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Gene expression profiles (GEPs) of immuno-oncologic pathways as predictors of response to checkpoint inhibitors in advanced NSCLC

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ABSTRACT

Background: Immune checkpoint inhibitors (ICIs) revolutionized non-small-cell lung cancer (NSCLC) treatment. However, improving patients' selection for this therapy is needed. Gene expression profile (GEP) is a promising biomarker tool. We assessed the predictive value of 48 onco-immune GEPs in an NSCLC real-world scenario.
Methods: Retrospective cohort of Brazilian NSCLC patients treated with ICIs in any line. GEP was assessed in FFPE tumor tissue using the nCounter PanCancer IO360 panel, comprising 770 cancer immune genes.
Results: The median age of the 135 patients was 61 years old, most male (57.8 %), history of smoking (83.6 %), ECOG-PS 0-1 (88.7 %), clinical stage IV (91.9 %) and adenocarcinoma (65.1 %). First-line ICI in 40 % of cases, alone or in combination with chemotherapy. The median follow-up was 28 months, overall survival after starting immunotherapy (post-immunotherapy survival – PIS) was 17.8 months, and real-world progression-free survival was 5.5 months. The GEP analysis was possible in 66 patients. We found that 14 different GEPs associated with PIS, namely *IDO1, PD-L2, Cytotoxicity, Cytotoxic Cells, IFN Downstream, CTLA4, PD-L1, TIGIT, Lymphoid, Immunoproteasome, Exhausted CD8, IFN Gamma, TIS* and *APM. TIS* and *IFN-γ* were the most significant GEPs associated with favorable outcomes. The median PIS for patients with high TIS expression was 29.2 versus 15.5 months (HR 0.42; 95 %CI; 0.17–0.67; $p < 0.05$) for those with low expression. Similar results were observed for IFN-γ.
Conclusions: : The *TIS (tumor inflammation signature)* and *IFN-γ* signatures constitute predictive biomarkers to identify patients with NSCLC patients who would possibly benefit from ICI therapies.

Introduction

Immune checkpoint inhibitors (ICIs) improved the survival rate of patients with advanced non-small cell lung cancer (NSCLC) and started a new era in lung cancer treatment [1]. However only a fraction of patients benefit from immunotherapy and some suffer from limiting side effects [2]. Thus, there is an urgent need to identify predictive biomarkers to select patients for this therapeutic modality adequately. Programmed death-ligand 1 (PD-L1) expression in tumor cells, tumor mutational burden (TMB), microsatellite instability (MSI) and tumor-infiltrating lymphocytes (TIL) are the most explored biomarkers, while gene expression profile (GEP) may be a promising biomarker [3].

In clinical practice, PD-L1 expression is the only adequately validated biomarker to select candidates for isolated immunotherapy for NSCLC in the first line (PD-L1 ≥50 %) [4]. Although there is a clear correlation between PD-L1 expression and the effectiveness of ICIs, this biomarker is considered imperfect due to several factors associated with antibodies used to evaluate its expression: differences in testing

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Table 1
Clinicopathological and treatment characteristics of 135 NSCLC patients treated with anti-PD-(L)1 therapy.

Clinicopathological and treatment characteristics	n	(%)
Median age = 61 years (33-81)		
Institution		
Barretos Cancer Hospital	**100**	**74.1**
A. C. Camargo Cancer Center	**35**	**25.9**
Gender		
Male	**78**	**57.8**
Female	**57**	**42.2**
Clinical stage (AJCC 8ª edition)		
III	**11**	**8.1**
IV	**124**	**91.9**
ECOG-PS		
0-1	**128**	**88.7**
2	**12**	**9.0**
3-4	**3**	**2.2**
Unknown	**2**	**-**
Histology		
Adenocarcinoma	**88**	**65.1**
Squamous cell carcinoma	**41**	**30.3**
Adenosquamous carcinoma	**3**	**2.2**
NSCLC-NOS	**3**	**2.2**
Molecular profile		
***EGFR*+ (del19 or L858R - PCR)**	**5**	**5.7**
***ALK*+ (IHC)**	**3**	**3.4**
***ROS1*+ (FISH)**	**1**	**4.1**
***BRAF V600E*+ (PCR)**	**1**	**3.5**
***KRAS*+ (PCR)**	**3**	**10.7**
Smoking history		
Never	**22**	**16.4**
Former	**49**	**36.6**
Ever	**63**	**47.0**
Unknown	**1**	**-**
Immune checkpoint inhibitor(s) used		
Nivolumab	**71**	**52.6**
Pembrolizumab	**33**	**24.4**
Atezolizumab	**14**	**10.4**
Cemiplimab	**2**	**1.5**
Avelumab	**1**	**0.7**
Durvalumab	**1**	**0.7**
Nivolumab + ipilimumab	**9**	**6.7**
Pembrolizumab + ipilimumab	**1**	**0.7**
Durvalumab + tremelimumab	**3**	**2.2**
Line of therapy		
First	**55**	**40.7**
Second	**38**	**28.1**
Third or beyond	**42**	**31.1**
Treatment strategy		
Anti-PD-(L)1 alone	**95**	**70.4**
Anti-PD-(L)1 + Anti-CTLA4	**3**	**2.2**
Anti-PD-(L)1 + chemotherapy	**27**	**20.0**
Anti-PD-(L)1 + Anti-CTLA4 + chemotherapy	**10**	**7.4**

ECOG-PS, performance status ECOG (Eastern Cooperative Oncology Group); NSCLC-NOS: non-small-cell lung cancer - not otherwise specified; PCR: polymerase chain reaction; IHC: immunohistochemistry; FISH: fluorescence in situ hybridization

platforms, differences in the assessed cells, differences in cut-off points [4,5], intra-tumoral expression heterogeneity and observer-dependent variability [6–8]. Recently, one of these limitations was mitigated by a study that demonstrated that including inflammatory cells in the analysis of PD-L1 expression (Combined Positive Score) does not alter the test results [9].

NSCLC is among the tumors with the highest mutational burdens, which has previously been associated with greater efficacy of ICIs [10]. However, no randomized clinical trial has shown its discriminatory ability regarding overall survival [11]. In addition, several factors limit its use in clinical practice: lack of a universal cutoff that defines high TMB [12], need for a large amount of tumor sample with acceptable pre-analytical quality, variations of bioinformatics pipelines, lack of harmonization between different platforms and lack of methods to convert TMB estimates between different panels [13]. Thus, the role of TMB as a predictive biomarker of response to ICIs in NSCLC remains to be seen and has not been used in clinical practice to select patients who are candidates for immunotherapy [14]. Although MSI is recognized as a predictive biomarker of response to immunotherapy, its frequency in lung cancer is rare, even when considering a mixed Brazilian population [15].

A high density of TILs has been associated with a better prognosis in different tumor types, including NSCLC [16]. The presence of TILs is considered a reflection of better recognition of the tumor by the individual's immune system, one of the factors that characterize the inflamed tumor phenotype. This phenotype is more sensitive to ICIs and therefore TIL density has been studied as a predictive biomarker [17]. An association was found between the CD8+/CD4+ TILs ratio and response to anti-PD1 treatment in NSCLC. In addition, tumors with low CD8+ lymphocyte counts had worse response rates (p=0.046) [18–20]. Further larger studies are needed to determine the usefulness of TIL as a predictive biomarker of response to ICIs.

GEP is an active area of research with studies suggesting potential utility as predictive biomarkers of response to ICIs [17]. Immune gene expression profiles, particularly those associated with IFN-γ signaling and T-cell activation, may have predictive value and have been associated with response to immunotherapy in several types of cancer [8,21,22]. In the POPLAR study, a phase II study that tested atezolizumab in the second-line for advanced NSCLC, patients who had high expression of an effector T-cell (Teff) gene signature in the tumor had better survival [23]. However, in the IMpower150, a phase III study of atezolizumab combined with chemotherapy and bevacizumab in the first-line setting for advanced NSCLC, patients benefited from immunotherapy regardless of the Teff gene-signature expression [24].

An 18-gene tumor inflammation signature (TIS) was recently reported to predict improved response to immune checkpoint blockade. Its predictive value of response to ICIs was evaluated in a prospective cohort of 58 patients with different primary tumors (38 lung cancers, five melanomas, ten renal cell carcinomas, four urothelial carcinomas and one colon cancer). The TIS score was significantly associated with response to anti-PD-1 therapy in the entire cohort (OR 2.64; 95 % CI; 1.4-6.0; p=0.008), as well as in the NSCLC patient population (OR 3.27; 95 % CI; 1.2-11.6; p=0.03). Patients whose tumor had a high TIS score (upper tertile) had higher overall survival both in the entire cohort (HR 0.37; 95 % CI; 0.18-0.76; p=0.005) and in the NSCLC population (HR 0.36; 95 % CI; 0.14-0.90; p=0.02) [25]. The signature has been analytically validated [25] and is currently under investigation in Research Use Only (RUO) and Investigational Use Only (IUO) approaches for performance as a predictive biomarker.

Therefore, to assess the feasibility and utility of GEP in routine clinical practice, we assessed the TIS score and other gene expression profiles in a real-world series comprised of Brazilian NSCLC patients treated with ICIs.

Patients and methods

Study design and population

This was a retrospective cohort study of advanced NSCLC patients from Barretos Cancer Hospital and ACCAmargo Cancer Cencer, Sao Paulo state, Brazil, who have received at least one cycle of anti-PD-(L)1 therapy with palliative intent and had a formalin-fixed paraffin-embedded (FFPE) tissue stored in the pathology departments. The tissue should have been collected before the first dose of immunotherapy. For response analysis, patients should have had at least one radiological image after immunotherapy initiation to assess response to treatment after immunotherapy initiation.

Eligible patients were 18 years of age or older, had histologically confirmed NSCLC, advanced disease, had started anti-PD-(L)1 therapy, either as monotherapy or in combination with chemotherapy, anti-

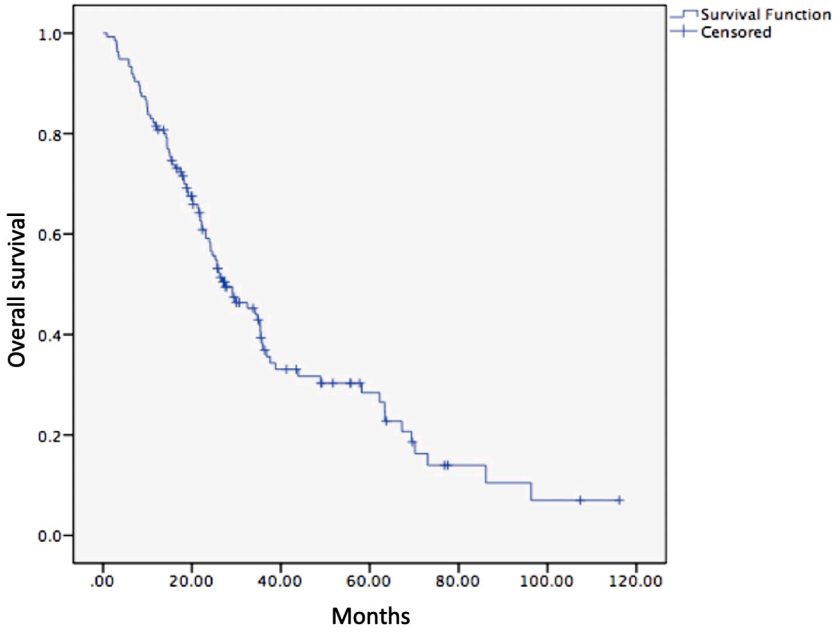

Fig. 1A. Kaplan-Meier curve for overall survival (OS). The analysis included the 135 NSCLC patients. With a median follow-up of 27.7 months, the median OS was 27.5 months.

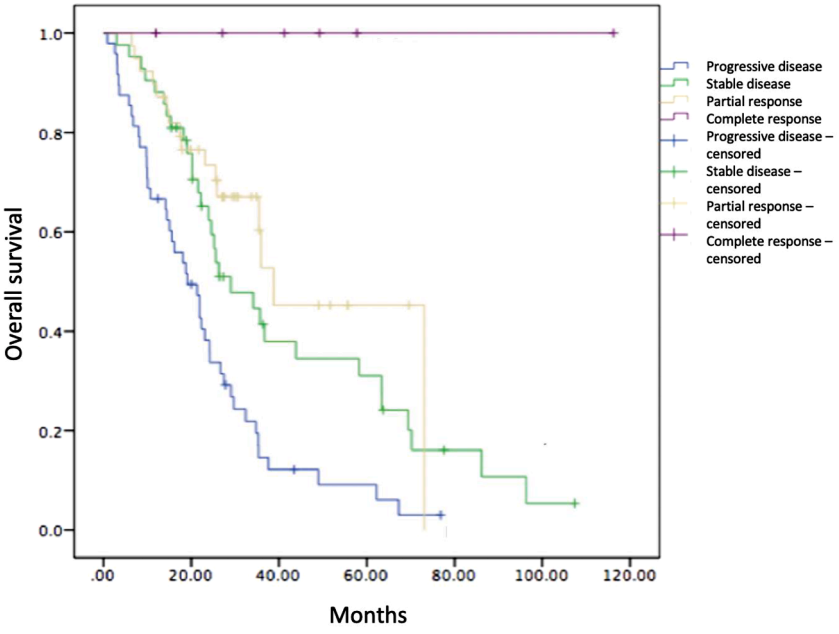

Fig. 1B. Kaplan-Meier curves for overall survival (OS) according to radiological response to immunotherapy: complete response *versus* partial response *versus* stable disease *versus* progressive disease.

angiogenic or anti-CTLA-4 treatment, and had at least one radiological response assessment after starting immunotherapy.

Clinical, demographic, radiological and pathological data were collected from patient's medical records. Histological diagnosis and staging of NSCLC were based on the 2015 World Health Organization Classification of Lung Tumors [26] and the 8th TNM Staging System of Lung Cancer [27], respectively. Tumor measurement was assessed at baseline and at least at one other time point after treatment initiation. All assessments were performed by investigators using RECIST version 1.1. Objective response rate (ORR) was defined as the proportion of patients with partial or complete radiological response to immunotherapy treatment. Real-world progression-free survival (rwPFS) was defined as the time from the date of first cycle of ICI to disease progression or death from any cause. Overall survival (OS) and post-immunotherapy survival (PIS) were defined as the time from the date of the first cycle of palliative systemic therapy and from the first cycle of palliative immunotherapy, respectively, to death from any cause.

The institutional review board approved the study protocol (CAAE 87212918.5.0000.5437) and a waiver for the written informed consent was obtained, given the study's retrospective nature.

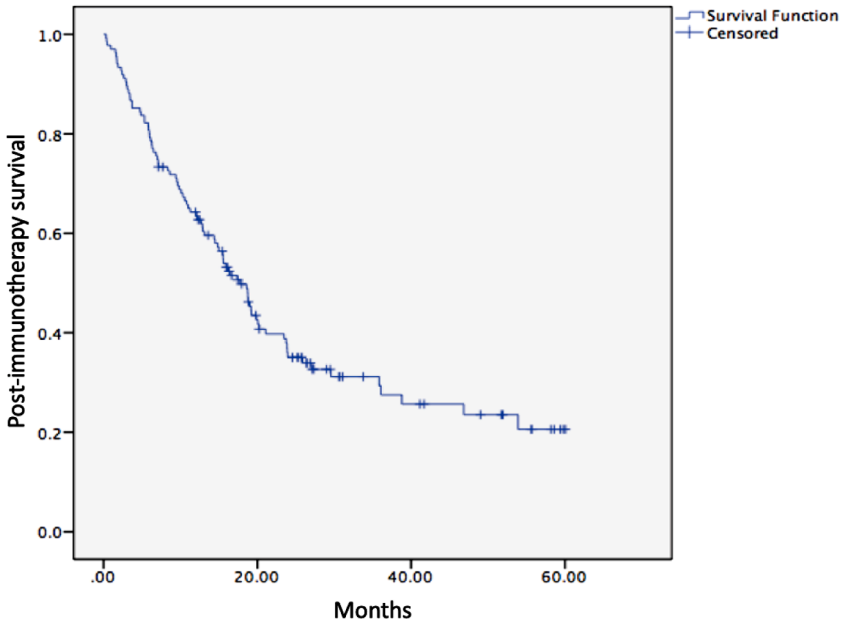

Fig. 1C. Kaplan-Meier curve for post-immunotherapy survival (months). The analysis included the 135 NSCLC patients. With a median follow-up of 27.7 months, the median post-immunotherapy survival was 17.7 months.

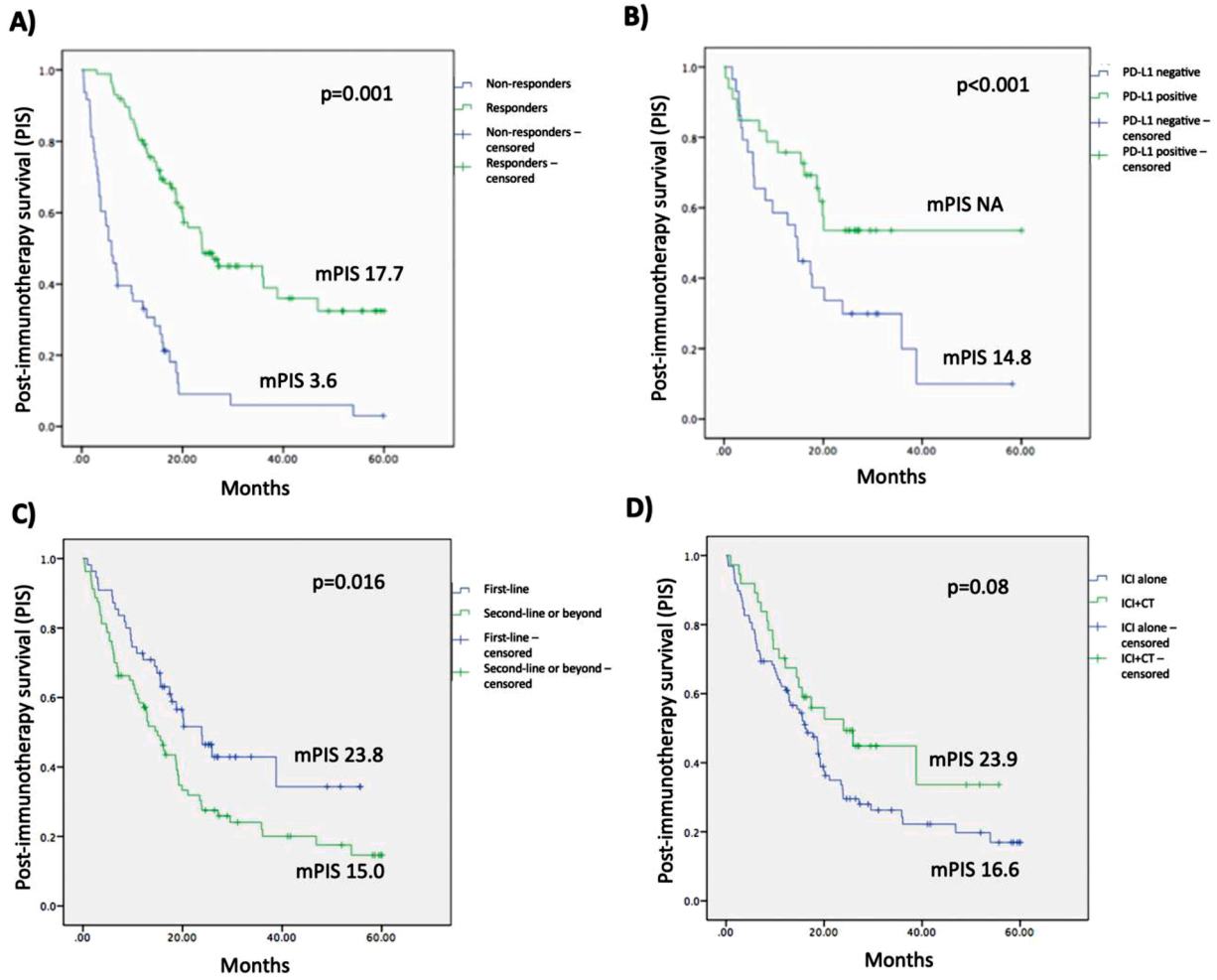

Fig. 2. Kaplan-Meier curves for post-immunotherapy survival (PIS) according to radiological response to immunotherapy (A), PD-L1 expression (B), line of treatment (C) and treatment strategy (D). mPIS = median post-immunotherapy survival; ICI = immune checkpoint inhibitor; ICI+CT = immune checkpoint inhibitor + chemotherapy

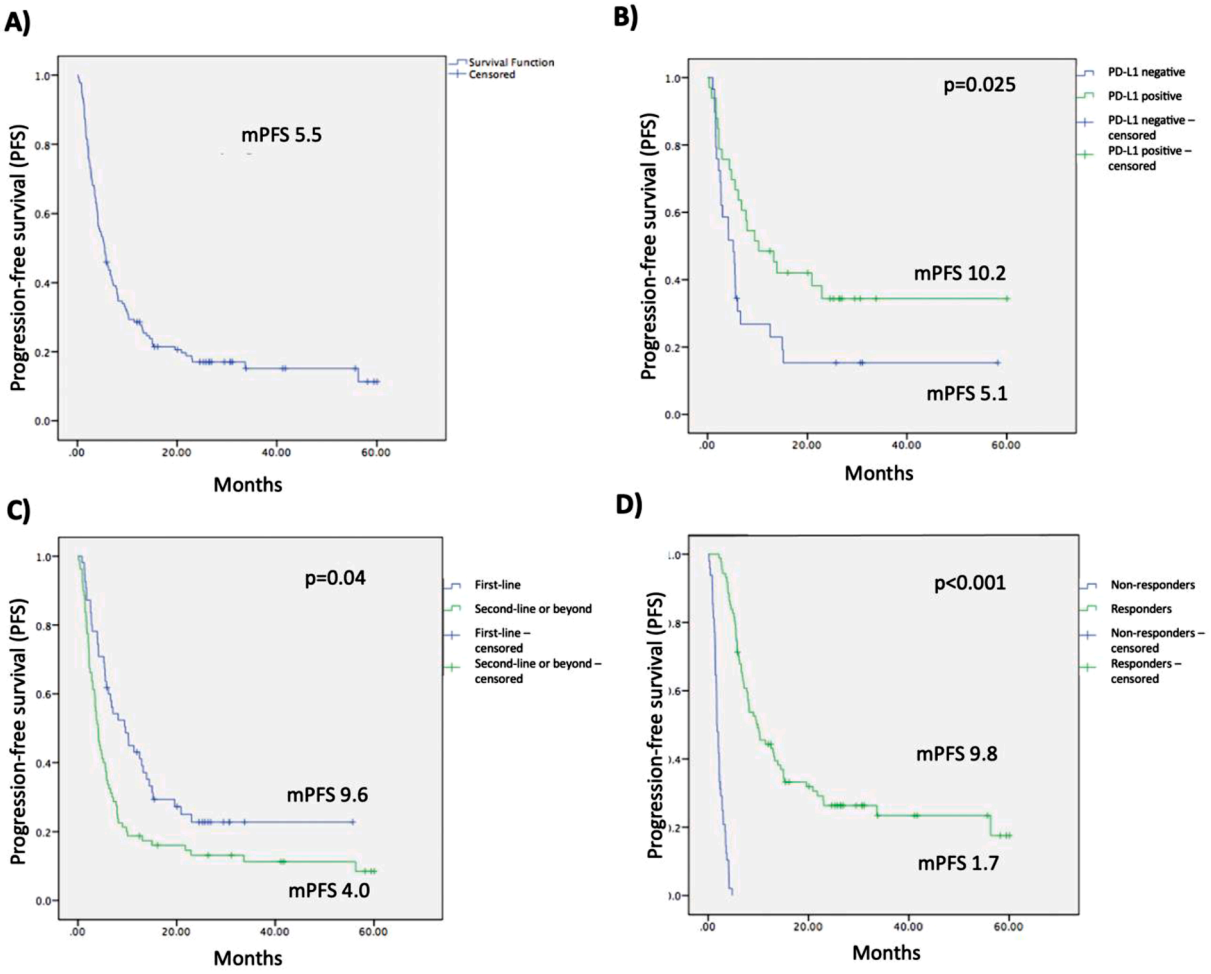

Fig. 3. Kaplan-Meier curves for progression-free survival (PFS) (A) and PFS according to PD-L1 expression (B), line of treatment (C) and radiological response (D). mPFS = median progression-free survival.

PD-L1 expression analysis

Immunohistochemistry staining for PD-L1 was performed on FFPE tumor tissue with Dako 22C3 pharmDx (Agilent Technologies/Dako, Carpinteria, CA, USA) [28] kit, following manufacturer's instructions. PD-L1 expression was measured by tumor proportion score (TPS) and categorized into low (<1 %), intermediate (1–49 %) or high (≥50 %) expression, as reported [9,29]. Neoplastic cells had to show partial or complete membrane staining to be counted as positive. A minimum number of 100 neoplastic cells were counted to consider a sample valid for its evaluation [30].

RNA isolation and GEP analysis by NanoString

RNA isolation was performed from formalin-fixed paraffin-embedded (FFPE) tumor samples, sectioned on slides with a thickness of 10μM as previously reported [31]. One slide was stained with hematoxylin and eosin (H&E) and evaluated by an experienced pathologist to identify and select the tumor tissue area. RNA was isolated using commercial kit (RNeasy FFPE Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The immune gene expression profile was evaluated through the panel *nCounter® PanCancer IO360* (NanoString Technologies) at the Laboratory of Molecular Diagnostic, Barretos Cancer Hospital (https://hadiagnostico.com.br). This panel comprises 770 genes (**supplementary material - Painel *PanCancer IO 360* Genes**) involved in the cancer immune response, both innate and adaptive. From 50 ng of RNA, the hybridization of the samples with the capture and reporter probes took place in a thermocycling machine at 65°C for 24 h, followed by purification and immobilization of the complexes formed (automated step - PrepStation equipment, NanoString Technologies). Cartridges were read in the *Digital Analyzer* (NanoString Technologies) considering 555 FOVs (fields of view).

Data analysis

Results were captured by the program nSolverAnalysis Software v4.0® (NanoString Technologies). Data were analyzed in the R-environment (R-project v3.2.1; The R Foundation, Viena, Austria) with a specific pipeline for pre-defined molecular GEPs (NanoString Technologies patent). All data analyses were conducted by Data Analysis Service (DAS) from NanoString Technologies.

Statistical analysis

For quantitative and qualitative data, descriptive statistics and tables were used. The Kaplan-Meier method and the log-rank test were employed for survival analysis. A stratified Cox regression model was used to calculate hazard ratios (HRs). Patients without an OS or PIS event were censored at the date of the last visit they were known to be alive.

For the association analysis between GEPs and survival, cutoff points

Table 2
Clinicopathological characteristics of the 66 NSCLC patients with tumor inflammation signature (TIS) analyzed.

Clinicopathological and treatment features	n	(%)
Median age = 62.5 years (36-81)		
Institution		
Barretos Cancer Hospital	42	63.6
A. C. Camargo Cancer Center	24	46.4
Gender		
Male	38	57.6
Female	28	42.4
Histology		
Adenocarcinoma	44	67.7
Squamous cell carcinoma	21	31.8
Adenosquamous carcinoma	1	1.5
ECOG PS		
0-1	58	90.7
2	5	7.8
3	1	1.5
Unknown	2	-
Molecular profile		
EGFR+ (del 19 or L858R - PCR)	4	10.5
ALK+ (IHC)	1	2.5
PD-L1expression (TPS)		
<1 %	20	52.6
1-49 %	3	7.9
≥50 %	15	39.5
Smoking history		
Never	11	16.7
Former	31	47.0
Ever	24	36.4
Line of therapy		
First	28	42.4
Second	18	27.3
Third or beyond	20	30.3
Treatment strategy		
Anti-PD-(L)1 alone	48	72.7
Anti-PD-(L)1 + Anti-CTLA4	1	1.5
Anti-PD-(L)1 + chemotherapy	9	13.6
Anti-PD-(L)1 + Anti-CTLA4 + chemotherapy	8	12.1
Immune checkpoint inhibitor(s) used in first-line		
Nivolumab	5	17.8
Nivolumab + ipilimumab + chemotherapy	5	17.8
Pembrolizumab + chemotherapy	3	10.7
Durvalumab + tremelimumab + chemothearapy	3	10.7
Atezolizumab + chemotherapy	3	10.7
Pembrolizumab	2	7.1
Cemiplimab	2	7.1
Nivolumab + Ipilimumab	1	3.6
Nivolumab + chemotherapy	1	3.6
Durvalumab + chemotherapy	1	3.6
Atezolizumab	1	3.6
Avelumab	1	3.6

PS: performance status; TPS: tumor proportion score

of 6 months for progression-free survival and 18 months for PIS were used. Statistical significance was considered for p-values ≤0.05 in all analyses. The date of 12/31/2020 was considered the cut-off date for data analysis.

Results

Clinicopathological features

A total of 135 NSCLC patients were included, 100 from the Barretos Cancer Hospital and 35 from the A. C. Camargo Cancer Center. Table 1 summarizes the clinicopathological characteristics of the patients included in the study. The median age at diagnosis was 61 years (ranging from 33 to 81 years). Most patients were males (57.8 %), clinical stage IV (91.9 %) and ECOG Performance Status ≤1 (88.7 %). Most patients had a smoking history (83.6 %) and 47 % were active smokers at the time of diagnosis. The predominant histology was adenocarcinoma (65.1 %) followed by squamous cell carcinoma (30.3 %). A subset of patients had somatic genetic alterations evaluated for *EGFR, ALK, ROS1, BRAF* or *KRAS*. Regarding treatment, most received immunotherapy in the first-line setting (40.7 %), with nivolumab being the most used anti-PD-(L)1 (52.6 %) (Table 1).

Effectiveness of immunotherapy

The ORR was 33.3 %, with 39 (28.9 %) partial responses and six (4.4 %) complete responses. Progressive disease as the best response to treatment was observed in 48 patients (35.5 %). The response rate among patients treated in the first line (n=55) and among those treated in the second line (n=80) was 54.5 % and 18.7 %, respectively ($p<0.001$). The response rate among patients treated with anti-PD(L)1 alone (n=95), anti-PD(L)1 combined with anti-CTLA4 (n=3) and anti-PD(L)1 combined with chemotherapy with or without anti-CTLA4 or anti-angiogenic (n=37) was 20.1 %, 33.3 % and 64.8 %, respectively ($p<0.001$).

At the time of data cutoff, 91 deaths had occurred (67.4 %). The median duration of follow-up for OS and PIS was 27.7 months, and for rwPFS was 28.2 months. The median OS was 27.5 months (Fig. 1A) and was higher among those who achieved an objective response. No deaths occurred among those with a complete response (Fig. 1B).

The median PIS was 17.74 months (Fig. 1C). It was higher among those who responded to treatment (17.7 versus 3.6 months; $p<0.001$), among patients with positive PD-L1 tumors (NA versus 14.85 months; $p<0.001$) and also higher among those treated with immunotherapy in first line (23.8 versus 15.0 months; p=0.016). Although there was a trend toward the benefit in favor of the combined treatment of immunotherapy and chemotherapy, there was no statistically significant difference (23.9 months versus 16.6 months; p=0.08) (Fig. 2).

The median rwPFS was 5.5 months, being higher among PD-L1 positive patients (10.2 versus 5.1 months; p=0.025), among those treated in the first line (9.6 versus 4.0 months; p=0.04) and treatment responders (9.8 versus 1.7 months; $p<0.001$) (Fig. 3).

Gene expression profile (GEP)

Of the 135 patients in the study, 38 did not have tumor samples available, 14 had samples with less than 60 % of tumor cells and five had an RNA concentration below the detection limit (<0.002ng/uL), being excluded from this analysis. Therefore, the gene expression profile was performed in 78 cases. Reliable results for further investigation were considered when the geometric mean of the housekeeping gene was above the geometric mean + 2 SD (standard deviation) of negative controls (**Supplementary** Figure 1). Thus, after applying the threshold of 32, 12 cases were considered unsuitable for analysis, leading to a final number of 66 subjects analyzed (Table 2). The median PIS, rwPFS, and ORR in this cohort of 66 patients were 17.74 months, 5.58 months, and 42.4 %, respectively.

For each case, it was evaluated 43 GEPs, which are weighted linear sums of gene expression, and the loss of five GEPs, which that measure the decreased expression of a gene within a pathway where genes are typically expressed at constant ratios (**supplementary material - PanCancer IO 360 Biological Signatures**). Among the 43 GEPs, 14 were significantly associated with PIS, namely *IDO1, PD-L2, Cytotoxicity, Cytotoxic Cells, IFN Downstream, CTLA4, PD-L1, TIGIT, Lymphoid, Immunoproteasome, Exhausted CD8, IFN Gamma, TIS* e *APM* (Fig. 4). Patients with high expression of *IFN Gamma* signature had a significantly higher PIS than those with low expression (medians of 29.2 versus 15.5 months; adjusted p-value=0.014). A similar result was observed with the *TIS* expression (Figs. 5 and 6). Moreover, we found five GEPs, which low scores were associated with better radiological response (*DC, Endothelial Cells, Macrophages, Neutrophils and T Cells*) (Fig. 7). For the *TIS* and *IFN Gamma* signatures there was no statistical difference between high and low scores regarding radiological response rate.

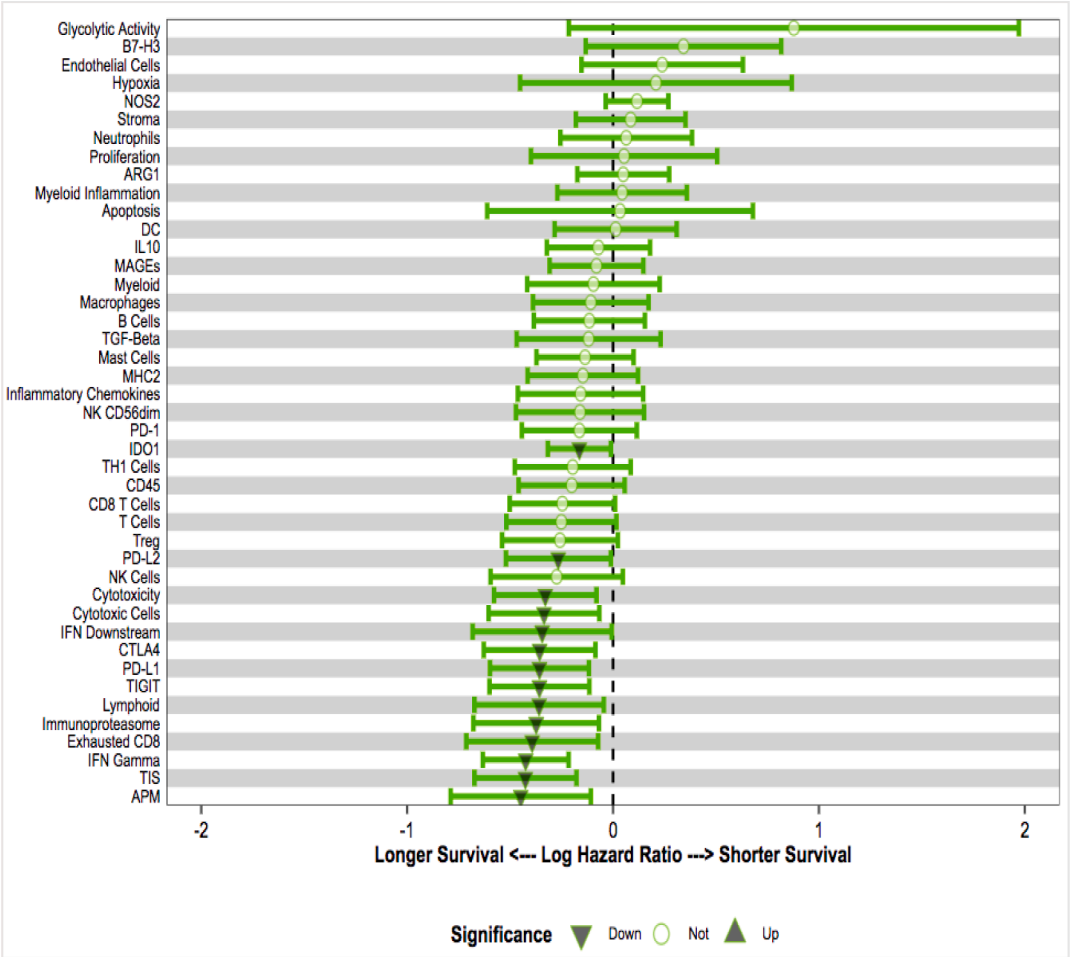

Fig. 4. Forest plot of post-immunotherapy survival (PIS) according to each gene expression signature. Long PIS means more than 18 months.

There were no cases with loss of expression of the *MMR Loss, MSI Predictor* and *APM Loss* signatures. About 50 % of patients showed loss of expression for the hypermutation signature, however, there was no clear clustering concerning radiological response, rwPFS or PIS. Among four cases with JAK-STAT pathway loss of expression, three radiological responses and longer PIS were observed (Fig. 8).

Discussion

Following the revolution of targeted advanced lung cancer therapies, immune checkpoint inhibitors (ICIs) emerged unmet medical needs. Anti-PD-(L)1 and anti-CTLA4 agents mainly benefit smokers and patients without actionable mutations. However, despite ICIs being approved for practically all cases of advanced NSCLC, only a fraction of these patients benefit from this type of treatment, which burdens the health system. In addition, they are not free from side effects. Following the concept of targeted therapy, the need to use predictive biomarkers of ICIs response in clinical practice is evident. Nevertheless, only PD-L1 expression has been used in clinical practice and has important limitations, with high-expressing tumors not responding to treatment and, eventually, tumors without PD-L1 expression showing lasting responses.

To explore genetic signatures as predictive biomarkers of response to PD-(L)1 inhibitors in a real-world scenario, we retrospectively included 135 patients with aNSCLC treated with these drugs at two Brazilian reference centers. This is the largest cohort of Brazilians with advanced NSCLC treated with immunotherapy evaluated for predictive biomarkers that we are aware of. The clinicopathological features reflect a typical NSCLC population with a median age of around 60 years and a predominance of males and smokers. However, only 40 % of these patients received immunotherapy in the first line of treatment, whereas today, most would have received it in the first line of treatment. This is justified by at least three reasons: [1] the historical moment, since most of these patients received immunotherapy treatment before the approval of these drugs in the first line of treatment; [2] most of these patients were treated in the context of public health, which makes access to first-line treatment unfeasible; and [3] most of these patients participated in an expanded access program for nivolumab from the second line of treatment. Moreover, among the 66 patients included in the GEP analyses, eight (12.1 %), were participants in randomized controlled trials [32–36], and, therefore, treated with immunotherapy or combinations not approved by regulatory agencies or treated in a different approved setting.

The clinical outcome results of our cohort are in line with the literature. About a third of the patients had a radiological response, which was more prominent when immunotherapy was performed in the first line. Ten patients were treated with combination chemotherapy with anti-PD-1 and anti-CTLA4. They had the highest response rate in the cohort, possibly because they received combination treatment and were all treated in the first line. The median overall survival observed is superior even to that observed in randomized clinical trials with first-line immunotherapy. This fact could be due to an immortality bias since the study allowed the inclusion of patients in any line of treatment. To minimize this study limitation, the primary analyses were carried out with post-immunotherapy survival, which takes into account the first

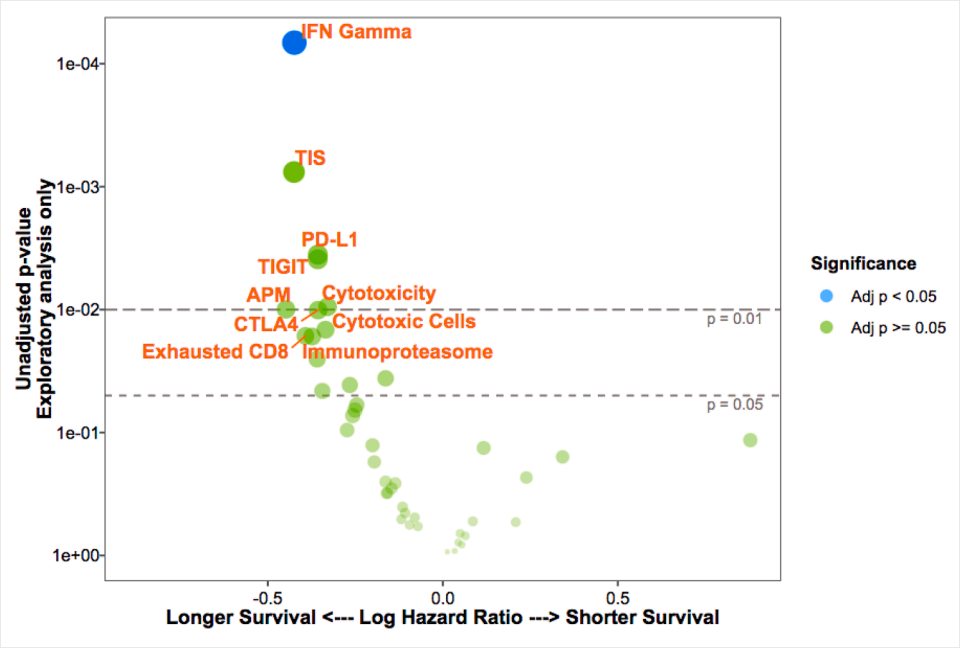

Fig. 5. Volcano-plot of post-immunotherapy survival displaying the hazard ratios and the p-value of each gene expression signature. Signatures with greater statistical significance appear higher on the graph (larger, darker dots), while signatures with more extreme hazard ratios appear further from the center of the graph. Rightmost subscriptions are associated with a reduced risk of an event relative to the baseline, and leftmost subscriptions are associated with a higher risk of an event relative to the baseline. The horizontal lines indicate the adjusted p-value of 0.01 and 0.05. When the adjusted p-values vary above 0.05, the limits are not shown on the graph.

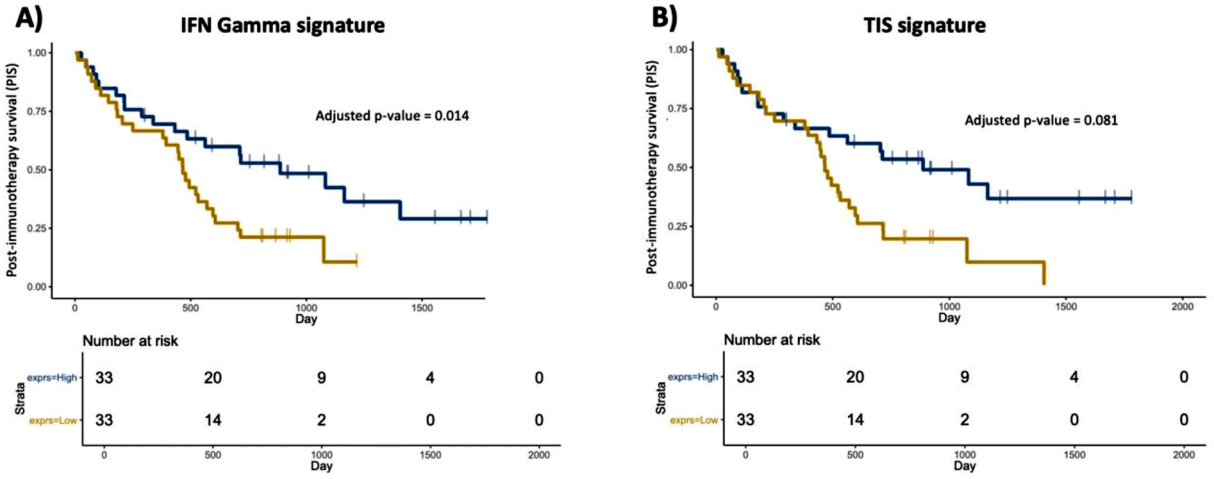

Fig. 6. Kaplan-Meier curves that showing the impact of the most significantly associated gene expression signature scores with post-immunotherapy survival (PIS).

day of immunotherapy as the start date of counting the survival time. As expected, patients with a radiological response to treatment had the highest overall survival. Interestingly, despite the long follow-up time, there were no deaths among patients with complete response. On the other hand, patients with disease progression had the best response, and those with PD-L1 negative tumors had poor survival.

The observed median post-immunotherapy survival is slightly higher than that reported in a Latin American study that included NSCLC patients treated with immunotherapy regardless of the line of treatment (12.7 months) [37], possibly due to a higher proportion of patients treated with first-line immunotherapy in our study.

When comparing post-immunotherapy survival according to treatment regimen (monotherapy versus combination therapy), there was a trend towards benefit for those treated with combination therapy. This data should be viewed cautiously since some confounding factors may have influenced this result. All patients treated with combination therapy did so in the first line, which may be responsible for the best survival curve. The progression-free survival results were as expected and are in line with the post-immunotherapy survival data.

The scarcity of tumor tissue limited the inclusions for molecular

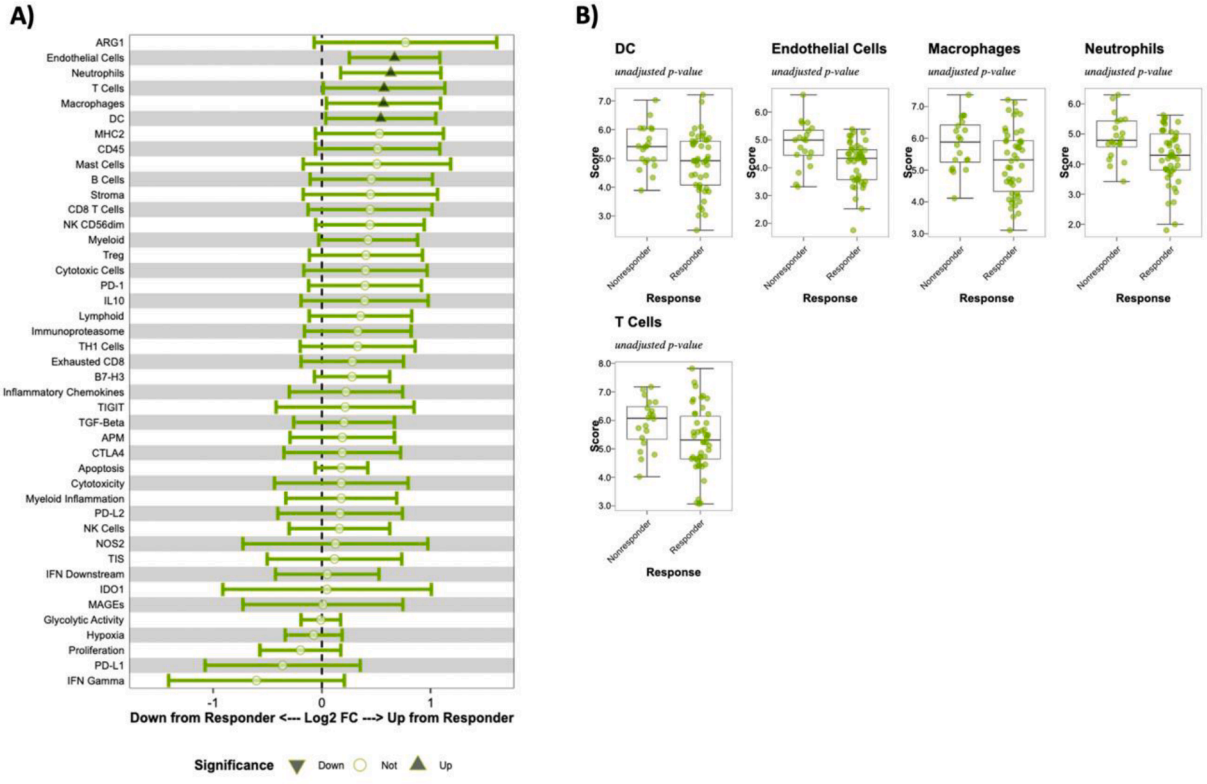

Fig. 7. (A) 'All Signatures' forest-plot shows the differential expression means and 95 % confidence intervals between response variables, for each signature on an unadjusted scale. The vertical axis is shown at fold change equal to zero, indicating equivalent expression between the groups. As the marker shifts from the center line there is an increase (shift to the right), or decrease (shift to the left), in the differential expression of that signature when compared to the baseline group (represented as the vertical line at zero). The shape of the marker in each box indicates whether there is a significant difference in the signature as assessed by univariate analysis (note that this significance is not adjusted for multiple comparisons). (B) Box plots of significant signatures related to radiological response.

profiling, ending with 66 with GEP analysis. The clinicopathological and therapeutic characteristics of these 66 patients are similar to the total study population. Among the 48 gene expression signatures evaluated, 14 discriminated between two different post-immunotherapy survival populations.

The most significant association with patient better outcomes, was with the IFN Gamma signature. These results are in accordance with the literature. A study with 92 NSCLS patients treated with durvalumab showed a higher response rate and improved progression-free survival and overall survival among patients whose tumors had IFN Gamma signature, regardless of PD-L1 expression [42]. The same findings have been observed in other solid tumors [8], reinforcing the immunotherapy-predictive role of the IFN Gamma signature.

Our study's second most statistically significant signature was the *TIS* (tumor inflammation signature*)*. It comprise 18 genes related to inflammatory cells of the tumor microenvironment and pro-inflammatory cytokines, which potentially discriminate ICIs response. Our findings are following previous studies that reported this signature associated with response to immunotherapies in different solid tumors [43].

Five signatures were composed of just one gene, namely the PD-L1, PD-L2, and CTLA-4 signatures, which encode the ICIs-related targets, and two others composed of the *IDO1* and *TIGIT* genes. Interestingly, early clinical studies with new agents that target IDO1 and TIGIT proteins demonstrate a promising activity, both as monotherapy and in combination with other agents [38–41].

Among the other signatures identified, the *Immunoproteasome* and *APM*, are complementary to each other and related to tumor immunogenicity. The first is related to the proteolytic activity of the proteasome, which increases the number of molecules (antigens) to be presented by the class I MHC complex to CD8+ T cells. The *APM* signature measures precisely the abundance of genes related to the MHC class I complex. A retrospective study included 51 chemotherapy-refractory NSCLC patients with monotherapy ICIs and evaluated the gene expression profile associated with the antigen presentation machinery [44]. Higher response rates, better progression-free survival, and overall survival were observed among those with high expression of these genes. In the same study, the APM score was even better than the inflamed tumor signatures. These same results were also observed in ICIs-treated melanoma patients [44].

The nCounter PanCancer IO360 panel also contains an *MMR (Mismatch repair) and MSI (Microsatellite instability) Predictor* signature, which were not associated with survival or response rate. These findings align with our previously reported absence of MSI by PCR-based approach in this population [15].

Conclusion

Immune checkpoint inhibitors revolutionized the treatment of NSCLC, yet patient selection criteria are not ideal. In this real-world scenario, we validated the predictive biomarker value of two major genetic signatures, *IFN Gamma* and *TIS,* significantly associated with

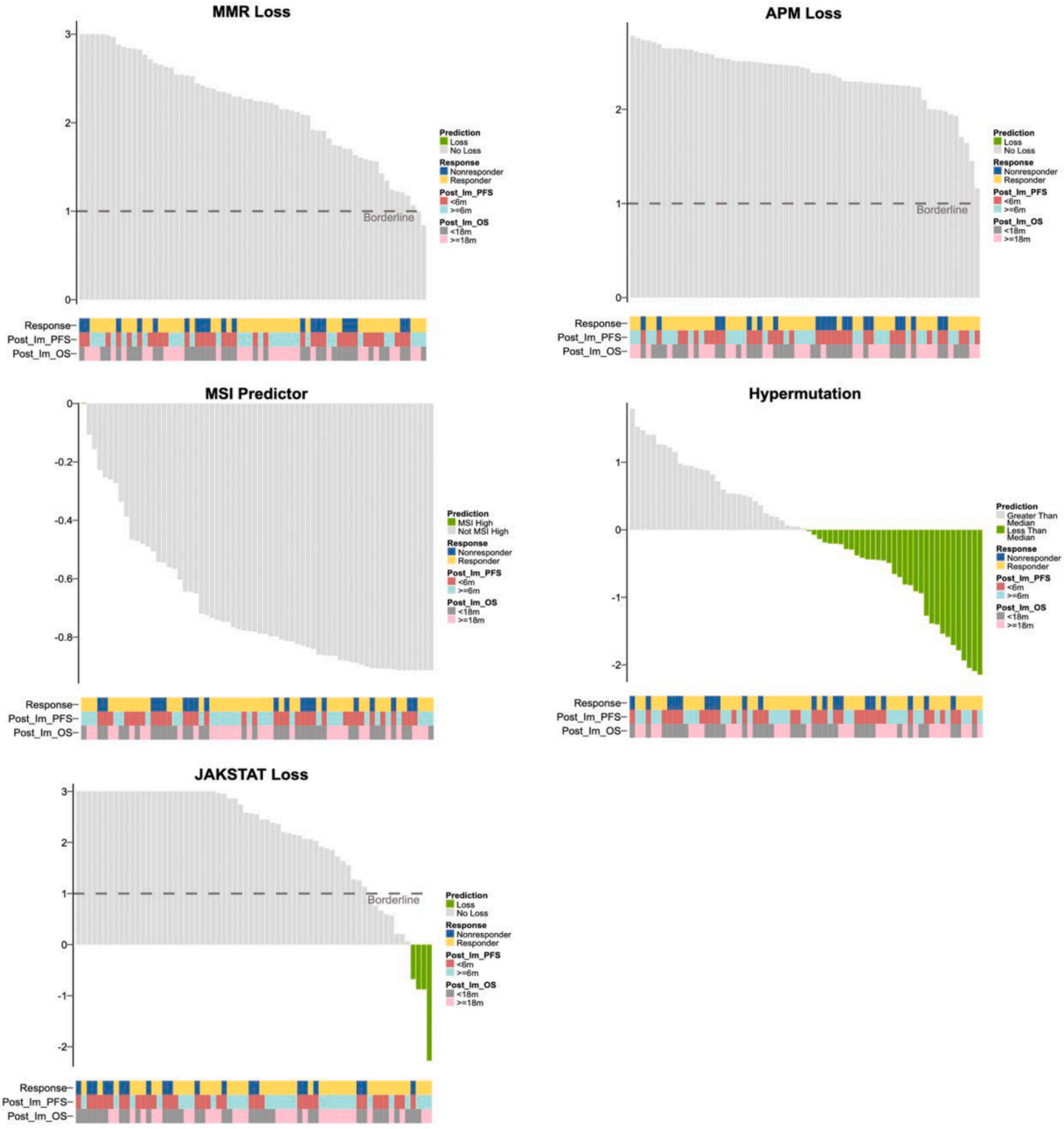

Fig. 8. The Loss Signature waterfall plot displays the selected loss signature for all samples. For AMP Loss, JAK-STAT Loss and MMR Loss, the line at which loss is significant falls at zero. These plots are re-scaled so that the scores are deviations from the threshold over which a loss of function is defined. The scores are then reversed for lower values to be depicted as a loss. Bars that fall below the zero line indicate a potential loss for that sample. Bars above the threshold do not indicate a loss. These samples fall under the borderline threshold which is drawn at 1. The hypermutation graph displays the hypermutation scores scaled to have a mean of zero and standard deviation of one and then outliers are truncated at ± 3 standard deviations. The MSI predictor is a combined signature from the MMR Loss signature and the hypermutation signature. Post_Im_PFS = post immunotherapy progression free survival; Post_Im_OS = post immunotherapy survival.

Brazilian NSCLC patients' survival with immunotherapy.

CRediT authorship contribution statement

Pedro De Marchi: Data curation, Investigation, Formal analysis, Writing – original draft. **Leticia Ferro Leal:** Investigation, Resources, Writing – review & editing. **Luciane Sussuchi da Silva:** Investigation, Resources, Writing – review & editing. **Rodrigo de Oliveira Cavagna:** Investigation, Resources, Writing – review & editing. **Flavio Augusto Ferreira da Silva:** Data curation, Writing – review & editing. **Vinicius Duval da Silva:** Data curation, Writing – review & editing. **Eduardo CA da Silva:** Data curation, Writing – review & editing. **Augusto O. Saito:** Data curation, Writing – review & editing. **Vladmir C. Cordeiro de Lima:** Data curation, Writing – review & editing. **Rui Manuel Reis:** Supervision, Project administration, Resources, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2023.101818.

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