.17–3.09, $p = 0.009$) than remaining in the same intermediate or poor class or experiencing an upstaging (Fig. 4B and Supplementary Fig. 2) (stratified model's

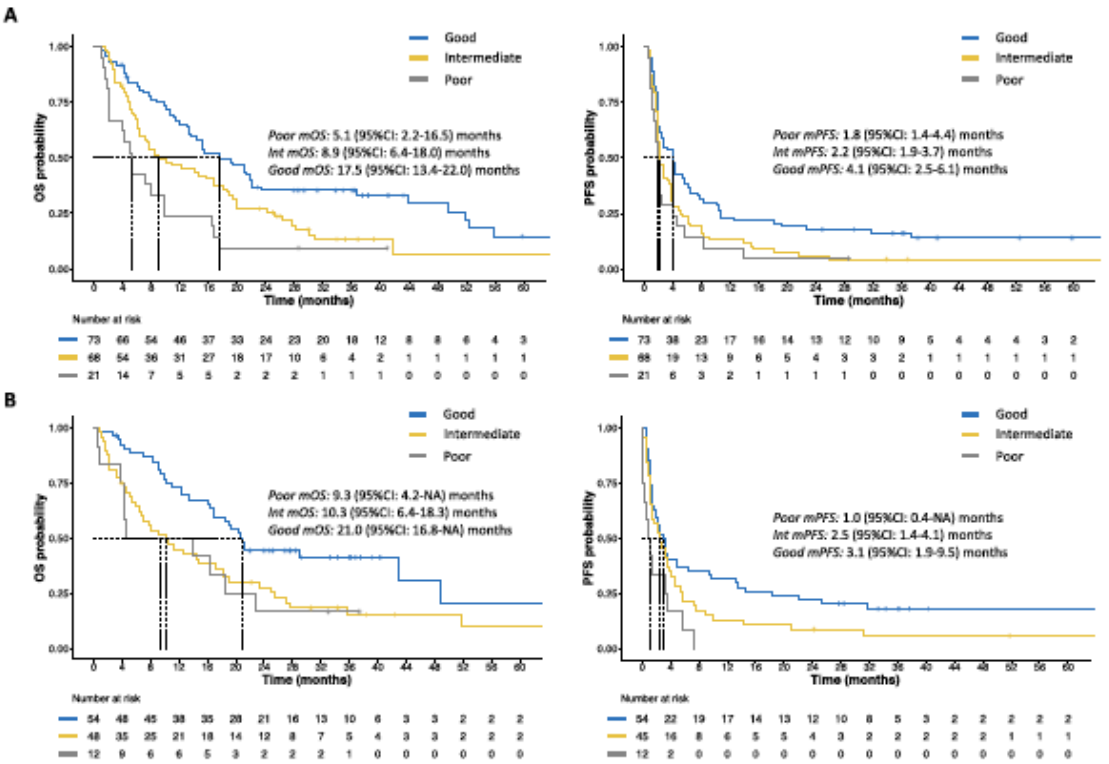

Fig. 3 Kaplan–Meier curves of OS and PFS according to LIPI score at C1D1 and C2D1. **A** OS and PFS curves of LIPI at C1D1; **B** OS and PFS curves of LIPI at C2D1. CI confidence interval; C: cycle; D: day; Int: LIPI score intermediate class; m: median; OS: overall survival; PFS: progression-free survival. Good, Intermediate and Poor are referred to LIPI score classes

AIC: 394.08). Consistently, in terms of PFS, those retaining a good prognostic score class between C1D1 and C2D1 or experiencing a downstaging experienced significantly better outcomes than all other cases (HR_{st}: 1.86, 95%CI 1.18–2.92, $p = 0.006$) (Fig. 4B and Supplementary Fig. 2) (stratified model's AIC: 452.14). We checked also if numerical score variations between the first two ICI treatment cycles, without considering the score class, could impact on prognosis. We did not observe any meaningful difference in PFS or OS (not shown).

Discussion

In this prospective cohort of patients with advanced solid tumors treated with ICI in a clinical trial at our Institution, almost 61% of patients progressed or died within 4 months from ICI initiation, with roughly 17% achieving an objective response and 18% completed at least one year on ICI treatment. These findings are in line with current evidence about immunotherapy outcomes [25, 26]. Several prognostic scores have been developed to improve ICI and/or phase I clinical trial candidates selection [9–18]. Nevertheless, these scores' capability of detecting rapid PD under ICI has not been tested and they have never been directly compared to predict objective responses and prognosis. Here, we performed a direct comparison of the most common scores in terms of PFS, OS, ORR, DCB and assessed the best predictor of a meaningful clinical benefit with ICI, considered to be a progression-free interval of more than 4 months. LIPI proved to be the best prognostic score, with the best performance in the identification of rapid progressions, with a solid association with OS and PFS beyond tumor type and relevant clinical features. In addition, LIPI score early dynamics between C1D1 and C2D1 were investigated and seemed to help improving prognostic prediction in terms of both PFS and OS beyond baseline score. Interestingly, the dNLR at baseline combined with its determination at C2D1 improved prediction of ICI outcomes in the context of advanced NSCLC, further suggesting that the early

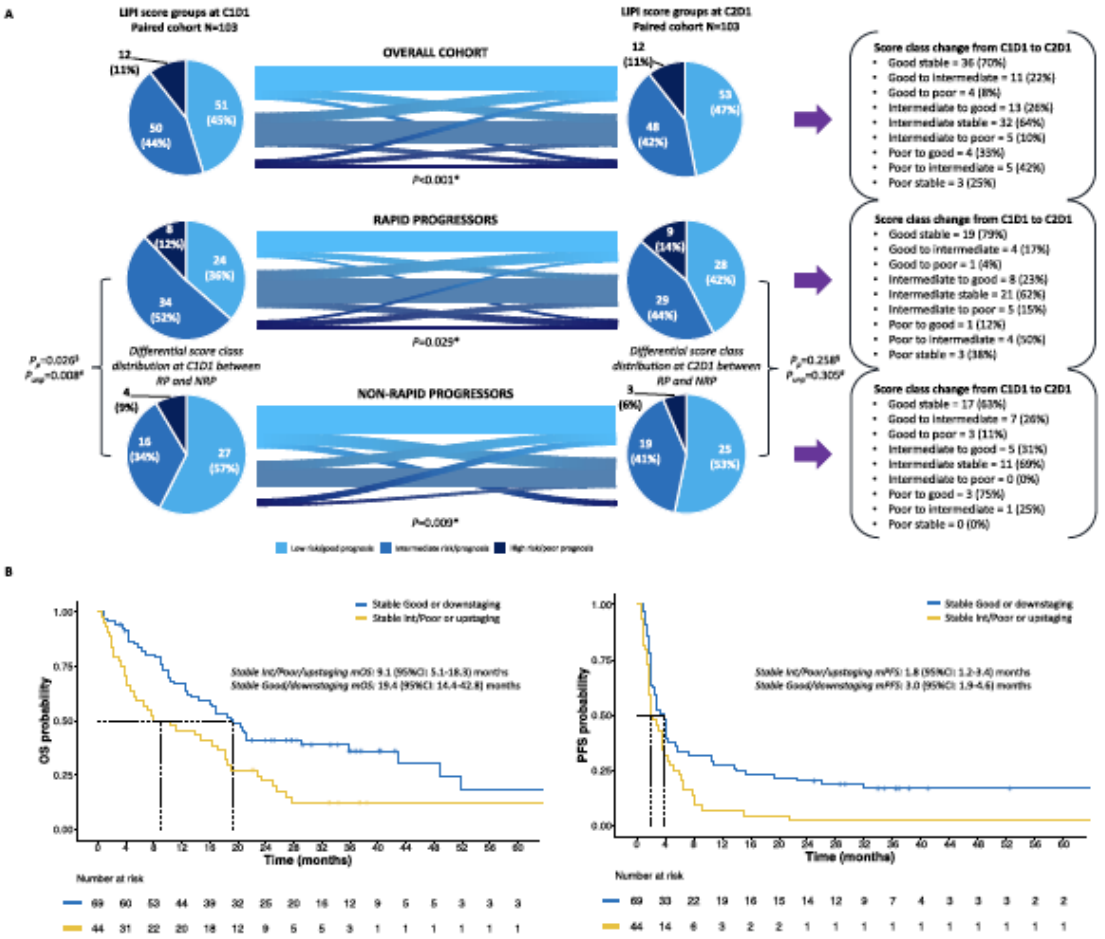

Fig. 4 LIPI score early dynamics and association with survival outcomes. **A** LIPI score distribution at C1D1 and C2D1 and dynamics. **B** OS and PFS curves of LIPI dynamics between C1D1 and C2D1. CI: confidence interval; Int: LIPI score intermediate class; m: median; NRP: non-rapid progressors; OS: overall survival; PD: progression of the disease; PFS: progression-free survival, P_p: p-values for the cohort with available C1D1 and C2D1 paired samples; P_{unp}: *p* values for the total cohort with LIPI scores available at C1D1 and/or C2D1; RP: rapid progressors; #: *p* values from χ^2 tests comparing LIPI good vs. LIPI intermediate/poor between RP and NRP; §*p* values from χ^2 tests comparing LIPI good vs. intermediate vs. poor between RP and NRP; **p* values from McNemar tests to assess LIPI dynamics in paired samples. Good, Intermediate and Poor are referred to LIPI score classes

assessment of inflammatory biomarker dynamics can be a valuable prognostic tool beyond baseline determination [27].

At present, only PD-L1 positivity, high microsatellite instability (MSI-H)/mismatch repair deficiency (dMMR) and high tumor mutational burden (TMB-H) are clinically-approved biomarkers for ICI patient selection [5–7]. However, all of these biomarkers present several limitations, including low frequency [28], heterogeneity among cancer types [6, 29–31] and costs. Other immunohistochemical or transcriptomic-based biomarkers are currently under investigation by our group and others (e.g. PD1 mRNA levels, IGG signature, tumor-infiltrating lymphocytes, tertiary lymphoid structures, LORIS) [8, 32–39]. Nonetheless, they are still far from reaching the clinical practice scenario. In this context, LIPI score emerges as a cheap and easy-to-detect blood-based biomarker which effectively stratified patients in three prognostic groups, independently of main clinicopathological factors. This score, assessed by detecting blood levels of LDH and dNLR (absolute neutrophil count/[white blood cell concentration – absolute neutrophil count]) [16], accounts for peripheral pro-inflammatory status[40–42], a known poor prognostic factor in patients with cancer [42, 43].

Higher categories of LIPI score are the reflection of higher LDH and dNLR blood levels, suggesting more peripheral chronic inflammation. Our experience is coherent with the results of a large meta-analysis involving almost 10,000 patients in 35 studies, where LIPI score robustly stratified patients receiving ICI into three groups with different survival outcomes [44].

Focusing on rapid progressions, we considered 4 months as an acceptable cut-off to detect a minimum clinically meaningful benefit by ICI treatment, taking into account the need for radiologic re-assessments for pseudo-progressions, toxicity, costs and life expectancy of these pretreated population (40% received the ICI in ≥ 3rd line and several prognostically unfavorable cancers included). LIPI score, with a sensitivity of 68.6%, was reasonably good at detecting RP, differently from all other tested scores, also independently of cancer type and relevant clinical factors differentially distributed between RP and NRP, as well as ECOG PS. To note, when accounting for the same clinical factors along with baseline LIPI, LIPI at C2D1 showed a strong and independent prediction of rapid PD beyond the baseline score, especially for what concerns the poor score category. In fact, a poor LIPI at C2D1 was associated to an 595% and a 863% increase in the odds of achieving rapid PD in comparison to a good or intermediate score. Furthermore, when stratifying for relevant clinical factors and LIPI score at C1D1, the Cox model including C2D1 showed a higher goodness of fit than the stratified Cox model including only baseline LIPI, suggesting a more refined prognostic accuracy, as also supported by landmark analysis of 12- and 24-month PFS/OS.

From a biological perspective, a downstaging in LIPI score category might be related to either a reduction in LDH and/or dNLR, which is associated to a lowering of neutrophils and/or an increase in lymphocytes levels. On one hand, LDH has been classically linked to tumor burden and cancer metabolism, but important immunosuppressive effects were also described in more recent years [45, 46]. Thus, its reduction might both directly reflect tumor response to treatment, as well as a reduction in immunosuppression, favoring response to ICI. On the other hand dNLR reduction due to lymphocytes increase suggests an effective ICI-promoted activation of adaptive immune response against the tumor [47]. This combination of factors could thus explain not only the baseline prognostic role of the score, but also the association of its dynamics and levels at C2D1 with better long-term outcomes and less rapid progressions. Importantly, when we assessed the prognostic impact of score dynamics by considering the numerical variations instead of LIPI score class change, we did not detect any meaningful impact on prognosis. This leads to the conclusion that it is more about "quality over quantity", meaning that the numerical change is not impactful in itself. Rather, it is the change in the score class that matters.

Our study is not exempt from limitations. First, missing hematologic parameters, especially LDH at C2D1, reduced the number of patients that could be tested for paired analyses, as well as the number of overall patients included in the multivariable and stratified regression models accounting for both LIPI at C1D1 and C2D1. We had no possibility of thoroughly assessing molecular features of included tumors and PD-L1 status was mostly unknown. However, most patients were treated in a ≥ 2nd-line setting and in tumors in which PD-L1 status was not mandatory for prescribing the therapy in Europe. Also, the kind of ICI was decided in sponsored trials. Additionally, being a single-center study, our findings require validation in an external cohort. Finally, some patients from the poor LIPI group achieved clinical benefit, possibly because automated neutrophil counts do not discriminate between the different subpopulations of neutrophils that could have protumor or antitumor functions [16, 48, 49]. Besides these limitations, our study confirms the effective prognostic role of baseline LIPI in ICI-treated patients in a pancancer context. LIPI might be especially useful to detect patients more or less likely to derive a clinically meaningful benefit from ICI, potentially serving either as a critical stratification factor or as an inclusion/exclusion criteria in ICI trials and with very limited costs; though further improvements in this research area are advisable. Moreover, early dynamics between C1D1 and C2D1 were able to identify patients with more favorable outcomes beyond baseline score. As far as we are concerned, this is the first study assessing LIPI score early dynamics and its association with survival. Only another study assessed LIPI dynamics, but evaluated the association with side effects and was exclusively conducted in a cohort of patients with NSCLC [50]. Another study from Mezquita et al. was only focused on dNLR, score dynamics were assessed differently, and the setting was restricted to NSCLC [27].These results merit further validation, as LIPI dynamics might become a useful inexpensive on-treatment tool to identify patients either benefiting from continuing immune-checkpoint inhibition or candidates to escalated or alternative therapeutic strategies in the context of adequately designed clinical trials.

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Authors' contributions J. García-Corbacho, F. Schettini and A. Prat conceived the study. J. García-Corbacho, A. Indacochea, I. Victoria, L. Angelats, L. Mezquita, B. Mellado, N. Viñolas, M. Noguè, T. Sauri, B. Adamo, J. Maurel, E. Pineda, L. Gaba, O. Reig and N. Basté participated in patient recruitment. J. García-Corbacho, A. Indacochea, I. Victoria, L. Angelats, D. Moreno and P. Galván collected study data. F. Schettini performed the statistical analyses. All authors interpreted study results. F. Schettini and J. García-Corbacho wrote the first manuscript draft. All authors revised and approved the final submitted manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the Corresponding Authors upon reasonable request.

Declarations

Conflict of interest J. García-Corbacho reports travel expenses from BMS and Pfizer and honoraria from AstraZeneca, GSK, Lilly, Regeneron, Roche and Pfizer. L. Mezquita reports grants/research support from Amgen, Inivata, AstraZeneca and Gilead, honoraria/consultation fees from Roche, Takeda, Janssen and MSD, lectures and educational activities for Bristol-Meyers Squibb, Roche, Takeda, AstraZeneca, MSD, Radonova and Janssen, travel accommodation/expenses from Bristol-Meyers Squibb, Roche, Takeda, AstraZeneca and Janssen. F. Brasó-Maristany reports patent application (PCT/EP2022/086493, PCT/EP2023/060810, EP23382703 and EP23383369). N. Basté participated in advisory boards for Nanobiotix, Merck Serono, MSD, BioNtech, Roche, and BMS. T. Saurí served as a consultant at AstraZeneca, BMS, Roche, MSD, AMGEN, and Daiichi Sankyo and received lecture fees from BMS and MSD; B. Mellado reports research funding from Janssen, Roche, Bayer, and Pfizer; speakers' bureau for Roche, Sanofi, Janssen, Astellas, Pfizer, Novartis, and Bristol-Myers Squibb; and travel and accommodation expenses from Janssen and Pfizer. Ò. Reig reports consulting or advisory role for BMS, EISAI, and Ipsen; and travel and accommodation expenses from Ipsen and Pfizer. L. Gaba reports advisory and consulting fees from GlaxoSmithKline, AstraZeneca, Merk Sharp Dohme, and PharmaMar, honoraria from GlaxoSmithKline, AstraZeneca, Merk Sharp Dohme, and PharmaMar for educational events/materials and travel expenses from GlaxoSmithKline, AstraZeneca, Merk Sharp Dohme. A. Prat reports advisory and consulting fees from AstraZeneca, Roche, Pfizer, Novartis, Daiichi Sankyo, and Peptomyc, lecture fees from AstraZeneca, Roche, Novartis, and Daiichi Sankyo, institutional financial interests from AstraZeneca, Novartis, Roche, and Daiichi Sankyo; stockholder and employee of Reveal Genomics; patents filed PCT/EP2016/080056, PCT/EP2022/086493, PCT/EP2023/060810, EP23382703 and EP23383369. F. Schettini reports honoraria from Novartis, Gilead, Veracyte and Daiichy-Sankyo for educational events/materials, advisory fees from Pfizer and Veracyte, and travel expenses from Novartis, Gilead and Daiichy-Sankyo. The other authors report no conflict of interest.

Ethics approval and consent to participate The study protocol was approved by the Ethic Committee of the HCB (IRB n. HCB/2017/0371) and was conducted according to the Declaration of Helsinki, good clinical practice guidelines and in compliance with applicable national and local laws. All patients signed an informed consent before entering the study.

References

1. Waldman AD, Fritz JM, Lenardo MJ (2020) A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol 20:651–668. https://doi.org/10.1038/s41577-020-0306-5
2. Akkın S, Varan G, Bilensoy E (2021) A review on cancer immunotherapy and applications of nanotechnology to chemoimmunotherapy of different cancers. Molecules 26:3382. https://doi.org/10.3390/molecules26113382
3. Ramos-Casals M, Brahmer JR, Callahan MK et al (2020) Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primers 6:38. https://doi.org/10.1038/s41572-020-0160-6
4. Schaft N, Dörrie J, Schuler G et al (2023) The future of affordable cancer immunotherapy. Front Immunol. https://doi.org/10.3389/fimmu.2023.1248867
5. Grossman JE, Vasudevan D, Joyce CE, Hildago M (2021) Is PD-L1 a consistent biomarker for anti-PD-1 therapy? The model of balstilimab in a virally-driven tumor. Oncogene 40:1393–1395. https://doi.org/10.1038/s41388-020-01611-6
6. Goodman AM, Kato S, Bazhenova L et al (2017) Tumor Mutational burden as an independent predictor of response to immunotherapy in diverse cancers. Mol Cancer Ther 16:2598–2608. https://doi.org/10.1158/1535-7163.MCT-17-0386
7. Marabelle A, Le DT, Ascierto PA et al (2020) Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 38:1–10. https://doi.org/10.1200/JCO.19.02105
8. García-Corbacho J, Indacochea A, González Navarro AE et al (2023) Determinants of activity and efficacy of anti-PD1/PD-L1 therapy in patients with advanced solid tumors recruited in a clinical trials unit: a longitudinal prospective biomarker-based study. Cancer Immunol Immunother 72:1709–1723. https://doi.org/10.1007/s00262-022-03360-9
9. Arkenau H-T, Barriuso J, Olmos D et al (2009) Prospective validation of a prognostic score to improve patient selection for oncology phase I trials. J Clin Oncol 27:2692–2696. https://doi.org/10.1200/JCO.2008.19.5081
10. Arkenau H-T, Olmos D, Ang JE et al (2008) Clinical outcome and prognostic factors for patients treated within the context of a phase I study: the Royal Marsden Hospital experience. Br J Cancer 98:1029–1033. https://doi.org/10.1038/sj.bjc.6604218
11. Chau NG, Florescu A, Chan KK et al (2011) Early mortality and overall survival in oncology phase I trial participants: Can we

improve patient selection? BMC Cancer 11:426. https://doi.org/10.1186/1471-2407-11-426
12. Day D, Guo C, Kanjanapan Y et al (2019) Survival in early phase immuno-oncology trials: development and validation of a prognostic index. JNCI Cancer Spectr 3:pkz071. https://doi.org/10.1093/jncics/pkz071
13. Templeton AJ, McNamara MG, Šeruga B et al (2014) Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. J Natl Cancer Inst 106:dju124. https://doi.org/10.1093/jnci/dju124
14. Riudavets M, Auclin E, Mezquita L (2020) Host circulating biomarkers for immune-checkpoint inhibitors: single-agent and combinations. Fut Oncol 16:1665–1668. https://doi.org/10.2217/fon-2020-0182
15. Yang T, Hao L, Yang X et al (2021) Prognostic value of derived neutrophil-to-lymphocyte ratio (dNLR) in patients with non-small cell lung cancer receiving immune checkpoint inhibitors: a meta-analysis. BMJ Open 11:e049123. https://doi.org/10.1136/bmjopen-2021-049123
16. Mezquita L, Auclin E, Ferrara R et al (2018) Association of the lung immune prognostic index with immune checkpoint inhibitor outcomes in patients with advanced non-small cell lung cancer. JAMA Oncol 4:351–357. https://doi.org/10.1001/jamaoncol.2017.4771
17. Bigot F, Castanon E, Baldini C et al (2017) Prospective validation of a prognostic score for patients in immunotherapy phase I trials: The Gustave Roussy Immune Score (GRIm-Score). Eur J Cancer 84:212–218. https://doi.org/10.1016/j.ejca.2017.07.027
18. Matos I, Villacampa G, Hierro C et al (2021) Phase I prognostic online (PIPO): A web tool to improve patient selection for oncology early phase clinical trials. Eur J Cancer 155:168–178. https://doi.org/10.1016/j.ejca.2021.05.040
19. Seymour L, Bogaerts J, Perrone A et al (2017) iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol 18:e143–e152. https://doi.org/10.1016/S1470-2045(17)30074-8
20. Eisenhauer EA, Therasse P, Bogaerts J et al (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45:228–247. https://doi.org/10.1016/j.ejca.2008.10.026
21. Wen PY, van den Bent M, Youssef G et al (2023) RANO 2.0: update to the response assessment in neuro-oncology criteria for high- and low-grade gliomas in adults. JCO 41:5187–5199. https://doi.org/10.1200/JCO.23.01059
22. Akaike H (1998) Information theory and an extension of the maximum likelihood principle. In: Parzen E, Tanabe K, Kitagawa G (eds) Selected Papers of Hirotugu Akaike. Springer, New York, pp 199–213
23. Burnham K, Anderson D (2002) Multimodal inference: a practical information-theoretic approach, 2nd edn. Springer, New York
24. Schettini F, Conte B, Buono G et al (2021) T-DM1 versus pertuzumab, trastuzumab and a taxane as first-line therapy of early-relapsed HER2-positive metastatic breast cancer: an Italian multicenter observational study. ESMO Open 6:100099. https://doi.org/10.1016/j.esmoop.2021.100099
25. Tan S, Li D, Zhu X (2020) Cancer immunotherapy: Pros, cons and beyond. Biomed Pharmacother 124:109821. https://doi.org/10.1016/j.biopha.2020.109821
26. Sehgal K (2021) Hyperprogression in patients with cancer receiving immune checkpoint inhibitors. JAMA Netw Open 4:e211839. https://doi.org/10.1001/jamanetworkopen.2021.1839
27. Mezquita L, Preeshagul I, Auclin E et al (2021) Predicting immunotherapy outcomes under therapy in patients with advanced NSCLC using dNLR and its early dynamics. Eur J Cancer 151:211–220. https://doi.org/10.1016/j.ejca.2021.03.011
28. Kavun A, Veselovsky E, Lebedeva A et al (2023) Microsatellite instability: a review of molecular epidemiology and implications for immune checkpoint inhibitor therapy. Cancers (Basel) 15:2288. https://doi.org/10.3390/cancers15082288
29. Schettini F, Corona SP, Giudici F et al (2021) Clinical, radiometabolic and immunologic effects of olaparib in locally advanced triple negative breast cancer: The OLTRE window of opportunity trial. Front Oncol 11:2496. https://doi.org/10.3389/fonc.2021.686776
30. Lawrence MS, Stojanov P, Polak P et al (2013) Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499:214–218. https://doi.org/10.1038/nature12213
31. Meléndez B, Van Campenhout C, Rorive S et al (2018) Methods of measurement for tumor mutational burden in tumor tissue. Transl Lung Cancer Res 7:661–667. https://doi.org/10.21037/tlcr.2018.08.02
32. Prat A, Pascual T, Juan M et al (2023) 1056P Efficacy of spartalizumab across multiple cancer types in patients with PD1-high mRNA expressing tumors (SOLTI-1904 ACROPOLI). Ann Oncol 34:S639–S640. https://doi.org/10.1016/j.annonc.2023.09.2195
33. Paré L, Pascual T, Seguí E et al (2018) Association between PD1 mRNA and response to anti-PD1 monotherapy across multiple cancer types. Ann Oncol 29:2121–2128. https://doi.org/10.1093/annonc/mdy335
34. Pesántez D, Indacochea A, Angelats L et al (2023) Unexpected Durable complete response with anti-PD-L1 blockade in metastatic undifferentiated pleomorphic sarcoma: a case report with host and tumor biomarker analysis. JCO Precis Oncol 7:e2300051. https://doi.org/10.1200/PO.23.00051
35. Paijens ST, Vledder A, de Bruyn M, Nijman HW (2021) Tumor-infiltrating lymphocytes in the immunotherapy era. Cell Mol Immunol 18:842–859. https://doi.org/10.1038/s41423-020-00565-9
36. Sautès-Fridman C, Petitprez F, Calderaro J, Fridman WH (2019) Tertiary lymphoid structures in the era of cancer immunotherapy. Nat Rev Cancer 19:307–325. https://doi.org/10.1038/s41568-019-0144-6
37. Chang T, Cao Y, Sfreddo H et al (2024) LORIS robustly predicts patient outcomes with immune checkpoint blockade therapy using common clinical, pathologic and genomic features. Nat Cancer 5:1158–1175. https://doi.org/10.1038/s43018-024-00772-7
38. Hernando-Calvo A, Cindy Yang S, Vila-Casadesús M et al (2024) Combined transcriptome and circulating tumor DNA longitudinal biomarker analysis associates with clinical outcomes in advanced solid tumors treated with pembrolizumab. JCO Precis Oncol 8:e2400100. https://doi.org/10.1200/PO.24.00100
39. Solis ES, Pascual T, Torres ES et al (2023) 2239P Gene expression profiling in tumors with and without tertiary lymphoid structures across cancer types. Ann Oncol 34:S1153–S1154. https://doi.org/10.1016/j.annonc.2023.09.1267
40. Petrelli F, Cabiddu M, Coinu A et al (2015) Prognostic role of lactate dehydrogenase in solid tumors: a systematic review and meta-analysis of 76 studies. Acta Oncol 54:961–970. https://doi.org/10.3109/0284186X.2015.1043026
41. Paramanathan A, Saxena A, Morris DL (2014) A systematic review and meta-analysis on the impact of pre-operative neutrophil lymphocyte ratio on long term outcomes after curative intent resection of solid tumours. Surg Oncol 23:31–39. https://doi.org/10.1016/j.suronc.2013.12.001
42. McMillan DC (2013) The systemic inflammation-based Glasgow prognostic score: a decade of experience in patients with cancer. Cancer Treat Rev 39:534–540. https://doi.org/10.1016/j.ctrv.2012.08.003
43. Laird BJA, Fallon M, Hjermstad MJ et al (2016) Quality of life in patients with advanced cancer: differential association with

performance status and systemic inflammatory response. J Clin Oncol 34:2769–2775. https://doi.org/10.1200/JCO.2015.65.7742
44. Guo Y, Pan Y, Wan J et al (2024) Prognosis stratification of cancer patients treated with immune checkpoint inhibitors through lung immune prognostic index: a meta-analysis and systematic review. BMC Cancer 24:523. https://doi.org/10.1186/s12885-024-12271-0
45. Miholjcic TBS, Halse H, Bonvalet M et al (2023) Rationale for LDH-targeted cancer immunotherapy. Eur J Cancer 181:166–178. https://doi.org/10.1016/j.ejca.2022.11.032
46. Claps G, Faouzi S, Quidville V et al (2022) The multiple roles of LDH in cancer. Nat Rev Clin Oncol 19:749–762. https://doi.org/10.1038/s41571-022-00686-2
47. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12:252–264. https://doi.org/10.1038/nrc3239
48. Sagiv J, Michaeli J, Assi S et al (2015) Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell Rep 10:562–573. https://doi.org/10.1016/j.celrep.2014.12.039
49. Liu S, Wu W, Du Y et al (2023) The evolution and heterogeneity of neutrophils in cancers: origins, subsets, functions, orchestrations and clinical applications. Mol Cancer 22:148. https://doi.org/10.1186/s12943-023-01843-6
50. Nelli F, Fabbri A, Virtuoso A et al (2024) Early changes in LIPI score predict immune-related adverse events: a propensity score matched analysis in advanced non-small cell lung cancer patients on immune checkpoint blockade. Cancers (Basel) 16:453. https://doi.org/10.3390/cancers16020453

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