Circulating microRNAs and PD-L1 tumor expression are associated with survival in advanced NSCLC patients treated with immunotherapy: a prospective study

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** These authors contributed equally to this study

Running title: Circulating microRNAs in response to immunotherapy

Abbreviation list:
bTMB: blood-based tumor mutational burden
CI: Confidence Interval
ctDNA: Circulating tumor DNA
CTLA4: Cytotoxic T-Lymphocyte Antigen 4
ddPCR: Droplet digital polymerase chain reaction
HR: Hazard-Ratio
ICIs: Immune-checkpoint inhibitors
LDCT: Low-dose computed tomography
MSC: microRNA-signature classifier
NGS: Next-generation sequencing
NSCLC: Non-small cell lung cancer
P: Patients with disease progression
PAD: Signature of presence of aggressive disease
PD: Signature of presence of disease
PD-1: Programmed death 1
PD-L1: Programmed death-ligand 1
PFS: Progression free survival
ORR: Overall response rate
OS: Overall survival
R: Responders
RAD: Signature of risk of aggressive disease
RD: Signature of risk of disease
RR: Relative risk of response
RT-qPCR: Reverse transcription quantitative polymerase chain reaction
SD: Patients with stable disease
TMB: Tumor mutational burden

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Statement of translational relevance

NSCLC is among the most responsive tumors to PD-1/L1 axis blockade therapy. Nonetheless, the true clinical benefit remains limited. Although PD-L1 expression by cancer cells and high tumor mutational burden (TMB) can be predictive of a favorable response, many patients who are negative for PD-L1 or have low TMB actually respond.

To identify biomarkers to improve patient selection, we focused on circulating microRNA. These small molecules released into the bloodstream by both nonimmune and immune cells play important roles in immune regulation. Here, we prospectively tested the efficacy of the already established plasma microRNA signature classifier (MSC), either alone or combined with PD-L1, as prognostic biomarker in advanced NSCLC patients treated with immune checkpoint inhibitors. The results showed that no patient with MSC high risk level responded to immunotherapy. Moreover, the combination of MSC and PD-L1 enabled the identification of a subgroup of patients reaching median progression-free and overall survival in 2 and 5 months, respectively.
ABSTRACT

Purpose: Immune-checkpoint inhibitors (ICIs) have improved the survival of NSCLC patients. However, only a subset of patients benefit from ICIs, and the role of PD-L1 as predictive biomarker is still debated. A plasma immune-related microRNA-signature classifier (MSC) was established in lung cancer screening settings in order to identify the lethal form of the disease in early stages. In the present exploratory study, we tested the efficacy of the MSC as prognostic marker in advanced NSCLC patients treated with ICIs.

Experimental Design: The MSC risk level was prospectively assessed in a consecutive series of 140 NSCLC patients before starting treatment with ICIs. Overall response rate (ORR), progression-free (PFS) and overall survival (OS) in strata of PD-L1 and MSC alone or combined were considered as end-points. Multiple plasma samples to monitor MSC risk level during treatment were also profiled.

Results: Adequate tissue and plasma samples were available from 111 (79%) and 104 (75%) NSCLC patients, respectively. MSC risk level was associated with ORR (P=0.0009), PFS (multivariate HR=0.31; 95%CI:0.17-0.56; P=0.0001) and OS (multivariate HR=0.33; 95%CI:0.18-0.59; P=0.0002). The combination of MSC and PD-L1 stratified patients into three risk groups having 39%-18%-0% one-year PFS (P<0.0001) and 88%-44%-0% one-year OS (P<0.0001), according to the presence of 2-1-0 favorable markers. During treatment, MSC risk level decreased or remained low until tumor progression in patients with responsive or stable disease.

Conclusions: The plasma MSC test could supplement PD-L1 tumor expression to identify a subgroup of advanced lung cancer patients with worse ORR, PFS and OS in immunotherapy regimens.
INTRODUCTION

Despite improvements in early diagnosis and new therapeutic strategies, the overall survival (OS) of lung cancer patients remains very low (1). The influence of the micro- and macroenvironment on cancer development is currently gaining increasing attention (2). Epithelial cancers, such as lung cancer, are now considered not simply the result of the abnormal growth of cancer cells but rather of complex interactions between cancer cells and stromal components (2, 3).

In this context, immune-checkpoint inhibitors (ICIs) targeting CTLA4 and the PD-1/L1 axis responsible for tumor immune evasion have drastically increased both progression-free survival (PFS) and OS in non-small cell lung cancer (NSCLC) patients (4-6). However, only a subset of patients respond to ICIs, and PD-L1 expression exhibits limited efficacy as a predictive biomarker (7). More recently, a high tumor mutational burden (TMB), as a surrogate for immunogenic tumor neoantigen presentation (8), was associated with a favorable response to ICIs in NSCLC patients irrespective of PD-L1 expression in both tissue and plasma sample (9-11).

To identify biomarkers for early lung cancer detection, we have trained and largely validated a circulating microRNA (miRNA) signature classifier (MSC) that can discriminate lung cancer with more aggressive features in lung cancer patients enrolled in low-dose computed tomography (LDCT) screening trials (12, 13). The MSC is composed of 24 miRNAs whose reciprocal ratios stratifies subjects into 3 risk levels, with the MSC high risk patients having worse prognoses than MSC intermediate or low risk patients (14). We have also shown that the MSC risk level was independent of tumor histology and mutational burden (15).

Since cells release miRNAs packaged in extracellular vesicles, these small circulating molecules may plausibly contribute to cell-to-cell communication by eliciting different functions according to the recipient cell type (16, 17). Indeed, alterations in miRNA levels
might lead to protumorigenic phenotypic changes in stromal cells such as fibroblasts (18),
macrophages (19), myeloid cells (20), dendritic cells (21) and T cells (22). Specifically for
miRNAs composing the MSC, their modulation in plasma was consistent with an
immunosuppressive conversion of immune cells, including neutrophils and macrophages (23).
Circulating miRNAs can thus be considered robust and reliable biomarkers reflecting changes
in tumor-host interaction (24).

The development of a minimally invasive blood-based tool able to supplement current
biomarkers to identify patients who could benefit or not from ICI treatment could be crucial
to improve the efficacy of these drugs. Here, we report the results of a prospective exploratory
study to evaluate the association between plasma MSC risk level and response to ICIs in
terms of overall response rate (ORR), PFS and OS in advanced NSCLC patients.

METHODS

Study design and patients’ characteristics

A consecutive series of 140 NSCLC patients was administered with ICIs from July 2015 to
May 2018 as a first-line (n=33), maintenance (n=5), second-line (n=71), or further line of
treatment (n=31). In detail, 126 patients were treated with anti-PD-1 (95 nivolumab and 31
pembrolizumab), 12 with anti-PD-L1 (3 avelumab, 3 atezolizumab and 6 durvalumab) and 2
with combined anti-PD-L1 and anti-CTLA4 (durvalumab+tremelimumab). Presence of liver
metastases was observed in 21 (15%) patients. Plasma and tissue samples were all collected
prior to starting immunotherapy and on treatment in a subset of patients. Samples were
prospectively analyzed to establish the plasma MSC risk level and tumor PD-L1 expression.
All patients were periodically evaluated until September 2018, and the response to treatment
was measured by radiological evaluation every 8 weeks after treatment initiation. According
to the RECIST 1.1 best response criteria, patients were classified as responders (R), patients
with stable disease (SD) and with disease progression (P). The study complied with the Declaration of Helsinki. All experimental protocols were approved by the Internal Review and Ethics Boards of the Istituto Nazionale Tumori of Milan and all patients provided informed consent.

**MicroRNA profiling of plasma samples**

Whole blood was collected in 10 ml K$_2$EDTA Vacutainer tubes and the plasma separated by two centrifugation steps at 1,258 g and 4°C for 10 min. Using a mirVana PARIS Kit (Thermo Fisher Scientific) or Maxwell RSC miRNA Tissue Kit (Promega), total RNA was extracted from 200 μl plasma samples and eluted in 50 μl of buffer. MicroRNA expression was determined by RT-qPCR using a 384-well microfluidic Custom Taq Array MicroRNA Card (Thermo Fisher Scientific) containing probes for the 24 miRNAs of interest, which were spotted in duplicate according to the protocol: miR-101-3p, miR-106a-5p, miR-126-5p, miR-133a, miR-140-3p, miR-140-5p, miR-142-3p, miR-145-5p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-197-3p, miR-19b-3p, miR-21-5p, miR-221-3p, miR-28-3p, miR-30b-5p, miR-30c-5p, miR-320a, miR-451a, miR-486-5p, miR-660-5p, and miR-92a-3p (MiRBase ID - v21). The Ct mean values to set an automatic baseline and a fixed threshold of 0.15 were then extrapolated using ViiA7 RUO software (Thermo Fisher Scientific).

**MSC algorithm**

The MSC test was performed following previously reported standard operating procedures with fixed parameters for prospective studies (25). Briefly, the effect of hemolysis on the MSC test was evaluated by both spectrophotometric and molecular analyses. In each plasma sample, the level of free hemoglobin was initially measured by the 414 nm/375 nm absorbance ratio (26). After miRNA profiling, 16 ratios between 4 hemolysis-related (miR-
miR-451a, miR-486-5p and miR-92a-3p) and 4 unrelated miRNAs (miR-126-5p, miR-15b-5p, miR-221-3p and miR-30b-5p) were then determined. Samples with a 414 nm/375 nm absorbance ratio higher than 1.4 and in which at least 50% of the miRNA ratios exceeded the respective cut-off values were considered hemolyzed and excluded from further analysis. For all samples passing quality control, the 4 signatures of risk of disease (RD), presence of disease (PD), risk of aggressive disease (RAD) and presence of aggressive disease (PAD) were determined (25). The respective MSC risk level was attributed to each sample as follows: low risk for $\text{RD}^{\text{neg}} \cap \text{PD}^{\text{neg}} \cap \text{RAD}^{\text{neg}} \cap \text{PAD}^{\text{neg}}$; intermediate risk for $\text{RD}^{\text{pos}} \cup \text{PD}^{\text{pos}} \cap \text{RAD}^{\text{neg}} \cap \text{PAD}^{\text{neg}}$; or high risk for $\text{RAD}^{\text{pos}} \cup \text{PAD}^{\text{pos}}$ (13).

**PD-L1 immunohistochemical analysis**

According to the kit instruction, 2.5/3 micron-thick sections were cut from paraffin blocks, dried, dewaxed, rehydrated, and unmasked with Dako PT-link EnVision™ FLEX Target Retrieval Solution (low pH – 30 min - 98°C). PD-L1 monoclonal antibody 22C3 (Dako) was incubated with the EnVision FLEX+ detection kit (Dako) in the Autostainer System (Dako). PD-L1 expression was measured as the percentage of positive neoplastic cells (PNCs) and patients stratified in 3 groups: PNC<1%, PNC 1%-49% and PNC≥50%. In a group of 43 patients with available material, PD-L1 expression was confirmed by using the monoclonal antibody SP263 (Ventana Medical Systems) on an automated Benchmark Ultra Platform (Ventana Medical Systems) according to the manufacture’s protocol.

**Statistical analyses**

The continuous variables were given as the mean values ± standard deviation (SD) and the categorical variables as numbers and percentages. Cohen's kappa statistic was used to analyze the interrater agreement of the categorical variables.
The ORR was estimated as the percentage of R among all patients in respective class. The relative risk of response (RR) and corresponding 95% confidence interval (CI) were calculated by 2x2 contingency table and Fisher’s exact test was adopted to calculate p-values (27). A 0.5 value was added to all cells in order to avoid computational problems caused by 0 value (28).

For PFS and OS end-points, the time to event occurrence was computed from the start date of treatment to the date when the event was recorded or was censored at the date of the last follow-up assessment in event-free patients. One-year survival curves were estimated using the Kaplan-Meier method and were compared by the log-rank test. Patients who discontinued therapy after one cycle due to adverse effects or clinical deterioration were not considered for PFS analysis.

The crude and adjusted hazard ratios and the corresponding 95% CIs were estimated using Cox proportional hazard models. Multivariate analyses were performed adjusting models for age, gender, smoking status and presence of liver metastasis. Since treatment with ICIs as first-line therapy was approved only for advanced NSCLC with PD-L1 expression on at least 50% of tumor cells (29), line of therapy was considered as covariate only in multivariate analysis in strata of MSC risk level.

All tests were two-sided, and a p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC), and the Kaplan-Meier curves were obtained using GraphPad Prism version 5.02 statistical software.

RESULTS

Molecular and clinico-pathological findings of the prospective series
A consecutive series of 140 advanced (all stages III-IV) NSCLC cases treated with ICIs was prospectively analyzed for the plasma MSC test and monitored for up to three years. The median PFS and OS for the whole series were 3.0 and 8.1 months, respectively (Supplementary Figure S1).

As reported in Table 1, female patients accounted for 34% of the series, and the overall average age was 66.2 years. The main histology type was adenocarcinoma, accounting for 65% of tumors. Twenty patients (14%) were never smokers, 78 (56%) former smokers and 42 (30%) current smokers. The MSC was evaluable in 111 (79%) patients: 26 (19%) were MSC high risk, 51 (36%) were MSC intermediate, and 34 (24%) were MSC low risk. Twenty-nine (20%) samples were not analyzable due to high hemolysis levels (25). Adequate tumor tissue samples for PD-L1 expression by 22C3 IHC assay were available for 104 (74%) patients: PNCs were <1% in 30 (21%) patients, between 1% and 49% in 36 (26%) patients, and ≥50% in 38 (27%) patients. For the 43 patients analyzed with both clones 22C3 and SP263, categorical data were consistent and included 17 with PNCs<1%, 21 with PNCs 1%-49% and 5 with PNCs≥50%. Response to treatment was assessed according to RECIST 1.1 criteria: 26 (19%) were classified as R, 33 (24%) as SD, 67 (48%) as P and 14 (10%) discontinued therapy after one cycle due to adverse effects (N=5) or clinical deterioration (N=9).

Overall, 131 (94%) patients had at least one plasma or tissue sample suitable for marker evaluation, while both samples resulted adequate for 84 (60%) patients (Figure 1). No interrater agreement (κ=-0.07) between MSC and PD-L1 was observed (Supplementary Table S1).

**Plasma MSC risk level and PD-L1 tumor expression are associated with ORR**

ORR in the whole series was 19% (26/140). No one of the 25 NSCLC patients with adequate plasma sample classified as R were MSC high risk level at the baseline. Indeed, among the 26
MSC high risk patients 5 (19%) were SD, 16 (62%) P and 5 (19%) discontinued ICI treatment after one cycle due to adverse effects (N=2) or clinical deterioration (N=3). Moreover, an opposite trend between R (0%-22%-38%) and P (62%-40%-35%) was observed among patients with MSC high-intermediate-low risk level. Overall, a significant reduction in ORR (RR=0.07; 95% CI: 0.00-1.05; P=0.0009) was observed by comparing MSC high vs. intermediate and low risk advanced NSCLC patients (Table 2). Considering PD-L1 tumor expression, a lower ORR was observed in patients with PNC<50% (RR=0.41; 95% CI: 0.20-0.83; P=0.0158).

The two markers were first combined considering the 131 patients with any data available, in terms of the presence or absence of any favorable marker: i.e., MSC intermediate and low risk level and/or PD-L1 ≥50%. In this scenario, the ORR was significantly lower in advanced NSCLC patients with no favorable markers as compared to those with 1-2 favorable markers (RR=0.11; 95% CI: 0.02-0.78; P=0.0024). Similar results were observed when considering the subgroup of 84 advanced NSCLC patients with both MSC and PD-L1 available (Table 2).

**Plasma MSC risk level and PD-L1 tumor expression are associated with patients survival**

Patients survival was evaluated according to plasma MSC risk level and PD-L1 expression on tumor cells both alone and combined. As shown in Figure 2A, lower one-year PFS was observed in the 21 lung cancer patients with a MSC high risk level than in the 47 with MSC intermediate and the 33 with MSC low risk levels (P<0.0001). As expected, the 34 patients with PD-L1 tumor expression ≥50% showed a better outcome than the 28 and 35 patients with PD-L1 signal <1% and 1%-49% (P<0.0001, Figure 2B). Similar results were observed when considering OS (Figure 3A-B).
Combining the two markers in the whole series, the one-year PFS was 24% in 88 patients with at least one favorable marker, while 32 patients with no favorable markers had disease progression within 5 months since ICI starting (Figure 2C; \( P < 0.0001 \)). Considering OS, 52% of subjects with favorable markers and the 11% without favorable markers were still alive one year after starting ICIs treatment (Figure 3C, \( P < 0.0001 \)).

Analysis was then restricted to the patients with both MSC and PD-L1 available data. This subset of patients could be stratified into three distinct risk groups showing 39%-18%-0% one-year PFS (\( P < 0.0001 \)) and 88%-44%-0% one-year OS (\( P < 0.0001 \)), according to the presence of 2, 1 or 0 favorable markers, respectively (Figure 2D and Figure 3D). For the mid group, no main differences in terms of OS were observed if the favorable marker was MSC intermediate and low or PD-L1<50% (Supplementary Figure S2).

**Multivariate analysis confirmed data on patients survival**

PFS and OS in strata of each marker were then analyzed by Cox proportional hazard models in univariate and multivariate analyses. The distribution of covariates across MSC risk level and PD-L1 tumor expression are described in Supplementary Table S2. In the whole series, MSC risk level, PD-L1 tumor expression as well as their combination remained significantly associated with both PFS and OS by univariate and multivariate analyses (Supplementary Table S3).

In the subgroup of patients with both markers available, patients with MSC intermediate/low risk level reported a significant reduction in disease progression and mortality as compared to those with high MSC risk level (Table 3). The corresponding multivariate HR were 0.35 (95% CI: 0.18-0.70; \( P = 0.0026 \)) and 0.28 (95% CI: 0.12-0.58; \( P = 0.0007 \)). Significant reduction in disease progression (multivariate HR=0.35; 95% CI: 0.19-0.63; \( P = 0.0006 \)) and mortality (multivariate HR=0.43; 95% CI: 0.21-0.88; \( P = 0.0211 \)) was also observed in patients with
PNC ≥50% as compared to those with PNC <50%. When the two markers were considered together, patients with at least one favorable marker reported a significant lower probability in disease progression (multivariate HR=0.25; 95% CI: 0.12-0.56; P=0.0006) and mortality (multivariate HR=0.28; 95% CI: 0.12-0.65; P=0.0034), as compared to those with no favorable markers.

**Plasma MSC risk level to monitor responsive and stable disease during treatment with ICIs**

For a subset of patients representative of each class of response to ICIs, longitudinally collected plasma samples were analyzed to monitor changes in MSC risk level during treatment: 4 R, 5 SD and 6 P were included. In 2 R patients the MSC decreased from intermediate to low risk level after starting ICI treatment (Supplementary Figure S3A-B). Two R MSC low risk patients remained MSC low risk in response to ICIs and in one of these MSC risk increased at tumor progression (Supplementary Figure S3C-D). A similar fluctuation of MSC risk level was observed in the 5 SD patients: 3 with MSC low risk level remained low until or shortly before disease progression (Supplementary Figure S3E-G). One MSC high risk decreased to intermediate risk and one who remained MSC intermediate risk had disease progression two weeks later (Supplementary Figure S3H-I). On the other hand, in patients with diseases progression the changes in the MSC risk level during treatment were not uniform across the 6 subjects analyzed (Supplementary Figure S3J-O).

**DISCUSSION**

With the advent of ICIs, new insights into the crosstalk between tumor and the host involving both innate and the adaptive immunity, are emerging (30). Indeed, looking for molecules that reflect an impaired crosstalk could represent a promising strategy to identify diagnostic,
prognostic and predictive biomarkers. PD-L1 is the only biomarker currently used in clinical practice but its role is still debated: different assays and cut-off have been used in the clinical trials (29, 31, 32); nonetheless ICIs efficacy was achieved also in PD-L1 negative tumors (33, 34). More recently, in advanced NSCLC patients enrolled in the CheckMate 227 clinical trial, TMB showed to be an independent PFS predictive factor, regardless of PDL-1 expression (9). In addition, a TMB blood-based assay (bTMB) was standardized in two large retrospective series and was still predictive for PFS. However, bTMB failed to predict OS and almost the 25% of patients did not achieved the minimum amount of circulating tumor DNA (ctDNA) for optimal assay performance or did not pass quality control steps (10).

In the present study, we have shown for the first time that a circulating miRNA signature classifier with prognostic value can supplement PD-L1 to identify patients with worse ORR, PFS and OS in a consecutive series of 140 advanced NSCLC cases treated with ICIs. A recent paper identified a signature of 7 miRNAs associated with 6-month OS in a retrospective series composed of 20 (training) and 31 (validation) advanced NSCLC patients treated with nivolumab (35). No overlap between the 7 miRNAs and the 24 composing the MSC was observed.

Since the use of ORR and PFS as surrogate end-points of OS is still a controversial issue in immunotherapy settings (36), we presented the results according to all 3 end-points. In a first data analysis according to ORR, the MSC test alone seemed to be able to identify a subset of non-responding patients corresponding to the 19% of the whole series. Nevertheless, the identification of a subgroup of patients with 0% one-year PFS and OS was achieved only by combining MSC and PD-L1. The added value of these markers was also confirmed by multivariate analysis, where the adjusted HR improved from 0.35 (PFS) and 0.43 (OS) of PD-L1 alone, to 0.25 (PFS) and 0.28 (OS) when combined with the MSC.
As for TMB, some limitations have also been identified in the present study. No adequate tumor tissue sample was available for PD-L1 expression in 26% of patients. In addition, the MSC testing efficiency was limited by the sensitivity of the assay to hemolysis (25), which affected 20% of the plasma samples analyzed. Nevertheless, when tissue and plasma samples were combined, the percentage of patients with adequate material for biomarker evaluation increased to 94%. Because ICI treatment is rapidly becoming a first-line therapy for NSCLC (29, 31), tissue and liquid biopsies are likely to become more easily accessible. Indeed in the present series, the percentage of inadequate plasma and tissue samples in first-line treated patients decreased to 13% and 3%, respectively.

Liquid biopsy based on ctDNA was recently assessed to monitor the response to immunotherapy in advanced NSCLC patients (37-39). Three studies measured the frequencies of mutations identified in the tumor tissue in plasma collected from a total of 73 advanced NSCLC patients by targeted next-generation sequencing (NGS) or droplet digital PCR (ddPCR). At baseline, ctDNA was found in the plasma samples of 42 (58%) patients and was not associated with response to ICIs. On the other hand, the analysis of longitudinally collected plasma samples demonstrated that ctDNA levels decreased in responding patients and increased in nonresponding patients, thus supporting its use as an “on treatment” biomarker. In our study, analysis on longitudinally collected plasma samples suggested that MSC risk level follows tumor response to treatment in R and SD patients, although not homogeneous in 6 P patients. These preliminary results must be further validated in larger series.

Immunotherapy with ICIs may provide long-term benefits in approximately 25% of NSCLC patients (40). Nonetheless, tumor progression or even hyperprogression is observed in nonresponding patients (41, 42). This first exploratory prospective study suggests that circulating miRNAs can supplement standard tissue biopsy in the clinical practice. Larger and
possibly randomized studies are needed to establish the efficacy of MSC to select a subgroup of patients who do not benefit of ICI treatment.

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Table 1: Clinico-pathological and molecular characteristics of 140 advanced lung cancer patients treated with immune checkpoint inhibitors. For continuous variables average and respective standard deviation are reported.

<table>
<thead>
<tr>
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<th>140 lung cancer patients treated with checkpoint inhibitors</th>
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<tbody>
<tr>
<td></td>
<td>N (%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>48 (34%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.2 ± 9.9</td>
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<tr>
<td>Smoking habit</td>
<td></td>
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<tr>
<td>Never</td>
<td>20 (14%)</td>
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<tr>
<td>Former</td>
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<tr>
<td>Adenocarcinoma</td>
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<tr>
<td>Squamous carcinoma</td>
<td>34 (24%)</td>
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<tr>
<td>Others</td>
<td>15 (11%)</td>
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<tr>
<td>Stage</td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
<td>110 (79%)</td>
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<tr>
<td>Plasma MSC risk level</td>
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<tr>
<td>High</td>
<td>26 (19%)</td>
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<tr>
<td>Intermediate</td>
<td>51 (36%)</td>
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<tr>
<td>Low</td>
<td>34 (24%)</td>
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<tr>
<td>PD-L1 expression</td>
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<tr>
<td>PNC &lt; 1%</td>
<td>30 (21%)</td>
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<tr>
<td>PNC 1%-49%</td>
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<td>PNC ≥ 50%</td>
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<td>36 (26%)</td>
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<td>Overall response rate to ICIs</td>
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<td>Responsive</td>
<td>26 (19%)</td>
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<tr>
<td>Stable disease</td>
<td>33 (24%)</td>
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<td>Disease progression</td>
<td>67 (48%)</td>
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<tr>
<td>Not evaluated</td>
<td>14 (10%)</td>
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MSC: microRNA signature classifier; PNC: positive neoplastic cells
**Table 2:** Results on overall response rate (ORR) and corresponding relative risk (RR). P-values by two-tailed Fisher exact probability test are reported

<table>
<thead>
<tr>
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<th>All patients</th>
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<td>PNC &lt;50%</td>
<td>66</td>
<td>15%</td>
</tr>
<tr>
<td>PNC ≥50%</td>
<td>38</td>
<td>37%</td>
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<td><strong>MSC and PD-L1</strong></td>
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<td></td>
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<tr>
<td>0 favorable markers</td>
<td>35</td>
<td>3%</td>
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<tr>
<td>1-2 favorable markers</td>
<td>96</td>
<td>26%</td>
</tr>
</tbody>
</table>

CI: Confidence Interval; MSC: microRNA signature classifier; PNC: positive neoplastic cells
Table 3: Results from the Cox proportional hazards models on progression free survival and overall survival in the subset of patients with both plasma MSC risk level and PD-L1 tumor expression available.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Progression Free Survival</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>MSC risk level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>16</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate/Low</td>
<td>62</td>
<td>0.33</td>
<td>0.18-0.62</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>0.29</td>
<td>0.15-0.58</td>
</tr>
<tr>
<td>PD-L1 expression</td>
<td>78</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>PNC &lt;50%</td>
<td>30</td>
<td>0.37</td>
<td>0.20-0.66</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0.39</td>
<td>0.21-0.88</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>MSC and PD-L1</td>
<td>78</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>0 favorable markers</td>
<td>66</td>
<td>0.24</td>
<td>0.11-0.49</td>
</tr>
<tr>
<td>1-2 favorable markers</td>
<td>72</td>
<td>0.27</td>
<td>0.20-0.34</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, smoking status, presence of liver metastasis and for MSC risk level also line of therapy

HR: Hazard Ratio; CI: Confidence Interval; MSC: microRNA signature classifier; PNC: positive neoplastic cells
Figure legend

**Figure 1:** CONSORT diagram of the prospective Apollo study evaluating the plasma microRNA signature classifier (MSC) and PD-L1 expression on tumor cells in 140 advanced lung cancer patients treated with immune checkpoint inhibitors (ICIs).

**Figure 2:** Kaplan-Meier curves reporting the progression free survival (PFS) of advanced lung cancer patients treated with immune checkpoint inhibitors in strata defined by the microRNA signature classifier (MSC) and PD-L1 tumor expression. Analysis of (A) 101 subjects with available plasma MSC risk level: 21 high vs. 47 intermediate vs. 33 low; (B) 97 subjects with available PD-L1 expression on tumor cells: 28 with PD-L1<1% vs. 35 with PD-L1 1%-49% vs. 34 with PD-L1≥50%; (C) 120 subjects with at least one marker available: 32 with no favorable markers vs. 88 with any favorable marker (MSC intermediate or low and/or PD-L1≥50%); and (D) 78 subjects with both markers available stratified according to the presence of 0, 1 or 2 favorable markers. P-values by log-rank test are reported.

**Figure 3:** Kaplan-Meier curves reporting the overall survival (OS) of advanced lung cancer patients treated with immune checkpoint inhibitors in strata defined by the microRNA signature classifier (MSC) and PD-L1 tumor expression. Analysis of (A) 111 subjects with available plasma MSC risk level: 26 high vs. 51 intermediate vs. 34 low; (B) 104 subjects with available PD-L1 expression on tumor cells: 30 with PD-L1<1% vs. 36 with PD-L1 1%-49% vs. 38 with PD-L1≥50%; (C) 131 subjects with at least one marker available: 35 with no favorable markers vs. 96 with any favorable marker (MSC intermediate or low and/or PD-L1≥50%); and (D) 84 subjects with both markers available stratified according to the presence of 0, 1 or 2 favorable markers. P-values by log-rank test are reported.
140 consecutive advanced NSCLC patients treated with ICIs

Tissue sample for PD-L1

Adequate material: N=104

End-point 1:
ORR in strata of MSC and/or PD-L1

End-point 2:
PFS in strata of MSC and/or PD-L1

End-point 3:
OS in strata of MSC and/or PD-L1

Liquid biopsy for MSC

Adequate material: N=111

PD-L1

84

MSC

27
Figure 2

A. MSC risk level: 101 patients

B. PD-L1 expression: 97 patients

C. Any marker available: 120 patients

D. Both markers available: 78 patients

P value < 0.0001
A  MSC risk level: 111 patients

MSC high  MSC intermediate  MSC low

B  PD-L1 expression: 104 patients

PD-L1 <1%  PD-L1 1%-49%  PD-L1 ≥50%

C  Any marker available: 131 patients

No favorable markers  Any favorable marker

D  Both markers available: 84 patients

0 favorable markers  1 favorable marker  2 favorable markers

Figure 3
Circulating microRNAs and PD-L1 tumor expression are associated with survival in advanced NSCLC patients treated with immunotherapy: a prospective study

Mattia Boeri, Massimo Milione, Claudia Proto, et al.

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