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Molecular and immunological features associated with long-term benefits in metastatic NSCLC patients undergoing immune checkpoint blockade

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ABSTRACT

Introduction: Immunotherapy is firmly established as a treatment regimen in various solid tumors, driven by its exceptional benefits in a selected group of patients. Despite widespread adoption of immune checkpoint blockade (ICB) across diverse solid tumors, the quest for a clinically informative biomarker for long-term benefit remains unmet.

Methods: A total of 49 patients with metastatic NSCLC treated with ICB were included. Long-term (LTR) and short-term responders (STR) were defined as those with a response to ICB lasting more than 24 months or less than 6 months, respectively. Longitudinal blood specimens were collected before ICB treatment initiation and early-on treatment. Plasma ctDNA next-generation sequencing panel (NGS) and serum proteomics were performed. GeoMx DSP on baseline tumor tissue was performed in a subset of patients.

Results: Our analysis revealed specific characteristics of LTR compared with STR, namely higher PD-L1 in tumor cells ($p = 0.005$) and higher incidence of irAEs ($p = 0.001$). Genomic features associated with lack of benefit from ICB included co-occurring mutations in KRAS/STK11 and TP53/KMT2D ($p < 0.05$). At a baseline, LTR patients exhibited higher serum levels of proteins related with apoptosis (CASP8, PRKRA), chemotaxis, immune proteasome, processing of MHC class I (S100A4, PSMD9, RNF41) and immune homeostasis (HAVCR1, ARG1) ($p < 0.05$). Protein spatial profiling of tumor samples showed higher levels of proteins linked with the presence of immune cells (CD45), T cells (CD8), antigen presentation (HLA-DR) and immune regulation proteins (PD-L1, IDO1) within the tumor and tumor stroma component ($p < 0.05$) in LTR patients. Serum longitudinal analysis identified a set of proteins that presented distinct dynamics in LTR compared to STR, making them interesting candidates to evaluate as early predictors of treatment efficacy.

Conclusions: Our multimodal analysis of patients with metastatic NSCLC treated with ICB identified clinicopathological and immunological features associated with long-term benefits. The presence of preexisting antitumor immunity emerged as a strong predictor of long-term benefits, providing insights for potential biomarkers and therapeutic strategies for enhancing ICB outcomes in metastatic NSCLC.

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Introduction

While lung cancer remains the leading cause of cancer-related mortality, recent years have witnessed a decline in mortality rates from this ominous disease.[1,2] The addition of immune checkpoint blockade (ICB) and targeted therapy to the lung cancer armamentarium highly contributed to this landmark in the lung cancer history.[3,4] More significantly, these developments have ignited a surge in translational studies aiming to expand and solidify these benefits for a broader patient population.[5] In stark contrast with most targeted therapies, there are no current biomarkers to identify and select patients that will derive the greatest benefit from ICB.[6,7] Previous clinical trials showed that high tumor and immune PD-L1 expression predict overall favorable response to ICB.[8,9] Yet, a significant fraction of patients with PD-L1 positive (≥1%) tumors do not respond to ICBs and, conversely, other studies have demonstrated exceptional responses in patients with PD-L1 negative (<1%) tumors,[6] thus highlighting the need for more robust and reliable biomarkers to predict response to ICB.

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Adding complexity to the field, different treatment combinations (anti-PD-1/PD-L1 combined with chemotherapy ± anti-CTLA4) have become standard of care in advanced non-small cell lung cancer (NSCLC), but again, are still without a biomarker that can assist clinicians to assign the right treatment to the right patient at the right time.[10–12] Nevertheless, a common observation across all studies, is the presence of two distinctive subsets of patients. The first subset faces an extremely limited prognosis, often succumbing to the disease within the initial six months of treatment, while the second subset, comprising approximately 15–20% of patients, showcases an exceptional response that defies the odds by remaining alive after a 5-year follow-up.[13–15]

On this theme, identification of patient-specific features that characterize long-term responders (LTR) to ICB remains an unresolved challenge. Recent studies interrogating the pathological and genomic features found that high PD-L1 (≥50% and ≥ 90%) and next-generation sequencing (NGS) profiling, while identifying patients most likely to respond to ICB, do not identify this restricted group of patients with an exceptional benefit. Instead, these patients are characterized by an extremely high tumor mutational burden (TMB) (>90th percentile).[16] A recent meta-analysis exploring mechanisms that predict sensitivity to ICB, found high clonal TMB, along with increased expression of specific cytokines (*CXCL9* and *CXLC13*) in the tumor microenvironment, as strong predictors of response to ICB across different tumor types,[17] however no insights on specific mechanisms driving long-term benefits were described. Previous work describing the immune landscape in localized lung cancer demonstrated early changes in intratumor immunity, as well as in blood specimens as predictors of benefit.[18,19] Overall, these findings point to the potential significance of host anti-tumor immunity as a pivotal factor in orchestrating sustained responses to ICB. More recently, work on the neoadjuvant setting,[20,21] has shown that neoadjuvant chemoimmunotherapy and anti-PD-1 plus anti-CTLA-4 strongly upregulated intratumor immune gene programs as demonstrated through longitudinal analysis. This underscores the role of 'immunomodulation' as a responsible mechanism for the higher rates of major pathologic responses observed in the neoadjuvant chemoimmunotherapy group. Thereby, it is plausible to hypothesize that a similar approach can be of great importance and provide valuable information in the metastatic setting. However, the scarcity of high-quality specimens from patients with long-term clinical follow-up has limited the pursuit of such studies until now.

Here, we report a comprehensive analysis of plasma and serum samples prospectively and longitudinally collected from metastatic NSCLC patients treated with ICB (anti-PD-1/PD-L1) along with digital spatial proteomic analysis of tumor tissue obtained at diagnosis. Our investigation explores the genomic, serum proteomic, and tumor proteomic profiles, aiming to elucidate the unique molecular and immunological landscape that distinguishes patients who experience an exceptional benefit from ICB.

Materials and methods

Patient cohort

A cohort of 49 metastatic non-small cell lung cancer (NSCLC) patients, that underwent treatment with immune checkpoint blockade (ICB) (anti-PD-1/PD-L1) between 2017 and 2022 at Hospital del Mar, Barcelona, Spain, were included in this work. This study was approved by the Institutional Review Board (IRB) at Hospital del Mar (2022/10627/I). Anti-PD-1/PD-L1 was administered as provided by local and European guidelines. Mutational status of key driver genes (*EGFR, KRAS, ALK, ROS1, RET, MET, HER2, BRAF, NTRK1/2/3*) was assessed in tumor tissue using Oncomine Precision Assay Thermofisher®. Detailed clinicopathological information including demographics, smoking history, presence of brain and liver metastases, as well as radiologic treatment response, overall (OS) and progression-free survival (PFS) for all cases are summarized in Table 1.

Patients were classified as long-term responders (LTR) or short-term responders (STR) based on the benefit obtained after ICB initiation. LTR were defined as patients achieving a clinical benefit from ICB for more than 24 months. In contrast, STR patients were classified as patients progressing to ICB within the first 6 months. This represents a selected population focusing on the two extreme scenarios (LTR and STR), while significantly underrepresenting the population that falls between two poles.

PD-L1 immunohistochemistry

PD-L1 expression was determined by two pathologists (IS and MG) using the tumor proportion score (TPS) following the International for the Study of Lung Cancer (IASLC) guidelines.[22] Briefly, viable tumor cells displaying partial or complete staining for PD-L1 membrane expression were considered relative to the total number of tumor cells, while positive immune cells and neoplastic cells showing only cytoplasmic staining were excluded.

Plasma ctDNA NGS

The Personal Genome Diagnostics elio™ plasma complete assay (PGDx/Labcorp Oncology, Baltimore, MD USA) was used for comprehensive genomic profiling of cell-free DNA (cfDNA). The assay is comprised of 521 solid tumor-related genes and detects somatic single nucleotide variants, insertions/deletions, gene fusions, gene amplifications, blood tumor mutational burden (bTMB), and microsatellite instability from plasma-derived circulating tumor DNA (ctDNA). Briefly, cfDNA was extracted from up to 4 mL of plasma and genomic libraries were prepared from 25 ng of cfDNA. Targeted capture was performed by hybridization to dsDNA probes. The captured libraries were sequenced using a targeted deep-sequencing approach with 150bp paired-end reads on the NovaSeq 6000 instrument with an S2 flowcell (Illumina, CA USA) targeting 25,000× depth across the target regions. Demultiplexing, base quality trimming, alignment, variant calling, and filtering using a machine learning approach were performed as previously described.[23]

Table 1. Descriptive table including relevant clinicopathological characteristics of the population included.

Patient's Characteristics	*N(%)*
Gender	
Male	38 (77.6%)
Female	11 (22.4%)
Age, median (range)	65.1 (44–82) years
Smoking status	
Never	2 (4.1%)
Former	39 (79.6%)
Current	8 (16.3%)
ECOG	
0	19 (38.8%)
1	23 (46.9%)
2	6 (12.2%)
3	1 (2.04%)
Histology	
Adenocarcinoma	35 (71.4%)
NOS	3 (6.12%)
Squamous cell carcinoma	11 (22.4%)
Molecular alterations in tumor tissue	
KRAS	15 (30.6%)
EGFR	1 (2.0%)
MET amplification	2 (4.1%)
None	31 (63.3%)
PD-L1 by IHC (TPS)	
<1%	18 (36.7%)
≥1%	23 (47.0%)
Not available	8 (16.3%)
TNM Stage 8th edition	
I	2 (4.08%)
II	3 (6.12%)
III	14 (28.6%)
IV	30 (61.2%)
Brain metastasis	
No	41 (83.7%)
Yes	8 (16.3%)
Liver metastasis	
No	44 (89.8%)
Yes	5 (10.2%)
TMB, median (range)	2.14 (0–16.4) mut/Mb
Line of treatment	
1st Line	19 (38.8%)
2nd Line	25 (51.0%)
3rd Line	5 (10.2%)
Radiotherapy	
No	41 (85.4%)
Yes	7 (14.6%)
Radiologic response	
CR	6 (13.3%)
PR	11 (24.4%)
SD	8 (17.8%)
PD	20 (44.4%)
Survival status	
Alive	18 (36.7%)
Death	31 (63.3%)
Exceptional responders	
Short-term responders (STR)	28 (57.1%)
Long-term responders (LTR	21 (42.9%)

NOS, not otherwise specified; PD-L1, Programmed death-ligand 1; TMB, tumor mutational burden, CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Serum proteomics profiling

Proteome analysis was carried out as previously described[24] using the Olink Proximity Extension Assay (PEA) (Olink Proteomics AB, Uppsala, Sweden), specifically, we used the explore 384 Oncology panel. In brief, pairs of oligonucleotide-labeled antibody probes bind to their target protein, and if the two probes are brought in proximity the oligonucleotides will hybridize in a pair-wise manner. The addition of a DNA polymerase leads to a proximity-dependent DNA polymerization event, generating a unique target sequence analyzed through Next Generation Sequencing. Known sequence reads are counted and translated into normalized protein expression (NPX) units through a quality control and normalization process developed and provided by Olink. Data were quality controlled and normalized using an internal extension control, to adjust for intra-run variation. The final assay read-out is presented in NPX values, which is an arbitrary unit on a log2-scale where a high value corresponds to a higher protein abundance. All assay validation data (detection limits, intra- and inter-assay precision data, etc.) are available on the manufacturer's website (www.olink.com).

Protein spatial profiling

Quality control (QC) for each sample was performed by a pathologist, using a newly sectioned hematoxylin-eosin (H&E) slide of the tumor specimens. A minimum cutoff of 20 cells was required for each biological segment: Tumor or Tumor stroma component (TSC) to select adequate regions for further evaluation with NanoString GeoMx Digital Spatial Profiling (DSP) protein assay. Following confirmation of QC, 5 µm thick sections were stained with the semi-automated GeoMx DSP standard protocol using the Leica Bond Rx autostainer system (Leica Biosystem). For identification of tumor and cells of the TSC, sections were stained with pancytokeratin (panCK), SYTO 13 (GeoMx Solid Tumor TME Morphology Kit, Cat# 121300301), CD3e (Clone 175UMAB54, AF647, Origen, catalog number: UM50048 concentration 0.25 µg/ml) and CD20 (Clone IGEL/773, A594, catalog number: NBP2–47840, concentration 0.25 µg/mL).

We used 49 immune related biomarkers with the following panels Supplementary table S1: GeoMx Immune Cell Profiling (Cat# GMX-PROCO-NCT-HICP-12), GeoMx IO Drug Target (Cat# GMX-PROMOD-NCT-HIODT-12), GeoMx Immune Activation Status (Cat# GMX-PROMOD-NCT-HIAS-12), Immune Cell Typing (Cat# GMX-PROMOD-NCT-HICT -12). Following completion of the scanning in the GeoMx DSP device, multiplex immunofluorescence image slides were visualized adjusting channel thresholds for each fluorophore. Selection of ROIs was performed using the rectangle or polygon selection tool, for 1 area of up to 5 ROIs of 660 × 785 µm on each core. Each region of interest (ROI) was segmented into compartments or areas of illumination (AOI), labeled as "Tumor" (panCK-positive) or "TSC" (panCK-negative) Supplementary Figure S1. Each segmented AOI was illuminated via ultraviolet light on the DSP device, photocleaving the oligonucleotides tags conjugated with antibodies present within each AOI. Released tags were quantitated in an nCounter and counts were mapped back to tissue location, providing a spatially resolved digital profile of analyte abundance.

Bioinformatic analysis

Serum proteomic analysis

Serum protein data was generated at Olink® core facility and reported as NPX units. Data QC was performed under the manufacturer's recommendations. Principal Component

Analysis (PCA), distribution plots, sample median and sample interquartile range (IQR) were plotted to visualize outliers. Sample 56 timepoint 2 was identified as an outlier and excluded from downstream analysis. Differentially expressed proteins were identified using limma package (version 3.54.2). Several linear models were fit: (1) LTR vs STR at baseline, (2) LTR vs STR at timepoint 2, (3) LTR vs STR of ΔNPX values (timepoint2 - baseline) and (4) a mixed effects model with treatment and timepoint as fixed effects and patient as random effects with duplicateCorrelation function. *p* values were adjusted for multiple comparisons using the Benjamini – Hochberg false discovery rate (FDR) approach. Due to lack of results with adjusted *p* value, proteins were considered differentially expressed when $p \leq 0.05$ and the fold change |FC| >1.5 (|log2FC| > 0.585).

ctDNA plasma analysis

Raw data were generated at the PGDx CLIA Laboratory located in Baltimore, MD USAcore facility. Median coverage across all samples was 17,010×, ranging from 9,974× to 26,381×. Median TMB was 2.142 mutations/Mb (mut/Mb; range 0–16.423). Genomic analysis was performed using maftools package (version 2.14.0). Incidence of specific mutations between LTR and STR groups was calculated using Fisher's Exact test and visualized using coBarplot function. Mutually exclusive or co-occurring mutations were detected using somaticInteractions function, which performs pair-wise Fisher's Exact test to detect such significant pair of genes.

Protein spatial profiling analysis

Protein Spatial Profiling data was generated at Translational Molecular Department at MDAnderson Cancer Center, Houston. Digital counts were normalized by Housekeepers proteins using the GeoMx® DSP Data Analysis Suite. The GeoMx® DSP platform was used for high-plex molecular profiling within predefined regions of interest (ROI) but does not provide single-cell resolution or spatial mapping of individual proteins. Differentially expressed proteins were identified using limma package (version 3.54.2). *p* values were adjusted for multiple comparisons using the Benjamini – Hochberg false discovery rate (FDR) approach. Due to lack of results with adjusted *p* value, proteins were considered differentially expressed when $p \leq 0.05$ and the fold change |FC| >1.5 (|log2FC| > 0.585).

Multi-omic data integration

MixOmics R package, and specifically DIABLO approach,[25] was used to explore and integrate biological data sets (baseline serum proteomics and baseline tissue proteomics – tumor and tumor stroma component compartments) with a specific focus on the long-term response to ICB.

Statistical analysis

Statistical analyses were conducted to evaluate associations among continuous and categorical variables. The Mann-Whitney U test was applied for categorical variables with two levels, while the Kruskal–Wallis test was utilized for variables with more than two levels. Fisher exact test was used to test for independence of two factors. Survival analysis was carried out using the Cox proportional-hazards model. Overall survival was calculated as the time between the date of the beginning of ICB and the date of death.

All statistical analyses were performed using the R and RStudio software, version 4.2.2 (2022-10-31).

Results

Cohort description and main clinicopathological features of long-term responders to ICB

With the aim to explore clinicopathological, molecular and proteomics features associated with long-term response to immune checkpoint blockade (ICB), we included 49 metastatic NSCLC patients that received anti-PD-1/PD-L1, comprising 21 patients achieving a long-term response (LTR; >24 months after starting ICB) and 28 short-term responders (STR) that progressed within 6 months of starting ICB Figure 1a. Relevant clinicopathological and molecular features are described in Figure 1b and Table 1. Overall, our cohort was comprised predominantly by male patients (77.6%), former or current smokers (95.9%) and 71.4% of the cases presented as adenocarcinoma histology Table 1.

Comparison between LTR and STR cohorts showed, as anticipated, a significant increase in overall survival probability for LTR patients ($p < 0.0001$) Figure 1c. Patients experiencing exceptional benefit from ICB had more frequently PD-L1 positive tumors by IHC ($p = 0.005$, Figure 1d), and had higher incidence of immune-related adverse events (irAEs) ($p = 0.001$, Figure 1f). Notably, no differences were observed in blood tumor mutational burden (bTMB) ($p = 0.313$, Figure 1e). Radiological response assessment was also significantly different among groups, with LTR patients achieving higher rates of complete and partial responses (28.6% and 47.6% against 0% and 4.2% in STR patients respectively). On the other hand, STR patients showed a higher frequency of progressive disease compared with LTR group (83.3% Vs 0%) ($p < 0.001$, Table 2). Presence of brain or liver metastasis at the time of ICB initiation did not show significant differences among LTR and STR groups ($p = 0.999$ and $p = 0.639$, respectively, Table 2).

Genomic features of long-term response to immune checkpoint blockade in patients with metastatic NSCLC

Plasma ctDNA assay was available for 31 patients (10 LTR and 21 STR). *TP53* mutation was the most common mutation observed in our cohort (45% of cases), followed by *KEAP1* and *KRAS* (32% and 29% of cases respectively) Figure 2a. bTMB in our cohort (median 2.142 mutations/Mb) was in line with tissue TMB estimates within TCGA lung cancer cohorts (Suplementary Figure S2A LUAD and LUSC). However, we did not observe differences in bTMB between LTR and STR groups ($p = 0.313$) and was also not associated

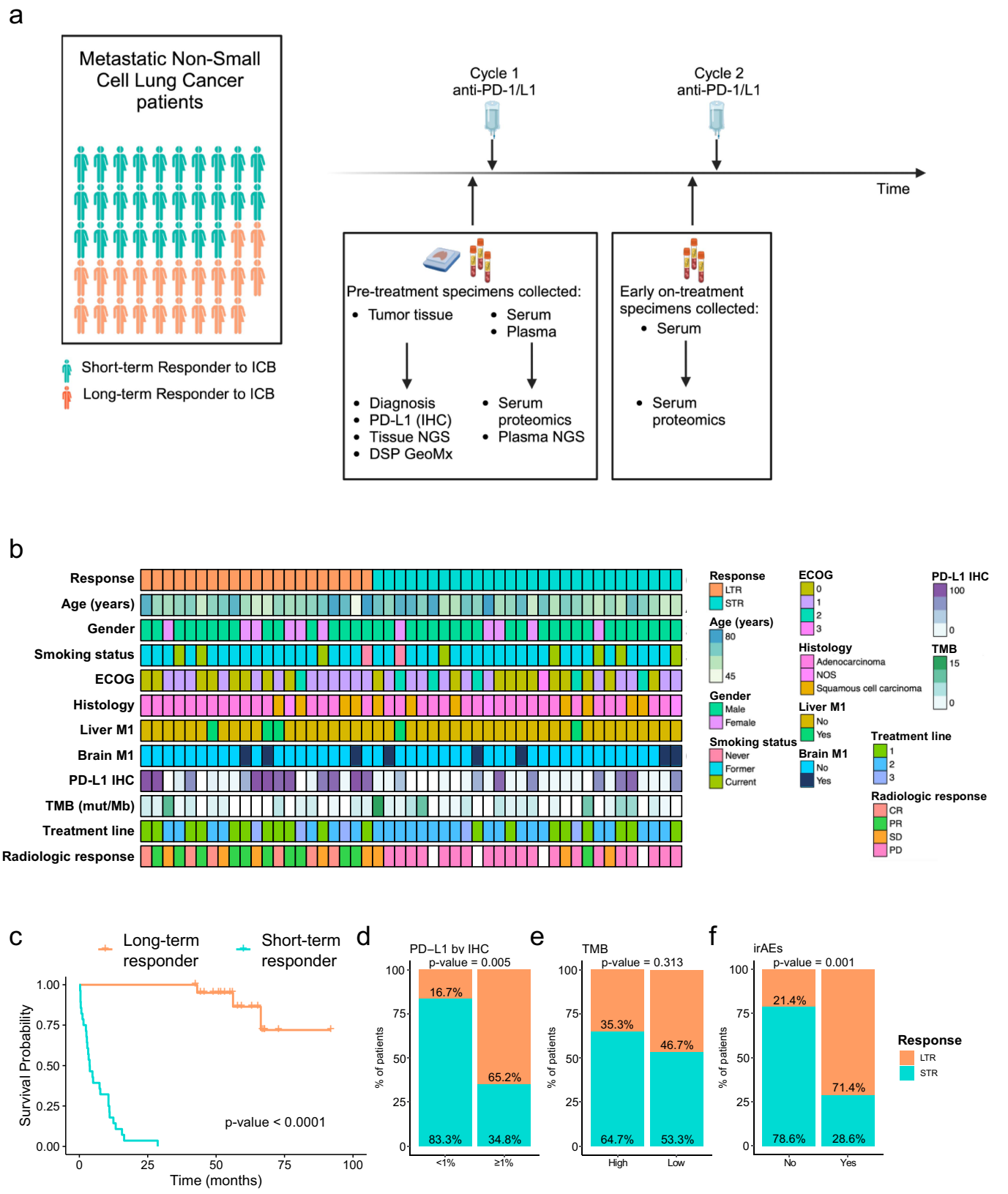

Figure 1. Study design and clinicopathological features from the population included. a) Overview of the study design, including timepoints for sample collection and subsequent analysis. Diagnosis work-up, tissue NGS panel and PD-L1 IHC was done at the time of diagnosis. Blood samples were obtained at baseline (pre-treatment) and early on-treatment (before the second cycle of ICB). b) Heatmap of patient demographics and clinicopathological characteristics. c) Survival analysis comparing long-term and short-term responders to ICB, visualized using Kaplan–Meier curves. d) Barplot comparing the proportion of LTR and STR patients in PD-L1 positive and negative tumors, e) stratify by baseline blood TMB (high and low based on the median – 2.142 mutations/Mb) and f) stratify by the incidence of irAes. *p* values were calculated using the Fisher exact test.

Table 2. Clinicopathological characteristics comparison between LTR (Long-term responders) and STR (Short-term responders) to immune checkpoint blockade.

	LTR *N = 21*	STR *N = 28*	p-value*
Gender			0.494
Male	15 (71.4%)	23 (82.1%)	
Female	6 (28.6%)	5 (17.9%)	
Age, median	64.8 years	65.2 years	0.868
ECOG			0.365
0	8 (38.1%)	11 (39.3%)	
1	12 (57.1%)	11 (39.3%)	
2	1 (4.76%)	5 (17.9%)	
3	0 (0.00%)	1 (3.57%)	
Smoking status			0.999
Never	1 (4.76%)	1 (3.57%)	
Former	17 (81.0%)	22 (78.6%)	
Current	3 (14.3%)	5 (17.9%)	
Histology			0.885
Adenocarcinoma	16 (76.2%)	19 (67.9%)	
NOS	1 (4.76%)	2 (7.14%)	
Squamous cell carcinoma	4 (19.0%)	7 (25.0%)	
Molecular alterations in tumor tissue			0.473
KRAS	6 (28.6%)	9 (32.1%)	
EGFR	0 (0.00%)	1 (3.57%)	
MET amplification	2 (9.52%)	0 (0.00%)	
None	13 (61.9%)	17 (64.3%)	
PD-L1 by IHC (TPS)			0.005
<1%	3 (16.7%)	15 (65.2%)	
≥1%	15 (83.3%)	8 (34.8%)	
TNM Stage 8th edition			0.493
I	1 (4.76%)	1 (3.57%)	
II	0 (0.00%)	3 (10.7%)	
III	7 (33.3%)	7 (25.0%)	
IV	13 (61.9%)	17 (60.7%)	
Brain metastasis			0.999
No	18 (85.7%)	23 (82.1%)	
Yes	3 (14.3%)	5 (17.9%)	
Liver metastasis			0.639
No	18 (85.7%)	26 (92.9%)	
Yes	3 (14.3%)	2 (7.14%)	
TMB, median (mut/Mb)	2.69	4.06	0.313
IrAEs			**0.001**
No	6 (28.6%)	22 (78.6%)	
Yes	15 (71.4%)	6 (21.4%)	
Radiotherapy			0.683
No	17 (81.0%)	24 (88.9%)	
Yes	4 (19.0%)	3 (11.1%)	
Radiologic response			**<0.001**
CR	6 (28.6%)	0 (0.00%)	
PR	10 (47.6%)	1 (4.2%)	
SD	5 (23.8%)	3 (12.5%)	
PD	0 (0.00%)	20 (83.3%)	
Survival status			**<0.001**
Alive	18 (85.7%)	0 (0.00%)	
Death	3 (14.3%)	28 (100%)	

NOS, not otherwise specified; PD-L1, Programmed death-ligand 1; TMB, tumor mutational burden, CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

with overall survival (cox proportional hazards regression model, $p = 0.323$).

Genomic differences between LTR and STR revealed a higher prevalence of *BRCA1* and *STK11* mutations within STR group ($p < 0.05$, for all mutations) Figure 2b. Additionally, we explored the role of co-occurring or mutually exclusive mutations between LTR and STR cohorts. Within the STR group, we identified co-occurring mutations in *KRAS/STK11* and *TP53/KMT2D*, which were significantly associated with lack of ICB efficacy. Mutually exclusive mutations in KRAS and TP53 were also more frequent found in STR group ($p <$ 0.05 for all) Figure 2c. In contrast, LTR group exhibited a higher incidence of co-occurring mutations in *KRAS/SPTA1* and *TP53/KEAP1* ($p < 0.05$ for all) Supplementary Figure S2B.

Additionally, we interrogated the correlation of specific mutations in our cohort and their association with overall survival. We observed that patients with tumors harboring *BRCA1* mutations displayed a significant reduction in overall survival ($p = 0.034$) Figure 2d. Furthermore, a trend toward a survival disadvantage was found in patients exhibiting co-mutation of *KRAS/KEAP1* and *KRAS/STK11* ($p = 0.185$ and $p = 0.164$ respectively) Supplementary Figure S3A.

Profiling of baseline and dynamic serum proteomic changes in patients undergoing immune checkpoint blockade

Longitudinal serum proteomic profiling, using the Olink® Explore 384-plex Oncology panel, was performed at baseline (prior to ICB) and previous to administration of second ICB cycle with the aim to interrogate the predictive value and the dynamic changes in serum proteins upon ICB treatment Figure 1a.

Baseline comparison between long-term (LTR) and short-term responders (STR) identified a majority of proteins ($n =$ 35) upregulated in LTR in contrast to only 4 proteins upregulated in STR. Among the proteins increased in LTR patients, we found proteins related to apoptosis (CASP8, PRKRA, CHAC2, CIAPIN1), cell cycle (CDKN2D, DCTN1, TACC3, SIRT2, USO1, SUTG1), autophagy (ATGA4) along with an increase of proteins involved in chemotaxis (S100A4), innate immune system (MME), immune proteasome and processing of MHC class I (PSMD9, RNF41), TCR signaling (STAT5B) and immune homeostasis (HAVCR1, ARG1) Figure 3a. Conversely, proteins upregulated in STR patients were related to angiogenesis and tumor suppressors (ADAMTS8, ADAMTS15), immune resting state (CD33) and regulation of T-cell proliferation (CD5).

At the time of second cycle of ICB (Timepoint 2), we identified 6 proteins upregulated in STR patients and only 2 in LTR (Figure 3b). Of note, only two out of 39 proteins differentially expressed at baseline were also different between groups at timepoint 2 (before 2nd cycle of ICB) (CD5 and ADAMTS15 in STR, Figure 3c), highlighting that proteomic changes after ICB treatment can be detected in peripheral serum. In STR, we observed the emergence of proteins related with fibroblast growth factor (FGF21), carcinogenesis (KLK4) and immune inhibitory receptors (PD-1). In contrast, two proteins showed higher relative expression in LTR patients after treatment compared to STR patients, suggesting a relative shift in protein expression profiles following treatment.

Indeed, evaluation of the change in expression (ΔNPX: timepoint 2 - baseline) between LTR and STR identified 12 proteins whose expression significantly increased in STR patients whereas decreased or remained the same in LTR patients (Figure 3d). These proteins were related to apoptosis (CIAPIN1), autophagy (ATGA4), cell cycle (SIRT2), proteins involved in chemotaxis (S100A4) and immune proteasome (PSMD9) compared with STR patients ($p < 0.05$ for all proteins).

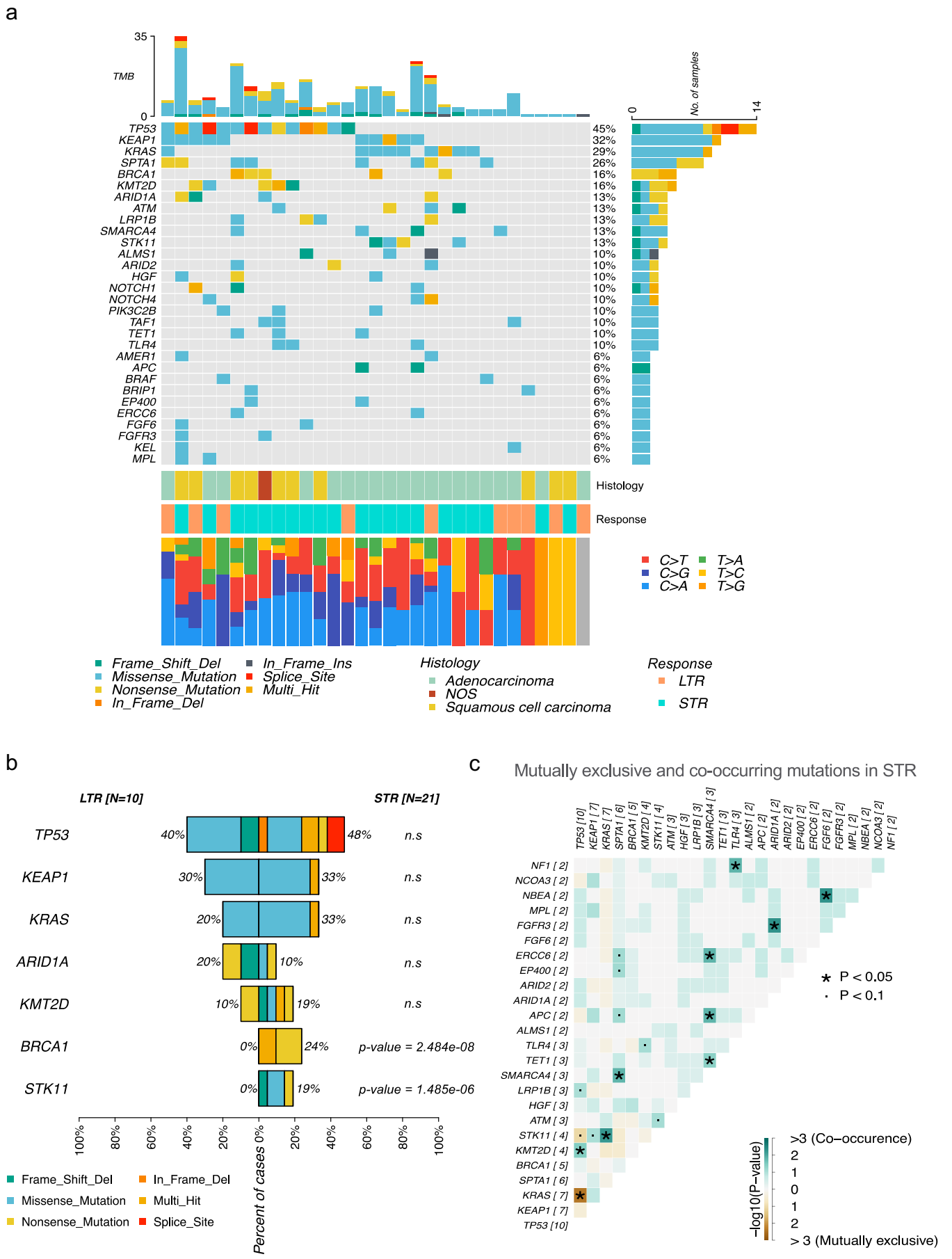

Figure 2. Distinct genomic landscapes of long-term and short-term responders (LTR Vs. STR). a) Oncoplot depicting the top 30 genomic alterations observed in our cohort. Blood TMB is displayed at the top. Columns denote samples annotated with histology, response to ICB and molecular features. b) Comparison of the mutation percentages between LTR and STR patients. c) Co-occurrence of mutations in the STR group where * denotes mutations that tend to be present simultaneously in this group. *p* values were calculated on the basis of the Fisher exact test. d) Kaplan-Meier survival analysis comparing patients harboring *BRCA1* mutations with their wild-type counterparts.

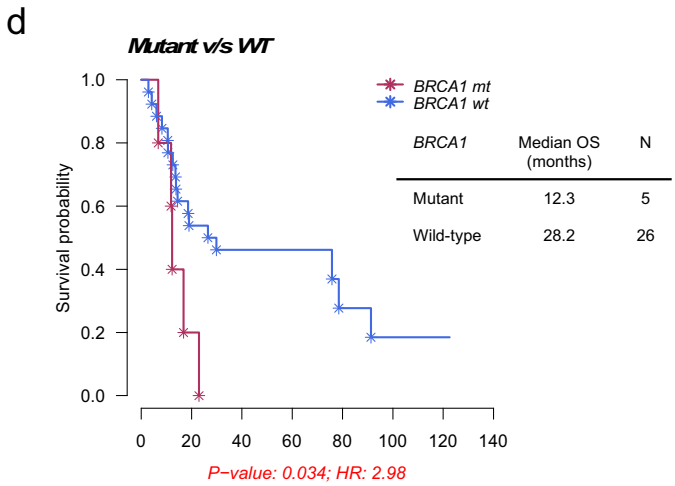

Figure 2. (Continued).

In line with these results, a mixed effect model of treatment and time revealed that most of the proteins differently expressed in serum tended to exhibit opposing trends when comparing the evolution from baseline to timepoint 2 across LTR and STR patients. Specifically, STR patients displayed higher levels at timepoint 2 for proteins related with cell cycle (YES1, DCTN1, STUG, SIRT2), apoptosis proteins (BIRC2, CHAC2), pro-tumoral inflammatory cytokines (ERBIN), T regulatory cells (FOXO3), and proteins that regulate T cell proliferation (HAVCR1), whereas the expression of these proteins either decreased or remained unchanged in LTR Supplementary Figure S4.

Tissue proteomic features linked to exceptional responders to immune checkpoint blockade

Building upon the earlier observations indicating an augmented immune system activation at the peripheral level, we next sought to interrogate the presence of proteins linked with immune activation at the tumor tissue. To this end, in a subset of patients ($n = 28$, LTR($n = 14$), STR ($n = 14$)), we conducted GeoMx Digital Spatial Profiling focused on proteins involved in tumor – immune system interactions within the tumor compartment (cytokeratin positive) and within the tumor stroma compartment (cytokeratin negative).

Comparison between LTR and STR patients revealed an augmented expression of immune proteins within LTR (in both compartments – tumor and TSC). These proteins were associated with immune checkpoints proteins (upregulation of PD-L1, IDO1, and downregulation of B7-H3 and OX40L), antigen presentation machinery (HLA-DR) and immune response (CD45, CD8) all suggestive of an augmented baseline host antitumor immune response in LTR patients Figures 4a, 4b. Illustrative cases of LTR and STR are shown in Figure 4c. Additional analysis comparing tumor PD-L1 expression (<0% Vs ≥ 0%), presence of brain and liver metastasis (Yes Vs No) at the time of ICB initiation was also performed Supplementary Figure S5.

Finally, in order to better understand the correlation between the serum and tissue proteomic data we used DIABLO (mixOmics)[25] as a framework for the integration of multiple datasets while explaining their relationship with a categorical outcome variable, in our work long-term response to ICB. As expected, TSC and tumor proteins were highly correlated. Interestingly, we found that serum proteins were also correlated to both tumor TSC compartments, suggesting that serum reflects the disease status and that could be used as biomarkers. Serum proteins related with tumor growth and proliferation (EGFL7, SIRT2, S100A4, MIA), release of tumor necrosis factor (NUCB2) and enhance of apoptosis (CHAC2) were negatively correlated (rho< −0.7, $p < 0.05$ for all proteins) with immune proteins detected within the tumor compartment using GeoMx DSP platform such as CD45, CD3, CD4, CD8, HLA-DR, CD56. Serum proteins linked with macrophage and T cell activation (AIF1), antigen presentation (APBB1P) and activation of immune system mediated by cGAS-STING pathway[26] (PQBP1) were correlated with higher abundance of immune check-point proteins within the tumor and the TSC compartments (LAG3, ICOS), T cell activation (GITR), T cell clonal expansion and survival (4-1BB) (rho >0.7, $p < 0.05$ for all proteins). Specifically, serum levels of AIF1, a protein induced by cytokines and interferons that promotes macrophage activation and T-lymphocytes, were positively correlated with tissue expression of FOXP3, and LAG3 in the STR group. Conversely, serum levels of SIRT2, an NAD-dependent deacetylase involved in cell cycle and mitotic processes, showed a negative correlation with tissue CD56 expression (NK cell marker). These findings suggest that serum proteins can act as mirrors of the tumor microenvironment, highlighting their potential as biomarkers for immune responses in the context of ICI therapy. Correlation and network plots are shown in Supplementary Figure S6 and S7, respectively.

Discussion

Our study focused on the collection and analysis of longitudinal samples from a particularly rare cohort of metastatic NSCLC patients – those who have exhibited a long-term response to immunotherapy. Our analysis revealed specific characteristics of these patients compared with short-term

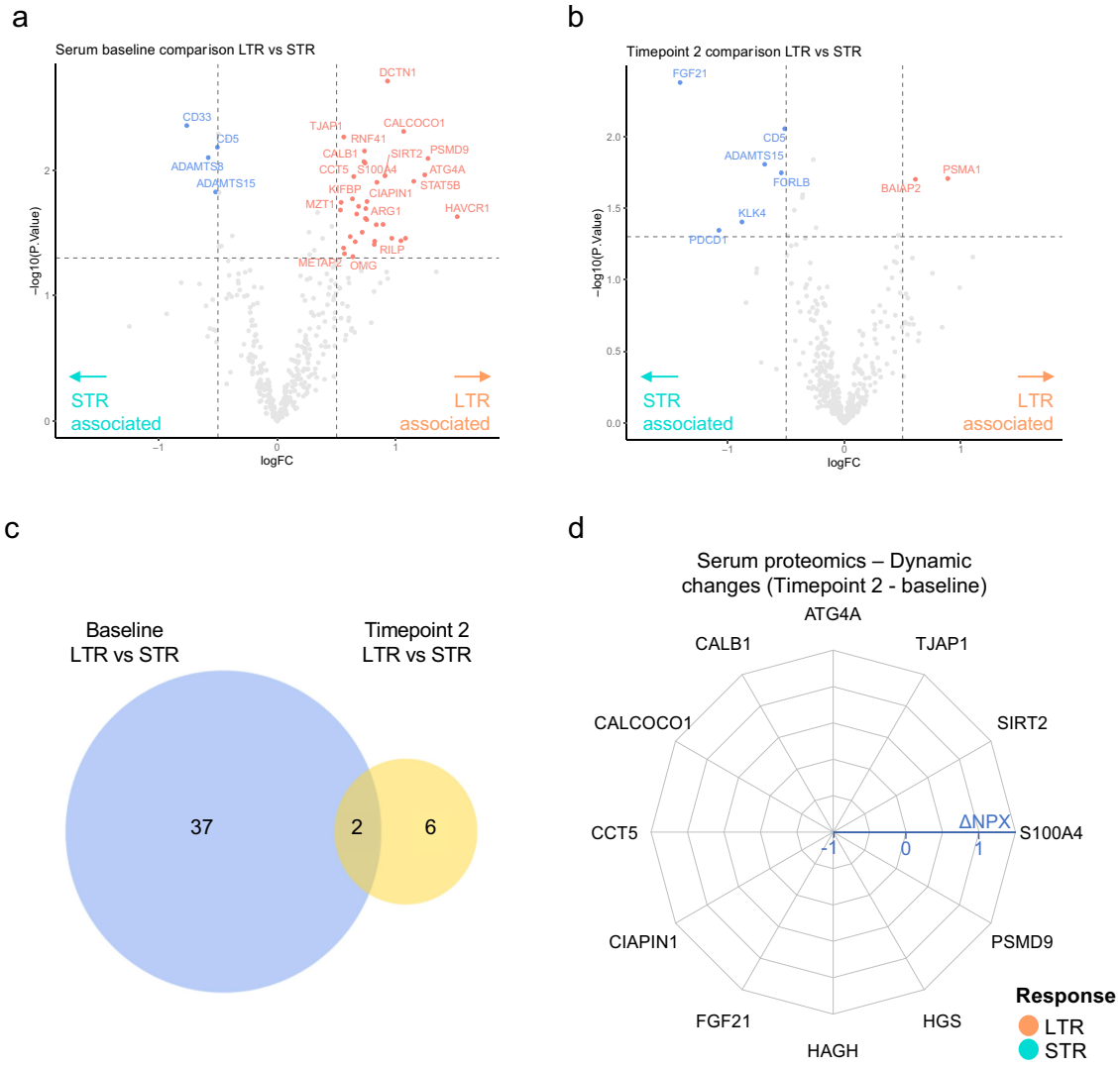

Figure 3. Profiling of baseline and dynamic serum proteomic changes in patients undergoing immune checkpoint blockade. a) Volcano plot showing baseline (pre-treatment) comparison between LTR and STR and b) at Timepoint 2 (early on-treatment, before administration of 2nd cycle). c) Venn diagram representing the overlap of proteins differently expressed at baseline and timepoint 2. d) Radarplot displaying the dynamic changes (mean ΔNPX) induced by ICB among LTR and STR patients. Differentially expressed proteins were selected on the basis of a statistical threshold of nominal p-value <0.05 and fold change |FC| >1.5 (|log2FC| > 0.585). A positive ΔNPX represents an increase of expression with time, whereas a negative value indicates a decrease.

benefit, such as higher expression PD-L1 in tumor cells, higher incidence of irAEs, as well as genomic features that predict lack of benefit to ICB, such as the co-occurrence of mutations in *KRAS, STK11*, *TP53* and *KMT2D*. Additionally, for the first time, we unveil disparities at a serum proteomic level, with LTR patients exhibiting higher abundance of proteins related with apoptosis, chemotaxis, immune proteasome, processing of MHC class I and immune homeostasis, all together suggestive of a preexisting anti-tumor immunity among LTR patients that can be identified at a peripheral level. This augmented anti-tumor immune response in LTR patients was further corroborated by our analysis of tumor tissue, which reveals higher expression levels of proteins linked with immune checkpoints proteins, antigen presentation machinery and immune response within the tumor microenvironment.

Pivotal clinical trials assessing the effectiveness of ICB in metastatic NSCLC have largely demonstrated a clinical benefit over previous standard platinum-doublet chemotherapy.[4,27,28] Remarkably, a consistent observation across these trials, marking a milestone in metastatic NSCLC, was the report of a restricted group of patients (10–20%) who remained alive beyond 5 years of follow-up.[13,15] However, and after a decade since the publication of the first results of ICB in NSCLC, no biomarker is available to correctly identify these patients prior ICB initiation. Tumor PD-L1 is the only biomarker in non-oncogenic driver metastatic NSCLC used for clinical decisions where its positivity enriches for a subset of patients more likely to respond to ICB.[16] Also, in the neoadjuvant setting, PD-L1 expression was positively associated with higher rates of complete pathologic responses.[8,9,29,30] In line with these results, in our cohort LTR patients exhibited a significant higher expression of PD-L1 (Figure 1d). Nevertheless, it is important to note that up to 16% of patients achieving an exceptional response to ICB were evaluated as PD-L1 negative tumors (Table 2), highlighting the need of more robust biomarkers.

Tumor mutational burden (TMB) was also proposed as a biomarker of response to ICB.[31,32] Under the approval of the US FDA, tumors harboring more than 10 mutations per

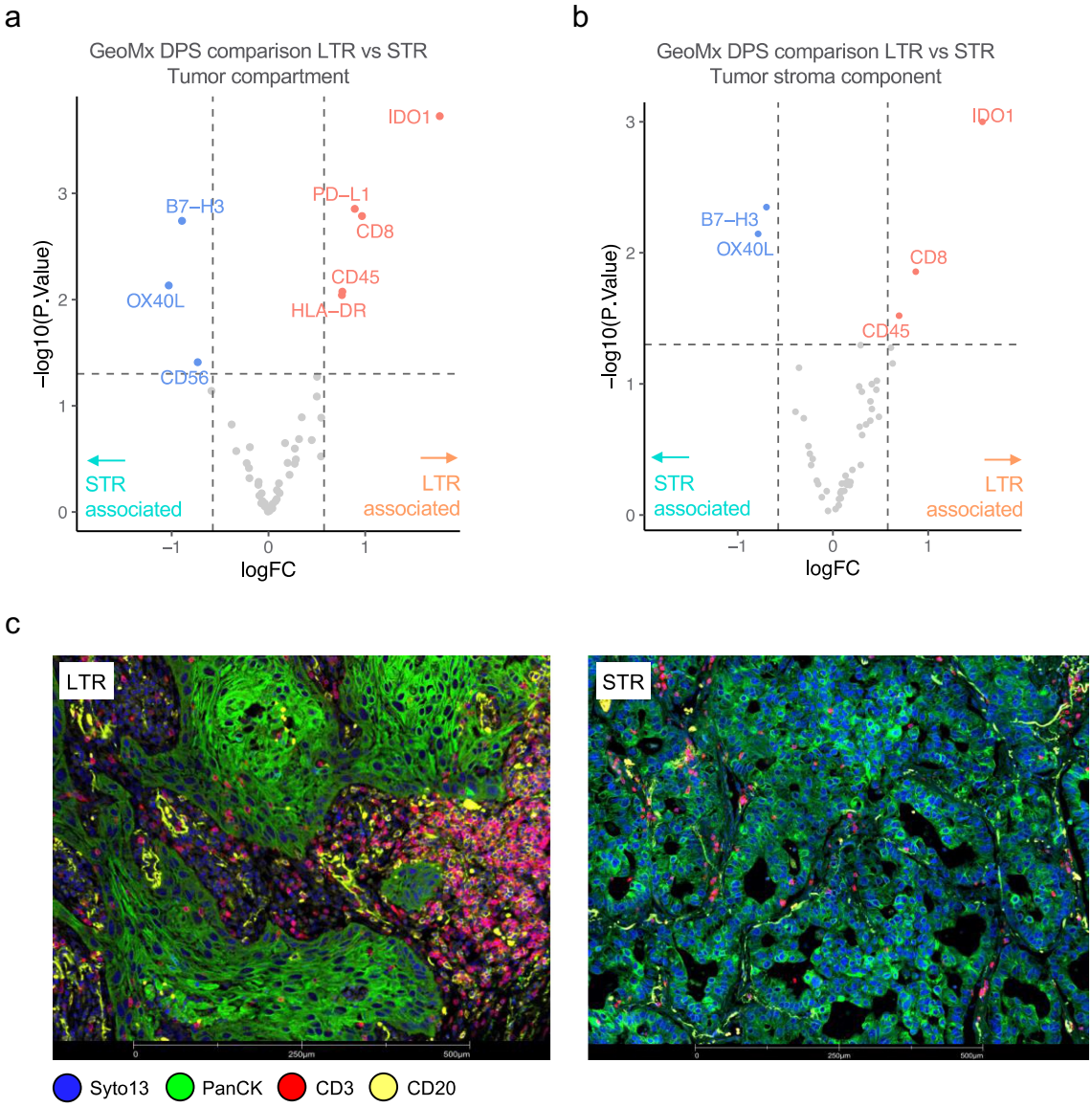

Figure 4. Tissue proteomic features linked to exceptional responders to immune checkpoint blockade. a) Volcano plot exhibiting elevated expression of immune related proteins within LTR patients within the tumor compartment and within b) the tumor stroma component (TSC). c) Representative images of mIF morphology staining of LTR (left) and STR (right) cases. Differentially expressed genes were selected on the basis of a statistical threshold of p-value <0.05.

megabase (independently of tumor primary site) can be treated with pembrolizumab (anti-PD-1).[33] Evidence suggests that a universal TMB threshold for all tumor types is somehow imperfect, and specific studies within each tumor type has been performed.[34] Particularly, in lung cancer, retrospective studies including more than 1500 patients treated with ICB found that patients with extremely high TMB (more than 19 mut/Mb) were associated with increasing improvements in response rates and survival outcomes.[35] In this line, Thummalapalli et al., showed that '*very high*' TMB (defined by TMB ≥ 90th percentile) strongly correlates with long-term benefit to ICB.[16] Of note, in our cohort, we do not find association between blood TMB and response to ICB, however, we acknowledge that the small sample size could impact and limited our ability to detect those differences.

Moreover, our genomic analysis showed that the presence of co-mutations in *KRAS/STK11* and *TP53/KMT2D* were more frequently found in STR patients. This goes in line with previous reports identifying *KRAS/STK11* and *KEAP1* mutations as markers of resistance to ICB in *KRAS*-mutant NSCLC.[36–38] *KMT2D* mutations were previously associated with worse survival outcomes in locally advanced and metastatic NSCLC.[39] In our cohort, we observe a higher frequency of *TP53/KMT2D* mutations within the STR patients. Of note, *BRCA1* mutation was found in higher percentage in STR group (24% Vs 0%, $p < 0.0001$). In line with our results, previous work by Remon et al. showed that the use of larger NGS panels identify higher incidence of *BRCA1* and *BRCA2* mutations and these mutations did not confer sensitivity to platinum-based chemotherapy. However, the role of ICB in *BRCA1*-mutant NSCLC is still unknown and it would be interesting to assess this potential association in larger cohorts.[40]

While current biomarkers to predict response to ICB, such as tumor PD-L1, TMB, tumor-associated immune cells among others are tissue-based, in our work we explored blood-based

biomarkers – serum proteomics. Previous works had shown the feasibility of this methodology to identify patients at higher risk of lung cancer,[41] and also to predict response and resistance to ICB in different tumor types.[42,43] In our work, serum proteomics analysis showed that LTR patients displayed higher level of proteins associated with apoptosis, chemotaxis, and components of the immune proteasome and processing of MHC class I. Collectively, these findings strongly indicate the presence of an enhanced preexisting anti-tumor immune response among LTR patients. Our findings align with previous research conducted at tumor tissue level, underscoring the importance of a preexisting immunity within the tumor microenvironment in tumor control.[18–20]

Our study assessed the changes in the serum proteome induced by ICB. In our cohort, at the time of cycle 2 of ICB short-term responders exhibit higher abundance of PDCD1 (PD-1) and CD5 compared to LTR, proteins associated with a negative regulation of T-cell proliferation and effector function. Moreover, dynamic changes evaluated as the difference in protein expression in early on-treatment compared to baseline, revealed a set of proteins that tend to decrease in LTR patients whereas they increase in STR patients, suggestive that higher protein levels could be associated with an uncontrolled tumor growth and lack of treatment benefit. These findings support the hypothesis that, by the time of administration of cycle 2 of ICB, patients exhibiting LTR may already be initiating an effective antitumor immune response and suggest that dynamic alterations in the serum proteome hold promise as an early biomarker long-term treatment benefit.

Lastly, with the aim to correlate the immune-related findings observed at a peripheral level (serum) with the tumor microenvironment and to compare the immune landscape between LTR and STR patients at a tumor tissue level, we performed digital spatial profiling (DSP GeoMx) in tumors from 28 patients. Tissue protein analysis showed an increase of immune checkpoints proteins (upregulation of PD-L1, IDO1, and downregulation of B7-H3 and OX40L), antigen presentation machinery (HLA-DR) and immune response (CD45, CD8) all indicative of an augmented baseline host antitumor immune response in LTR patients. Our findings suggest that the B7-H3 and the OX40-OX40L axis plays a critical role in T cell activation, and its dysregulation in STR tumors may impair anti-tumor immunity. This highlights the potential of targeting these pathways as a complementary strategy to enhance the efficacy of immune checkpoint inhibitors. These findings align with our findings at peripheral level and previous reports indicating the presence of preexisting anti-tumor immune response in patients who benefit from ICB alone or in combination with chemotherapy.[9,20,21,44,45]

Our study is not without limitations. First, our findings should be interpreted with caution due to the small sample size and lack of significance after adjusting for multiple testing in the proteomics analyses. Thus, our findings warrant validation in future studies that include larger cohorts. Nonetheless, it is important to mention, that our cohort comprises a quite rare clinical scenario of patients achieving a long-term benefit from ICB. Assembling larger cohorts with prospectively collected specimens in this context may be challenging. Our study reveals that only a limited number of DE serum proteins are conserved between timepoints, a finding that remains unexplained by our current data. This limitation underscores the need for future larger-scale studies to explore the underlying reasons for this variability. It is worth noting that our study, at peripheral and tissue proteomic levels focused on a specific subset of proteins. Consequently, we cannot rule out the potential contributions of additional proteins as predictive markers for long-term responses to ICB.

In conclusion, in our work we compile a richly annotated cohort of patients that achieve long-term benefits from ICB where longitudinal specimens were collected, allowing for a comprehensive comparison with patients who do not benefit from ICB. Genomic and proteomic profiling unveiled an amplified preexisting anti-tumor immunity that characterize long-term responders. Additionally, early changes observed upon ICB initiation strongly suggest the induction of an antitumor immune response, which holds promise as a potential biomarker for the early identification of long-term responders to ICB. These findings underscore the importance of further investigation in larger cohorts and future clinical trials to validate the clinical potential as a biomarker.

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Data availability statement

Raw data were generated at Olink core facility and PGDx CLIA Laboratory. Processed data are available from the authors and derived data supporting the findings of this study will be made available online upon the publication of this study. Individual, de-identified participant data and data dictionary can be made available upon reasonable request. Requests can be made to pedrorocha@vhio.net.

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