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Large-scale profiling of extracellular vesicles identified miR-625-5p as a novel biomarker of immunotherapy response in advanced non-small cell lung cancer patients

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CONTENTS

PART I - Immunotherapy for Non-small Cell Lung Cancer

1. Introduction ……4

2. Immune Checkpoint Inhibitors……6

3. Anti PD-1/PD-L1 antibodies…… 8

4. Combination strategies…… 11

5. Treatment of metastatic NSCLC…… 16

6. Immunotherapy biomarkers…… 19

    6.1. Tissue biomarkers ……19

    6.2. Liquid biopsy and emerging blood-based biomarkers …… 21

    6.3. Focus on MiRNA …… 23

7. Conclusions …… 25

PART II - The Study

Large-scale profiling of extracellular vesicles identified miR-625-5p as a novel Biomarker of immunotherapy response in advanced non-small cell lung cancer patients

1. Introduction ……27

2. Materials and methods …… 28

3. Results ……31

4. Discussion …… 40

5. Conclusions …… 43

6. Supplementary figures and tables ……44

References…… 51

Immunotherapy for Non-small Cell Lung Cancer

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Based on GLOBOCAN 2020 estimates produced by the International Agency for Research on Cancer, with 2.2 million new cancer cases and 1.8 million deaths, lung cancer represents approximately one in 10 (11.4%) cancers diagnosed and one in 5 (18.0%) deaths. Its incidence is higher in industrialized and transitioned countries, reflecting the high burden of risk factors, including mainly tobacco smoking but also other inhalable agents such as asbestos and air pollutants. However, this pattern may well change as the tobacco epidemic grows in the low-income and middle-income countries (LMICs) [1].

In Italy, lung cancer is named the "big killer" since it represents and the first cause of cancer mortality for decades. It is the third most common neoplasm in both sexes; the incidence is higher among men, but is steadily increasing among women due to growing smoking attitude over the past 30 years [2].

The poor prognosis is due to biological aggression, the high tendency to metastasize and the long asymptomatic latency. For these reasons, most patients have metastatic disease at the time of diagnosis and less than 20% of them are alive at 5 years [3].

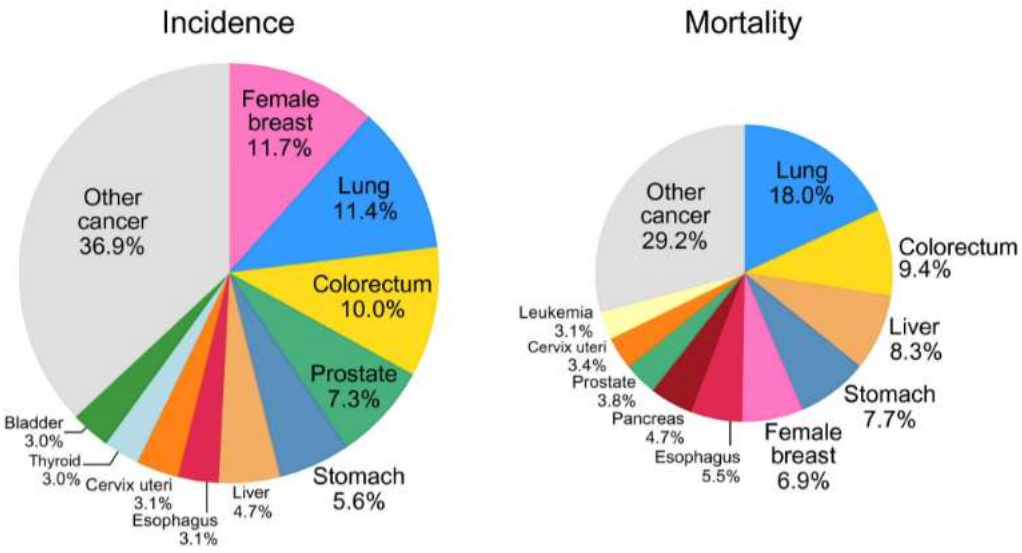


Figure 1. Distribution of Cases and Deaths for the Most Common Cancers in 2020 for Both Sexes [1]

The treatment depends on tumor histology and molecular profile. Lung tumors are categorized into two major histological groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 80 to 85 percent of cases, and includes two histologic subtypes: squamous cell carcinoma and non-squamous carcinoma, mainly adenocarcinoma [4]. A subset of NSCLC hosts a single driver anomaly that dictates cell growth, survival, and metastasization. This phenomenon, called oncogene-addiction, more often occurs in adenocarcinoma histology, in non-smokers, Asian ethnicity, young, and female patients. Mutations on EGFR, ALK, ROS-1, BRAF and novel emerging drivers, are therapeutic target for Tyrosine-kinase Inhibitors (TKI) and several studies have shown greater efficacy and better safety profile of these drugs than chemotherapy. As a result, the target therapies have become the standard of care for oncogene-addicted NSCLC [5].

However, most patients have non-oncogene-addicted disease and cannot benefit from target therapies. Until a few years ago, chemotherapy was the only chance of treatment, albeit resulting in a limited effect on survival rates.

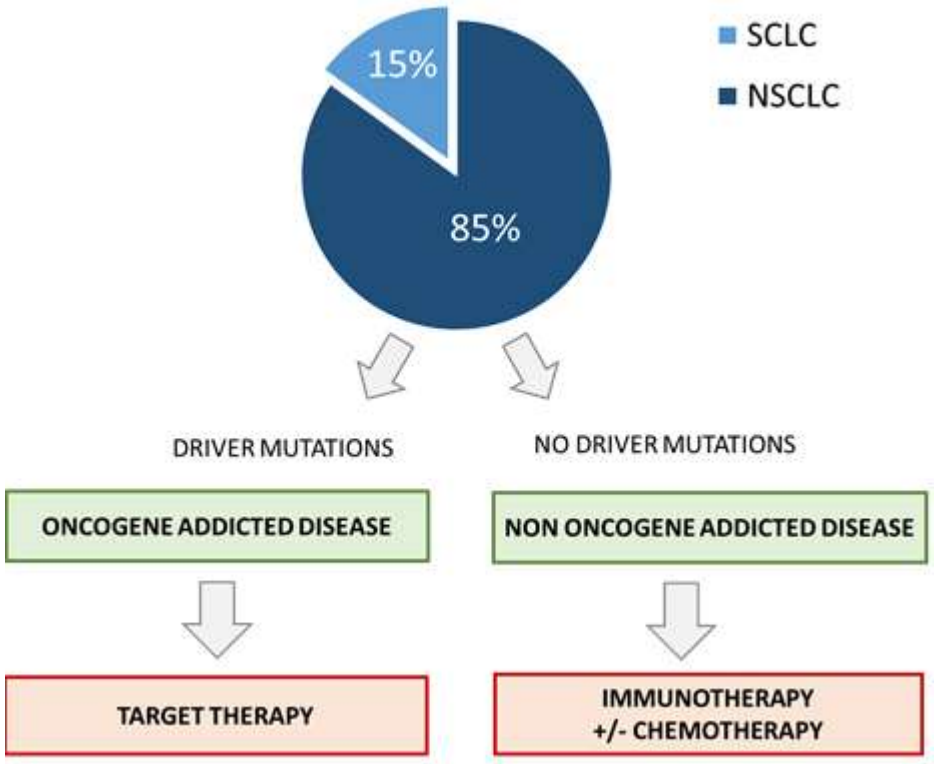


Figure 2. Lung cancer treatment based on histology and molecular profile.

The treatment landscape is dramatically changed thanks the advent of anticancer immunotherapy. The use of the Immune Checkpoints Inhibitors (ICIs) has revolutionized the management of advanced NSCLC and improved patients prognosis, achieving survival rates 5 times higher than those achieved with chemotherapy [6,7]. In recent years, ICIs have shifted the treatment paradigm and ushered in a new historic time for patients and clinicians: the era of immunotherapy.

2. IMMUNE CHECKPOINTS INHIBITORS

Modern Immuno-Oncology (IO), consists of different strategies such as vaccines, CAR-T and immunomodulators, aimed at mobilizing and activating the immune system to recognize and destroy cancer cells [8-10]. In current clinical practice, the main therapeutic approach is the use of monoclonal antibodies that act on specific key modulators of T-cell immune response against cancer. These molecules, also known Immune Checkpoint (IC), are present on T cells, antigen-presenting cells (APCs) and tumor cells. Their interaction activates either inhibitory or activating immune signaling pathways. [11-13]. Most relevant checkpoints are the inhibitory pathways consisting of cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), programmed cell death receptor-1 (PD-1), and programmed cell death ligand-1 (PD-L1).

CTLA-4 is expressed on T lymphocytes and has a major role in downregulation of the immune response. Binding to costimulatory molecules (CD80 / CD86) on antigen presenting cells results in lymphocytic inhibition. Therefore, agents acting on CTLA-4 halt this mechanism and release the T-cells activation [14]. Ipilimumab, an anti-CTLA-4 antibody, was the first immune checkpoint inhibitor (ICI) to enter clinical practice, receiving FDA approval as monotherapy for late-stage melanoma in 2011 [15, 16]. In subsequent years, immune checkpoint inhibitors (ICIs) dominated clinical research and the therapeutic scenario in many malignancies. For NSCLC patients, the greatest successes have been achieved with inhibitors of the PD-1/PD-L1 axis. PD-1 is a surface protein of activated T cells, natural killer (NK), B- lymphocytes, and APC cells. Tumor cells express the ligands, PD-L1 and PD-L2. Their interaction plays a key role as inhibitor of both adaptive and innate immune responses. It prevents the proliferation, differentiation and activation of T-cells, hinders cytokine production and

can inhibit signaling through B cell receptor [17, 18]. Anti-PD-1 / PD-L1 antibodies offered significant advantages over chemotherapy for NSCLC patients, including a favorable safety profile, increased antitumor activity, induction of long-lasting responses, and improved survival. Several trials have evaluated their efficacy as monotherapy as well as in combination with other agents, and led to upset the algorithms of treatment [19].

New immune checkpoints targets are under study, like lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), V-domain Ig suppressor of T cell activation (VISTA). Preliminary data promises further practice changing in the near future [20].

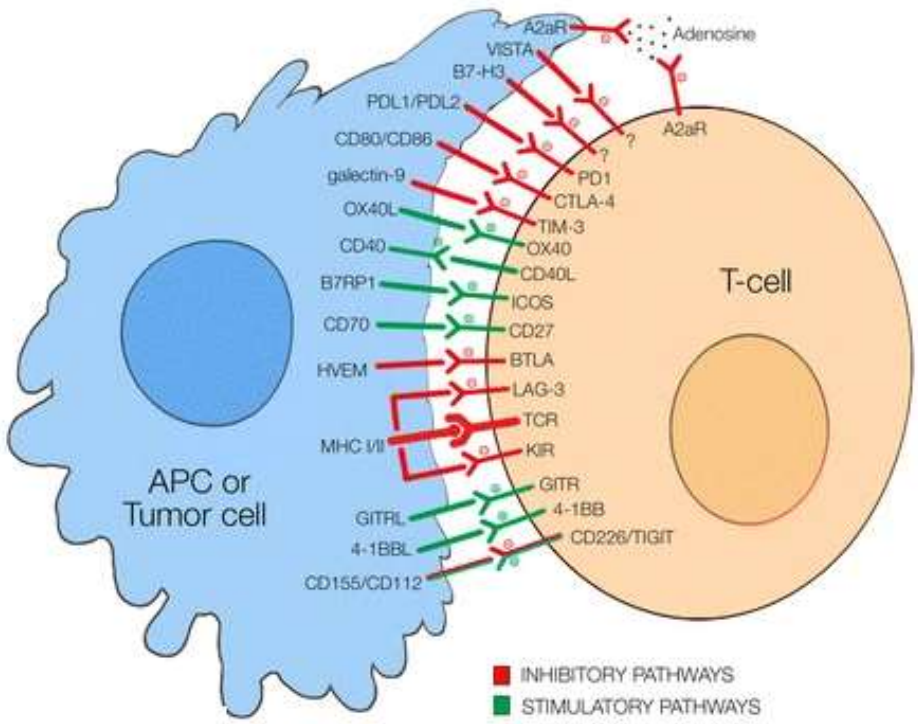


Figure 2. Immune checkpoints: inhibitory and stimulatory pathways [13].

Although ICI have become standard of care, some areas of unmet need remain. For example, we should optimize the monitoring and measuring the response to treatment since there may be atypical response patterns, such as pseudoprogression and hyperprogression, albeit rare [21]. We should redefine the absolute contraindications

to treatment since not all autoimmune disorders certainly interfere with ICI. The mechanisms underlying immune-related adverse events (IrAes) also remain unclear. Due to the exuberant immune stimulation, ICI can cause inflammatory events that resemble autoimmune diseases, especially thyroiditis, skin disorders, colitis and pneumonitis. Any organ can potentially be affected, including kidney (nephritis), liver (hepatitis), musculoskeletal and nervous systems [22, 23]. Serious events may cause treatment discontinuation and require management with high doses of steroids or immunosuppressive agents. Then, the IrAE prediction, recognition and monitoring are crucial but are still matter of debate.

Last but not least, some patients do not respond to treatment, and only a minority have durable response. Combination therapy approaches were born to increase response rate and improve survival, but the identification of predictive biomarker is essential for patient selection and distinguishing responders from non-responders to immunotherapy [24].

3. ANTI PD-1/PD-L1 ANTIBODIES

Blockade of the PD-1/PD-1 axis represents the mainstay of the current immunotherapy of NSCLC. Anti PD-1 and anti-PD-L1 antibodies have demonstrated an improvement in overall survival (OS) compared to chemotherapy (ChT) in the treatment of metastatic NSCLC (mNSCLC) both as monotherapy and in combination strategies.

Nivolumab is a fully human IgG4 monoclonal antibody against PD-1. In two large, international phase III trials, CheckMate 017 [25] and CheckMate 057 [26], Nivolumab showed improved survival over docetaxel after a first-line platinum-based ChT in squamous and non-squamous NSCLC, respectively. Median overall survival (mOS) was more than two months longer in the Nivolumab groups: mOS was 9.2 months versus 6.0 months in squamous NSCLC, 12.2 months versus 9,4 in nonsquamous NSCLC. Based on these results, Nivolumab was the first ICI receiving FDA approval in the second line treatment for advanced disease.

Subsequently, two other ICIs confirmed the superiority of immunotherapy over docetaxel in pretreated patients. Atezolizumab is a monoclonal antibody that binds to PD-L1. In the OAK trial [27], patients previously treated with one to two ChT

regimens were randomized to receive Atezolizumab or docetaxel. OS was significantly higher in the atezolizumab group compared to the docetaxel group (13.8 months [95% CI, 11.8–15.7] vs 9.6 months [8.6–11.2]). The study showed survival benefit regardless of PD-L1 expression or histology. Thus, like Nivolumab, Atezolizumab was approved as second-line therapy regardless of PD-L1 status.

Pembrolizumab, a highly selective anti-PD-1 humanized monoclonal antibody, is the first ICI to show differentiated efficacy based on PD-L1 expression. The Keynote 010 trial [28] was conducted on patients with previously treated NSCLC and PD-L1 expression on at least 1% of tumour. Pembrolizumab showed to improve OS compared with docetaxel in the total population, but with a huge benefit especially in patients with at least 50% of tumour cells (TC) expressing PD-L1. Consequently, the Keynote 024 [29], tested Pembrolizumab as first-line treatment in patients with PD-L1 expression on ≥ 50% of TCs. Immunotherapy was associated with significantly longer progression-free survival (PFS) and OS, and better safety profile, than platinum-based doublet ChT. New 5-year data showed roughly doubles survival rates: median OS was 26.3 months (95% CI, 18.3 to 40.4) for Pembrolizumab and 13.4 months (9.4-18.3) for chemotherapy (hazard ratio, 0.62; 95% CI, 0.48 to 0.81) [30]. These results establish Pembrolizumab as standard first-line therapy in patients with high PD-L1 expression.

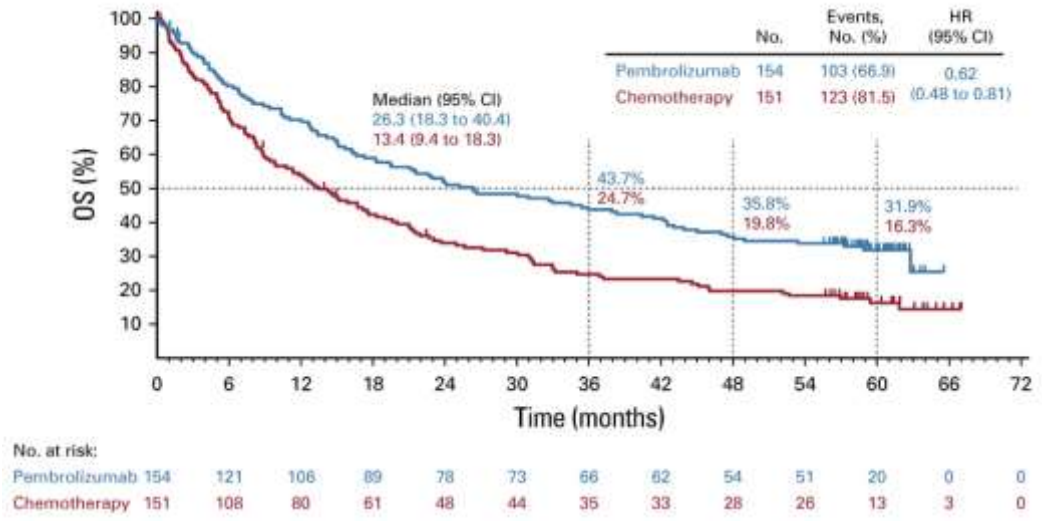


Figure 3. Kaplan-Meier estimates of OS in the pembrolizumab group and the ChT group for first-line treatment of metastatic NSCLC with PD-L1 Tumor Proportion Score ≥ 50% Keynote 024 trial [30]

Recently, another anti PD-1 antibody, Cemiplimab, showed survival benefit in the first-line treatment of mNSCLC with PD-L1 of at least 50% [31]. Median OS was not reached (95% CI 17·9-not evaluable) in the Cemiplimab group, while it was 14.2 months in patients treated with chemotherapy (11·2-17·5, hazard ratio [HR] 0·57 [0·42-0·77]; p=0·0002).

Similarly, Atezolizumab significantly prolonged overall survival vs chemotherapy in patients with high PD-L1 expression (PD-L1 expression on at least 50% of tumor cells or at least 10% of tumor-infiltrating immune cells). Median overall survival in this group was 20.2 months vs 13.1 months (stratified hazard ratio for death, 0.59; 95% confidence interval [CI], 0.40 to 0.89; P=0.01) [32].

All of these ICIs were compared with chemotherapy and received regulatory approval. In the absence of head-to-head trial between antibodies, it is difficult to determine which is the best treatment. Therefore, clinicians have more immunotherapeutic options in both first- and second-line treatment for stage IV NSCLC.

To date, only an anti-PD-L1 antibody, Durvalumab, was approved for non-resectable stage III NSCLC. In the PACIFIC trial, 713 patients received durvalumab (n = 476) or placebo (n = 237) as consolidation therapy following chemoradiation. The study found that durvalumab significantly prolonged overall survival, as compared with placebo (stratified hazard ratio for death, 0.68; 99.73% CI, 0.47 to 0.997; P=0.0025) [33]. Updated exploratory analyses demonstrated sustained survival benefit: estimated 4-year OS rates were 49.6% versus 36.3% for durvalumab versus placebo [34].

Advances in the treatment of advanced disease anticipated trials of immunotherapy in the early-staged NSCLC. Ongoing studies are evaluating the role of PD-1/PD-1 blockade as adjuvant or neoadjuvant treatment [35]. The superiority of ICI over conventional treatments has accumulated evidence in non-oncogene addicted disease. Efficacy data in tumors harboring oncogenic drivers are limited. In these cases, target therapies remain the standard of care, and immunotherapy should only be considered at the failure of other treatment options [36]. Conversely, all patients with non-oncogene addicted disease could benefit from ICI treatment, with the exception of those having contraindication (active autoimmune disorders). However, despite the advances, only a minority of tumors respond to single-agent ICI. In order to improve efficacy, the combination of chemotherapy and immunotherapy was expected to

increase the responders' rates and further prolong survival [37]. Combining ICIs with chemotherapy exploits the additive effect but also has the potential for synergy through several mechanisms. In particular, chemotherapy could elicit immune response against cancer by increasing antigen presentation to T cells, inducing immunogenicity and relieving tumor-induced immunosuppression [38-40].

4. COMBINATION STRATEGIES

Several randomized clinical trials (RCT) have demonstrated an OS benefit for the addition of Anti-PD-1/PD-L1 antibodies to platinum-based chemotherapy, providing new frontline treatment strategies for metastatic NSCLC.

The KEYNOTE-189 study [41] is a randomized double-blind phase 3 trial in non-squamous patients. Pembrolizumab in combination with platinum and pemetrexed improved response rates (RRs) from 18,9% to 47,6% and increased PFS reaching median value of 8.8 months in the pembrolizumab-combination group versus 4.9 months in patients treated with chemotherapy alone. 12-month overall survival was 69.2% versus 49.4% (hazard ratio for death, 0.49; 95% CI, 0.38 to 0.64; P<0.001) [41]. After a follow up of 4 years, the median OS was more than double in the pembrolizumab arm versus the chemotherapy-alone arm: 22.0 months versus 10.6 months, respectively (HR, 0.60; 95% CI, 0.50-0.72). OS benefit was irrespective of PD-L1 expression, with the greatest improvement in patients with a PD-L1 TPS of at least 50% [42]. Superiority of combination strategy was also shown in squamous NSCLC. In the KEYNOTE-407 trial [43], the addition of pembrolizumab to carboplatin and paclitaxel or nab-paclitaxel improved median OS from 11.3 to 15.9 months after a median follow-up of 7.8 months (HR for death 0,64). survival and response rates remained improved at longer follow-up. Three-year OS rates were 29.7% and 18.2% in pembrolizumab/chemotherapy and placebo/chemotherapy, respectively [44]. Also in this trial, survival benefit was consistent regardless of the level of PD-L1 expression.

Combinations of platinum-based ChT doublet and anti-PD-L1 antibodies, including both Atezolizumab and Durvalumab, have been troubled in development. In two randomized phase 3 trials, Atezolizumab plus chemotherapy for first-line treatment of

metastatic NSCLC showed to improve PFS but did not meet OS endpoint [45,46]. Instead, the IMpower130 trial, a multicentre, randomised, open-label, phase 3 trial, showed both PFS and OS superiority of Atezolizumab in combination with carboplatin plus nab-paclitaxel chemotherapy compared with chemotherapy alone as first-line treatment for metastatic non-squamous NSCLC [47].

Overall, even in the absence of statistical significance, a favorable survival trend is observed in all combination strategies compared to alone chemotherapy. In all these trials, the OS benefit was seen across all relevant patient subgroups, including those with low or negative PD-L1 expression. Furthermore, although immunotherapy adds IrAEs to chemo-related toxicities, combination treatments have shown acceptable tolerance profiles. Then, anti-PD-1/PD-L1 antibodies plus platinum-based doublet Cht became a standard first-line treatment of metastatic NSCLC. However, combination regimes were always compared to chemotherapy alone. No direct comparison has been made with Pembrolizumab in patients with PD-L1 TPS of ≥ 50%. So, single-agent Pembrolizumab remains an appropriate option in this setting. Indeed, for the same reason, in Italy the addition of Pembrolizumab to platinum-based chemotherapy received the regulatory approval only in patients with PD-L1 expression less than 50%, making the use of Pembrolizumab alone as the only one immunotherapeutic chance for patients with higher PD-L1 expression.

Recent advances derive from ICI combinations, especially anti-PD-1 and anti-CTL-4 antibodies. It has been found that the simultaneous CTLA-4 and PD-1 / PD-L1 blockade results in a synergistic and enhanced immune response against cancer: inhibition of CTLA-4 mainly act on the lymph nodes and can upregulate PD-1 expression favoring the activation of T cells, therefore, it exerts a priming effect for the PD-1 blockade which improves the function of effector T cells in the tumor microenvironment [48,49]. The efficacy of dual immunotherapy has already been explored in large RCTs. When compared to chemotherapy, it appears to provide controversial results, showing survival benefit especially in some subgroups of patients. When combined with chemotherapy it clearly and significantly improved overall survival. In the POSEIDON trial, patients with metastatic NSCLC who received a combined regimen of durvalumab (anti-PD-1), tremelimumab (anti-CTL-4), and platinum based ChT experienced a statistically significant improvement in

terms of PFS and OS compared to patients who received chemotherapy alone. In the same trial, durvalumab plus chemotherapy without tremelimumab showed a positive trend for OS but did not reach statistical significance [50].

Checkmate 568, an open-label phase II trial, successfully tested the efficacy and safety of Nivolumab plus low-dose ipilimumab as a first-line treatment of advanced/metastatic NSCLC [51]. Survival benefit over chemotherapy was confirmed in the CheckMate 227 trial, and was independent of PD-L1 expression level [52].

A recent phase 3 trial (Checkmate 9LA) explored the effect of nivolumab plus ipilimumab combined with two cycles of platinum-based chemotherapy in the first-line setting for mNSCLC. Immuno-chemotherapy provided a significant improvement in overall survival and had a favorable safety profile. At a median follow-up of 13.2 months, median OS was 15.6 months in patients treated with combined strategy group vs 10.9 months in patients treated with chemotherapy alone (HR = 0.66, 95% CI = 0.55–0.80). Overall survival benefit in the nivolumab/ipilimumab plus chemotherapy group was observed in both squamous and nonsquamous histologies and across all PD-L1 expression levels [53]. These new data broaden first-line treatment options for metastatic NSCLC [54].

Another dual immunotherapy consisting of anti-PD-L1 (Durvalumab) and anti-CTLA-4 (Tremelimumab) antibodies was investigated in two phase 3 randomized clinical trials. In the Mystic trial, patients with untreated mNSCLC were randomized to receive treatment with durvalumab, durvalumab plus tremelimumab, or chemotherapy. Despite treatment with durvalumab resulted in a reduced risk of death in patients with PDL-1 expression on at least 25% of tumor cells, the study did not meet its primary end points of improved OS with immunotherapeutic strategies vs chemotherapy [55]. The POSEIDON trial showed that durvalumab plus chemotherapy significantly improved PFS while a positive trend for OS did not reach statistical significance. Instead, durvalumab combined with tremelimumab and chemotherapy versus chemotherapy alone improved both PFS and OS (median OS of 14.0 months versus 11.7 months) [56].

These results highlight the usefulness of immunotherapeutic approaches in the first-line treatment for mNSCLC patients. In particular, combination strategies showed to improve survival, regardless of PD-L1 expression and histology.

TRIAL	DETAILS	HISTOLOGY	PD-L1 Expression	OS
Anti PD-1/PD-L1 single agent				
KEYNOTE 024	Pembrolizumab vs. Platinum-based chemotherapy	NSCLC	PD-L1 ≥ 50%	Median OS: 26.3 months vs. 13.4 months
IMpower110	Atezolizumab vs. Platinum-based chemotherapy	NSCLC	PD-L1 ≥ 50%	Median OS: 20.2 months vs. 13.1 months
EMPOWER-Lung 1	Cemiplimab vs. Platinum-based chemotherapy	NSCLC	PD-L1 ≥ 50%	Median OS: not reached vs 14.2 months
Combination strategies				
KEYNOTE 189	Pembrolizumab + Pemetrexed-platinum vs. Placebo + Pemetrexed-platinum	NS-NSCLC	All comers	Two-year Median OS: 22.0 months versus 10.6
KEYNOTE-407	Pembrolizumab + Carboplatin and paclitaxel vs Placebo + Carboplatin and paclitaxel	Squamous NSCLC	All comers	Two-year Median OS: 17.1 months vs. 11.6 months
IMpower130	Atezolizumab + Carboplatin plus nab-paclitaxel vs Carboplatin plus nab-Paclitaxel	NS-NSCLC	All comers	Median OS: 18.6 months vs. 13.9 months
Poseidon	Durvalumab + Tremelimumab + CT vs Platinum doublet chemotherapy	NSCLC	All comers	Median OS:14.0 months vs. 11.7 months
CheckMate 227	Nivolumab + Ipilimumab vs. Platinum doublet chemotherapy	NSCLC	PD-L1 expression level of 1% or more	Median OS:17.1 months vs. 13.9 months
CheckMate 9LA	Nivolumab + Ipilimumab vs. Platinum doublet chemotherapy	NSCLC	All comers	Median OS:15.9 months vs. 10.9 months

Table 1. First-line immunotherapy for mNSCLC: phase 3 trials showing OS benefit from ICIs

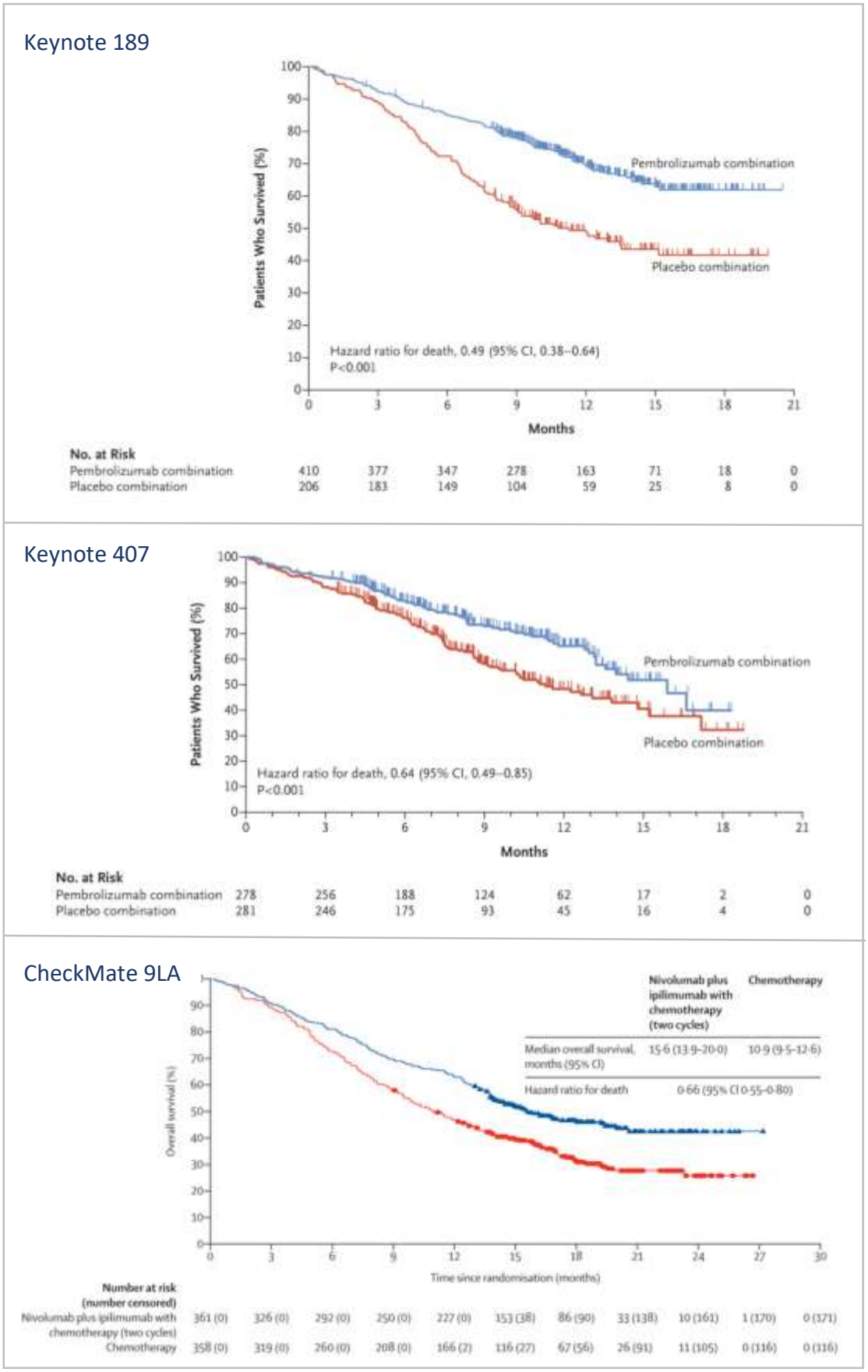


Figure 4. Overall Survival in Keynote 189, Keynote 407, CheckMate 9LA

5. TREATMENT OF METASTATIC NSCLC

ICIs have quickly revolutionized the treatment paradigm for the IV stage NSCLC. Single agents and combination strategies have both demonstrated great clinical activity and manageable side effects. The survival improvements shown in several phase 3 RCT have led to multiple regulatory approvals. As a result, clinicians have more immunotherapeutic options in treating metastatic disease.

In the current algorithms, the distinction between non-oncogene addicted and oncogene addicted disease remains a cornerstone. Target therapies are the unquestioned standard of care for patients with actionable mutations and immunotherapy should only be considered upon failure of other treatment options. A potential ICI activity in oncogene-addicted NSCLC was documented in the phase 3 IMpower150 study: the addition of atezolizumab to bevacizumab plus chemotherapy resulted in a survival improvement in NSCLC patients, including patients with EGFR mutations or ALK translocations [57]. Although the role of immunotherapy remains controversial in this setting, combination strategies with target therapies may increase antitumor activity and represent a new field of research. However, data on the efficacy of combining PD-1 inhibitors with Tyrosine-kinase Inhibitors (TKIs) are mainly obtained from subgroup analysis and revealed a limited effect [58]. To date, combinations with anti-PD-1/PD-L1 antibodies and anti-EGFR TKIs have failed to improve survival and have not be found feasible due toxicity [59,60]. Other combinations with anti-EGFR TKIs or ALK inhibitors are currently being investigated in ongoing phase I-II clinical trials [56]. Further research should focus on emerging oncogenic aberrations, including RET fusions, ROS and NTRK rearrangements, MET, BRAF and KRAS mutations, for which the role of immunotherapy is poorly explored.

Furthermore, the advent of new genomic diagnostic tools, including Next Generation Sequencing (NGS), has broadened knowledge of cancer biology and improved the acquisition of molecular targets [61, 62]. By providing a comprehensive molecular profile, NGS also offers the opportunity to identify potential biomarkers for immunotherapy, both from tissue biopsies and from blood samples [63,64]. Based on recent evidence, international guidelines already recommend the use of NGS to extend

the molecular profile, especially in order to favor access to target therapies [65]. Instead, the predictive and prognostic role for patients treated with immunotherapy has not yet been clarified. Although it is not routinely used in clinical practice and only a few Centers can benefit from it, in the near future NGS could become a standard method as a molecular diagnostic tool for NSCLC.

Therefore, immunotherapy currently plays a marginal role in oncogene-addicted disease, but most patients do not have a molecular target and do not receive target therapies (non-oncogene addicted disease). In these cases, ICIs are standard of care for metastatic NSCLC, either as a single agent treatment or in combination strategies. Monotherapies in pre-treated patients have been widely used in the past, but are now outmoded as ICIs are placed at the first-line setting.

Combined therapy has shown to improve the effectiveness of immunotherapy, expanding the beneficiary population, and overcoming drug resistance. Combinations of ICIs with platinum-based doublet chemotherapy represent appropriate therapeutic choices in both squamous and non-squamous histology, regardless of PD-L1 expression. They are superior to chemotherapy alone even in PD-L1 negative patients, but their efficacy is greater in PD-L1 positive patients. However, there is no RCT comparing the combination approach and anti-PD-1/PD-L1 single-agent in patients with PD-L1 expression $\geq$ 50% on TCs. As a result, ICI monotherapy remains a therapeutic option in this setting especially in patients unfit for chemotherapy [66, 67]. In this regard, most clinical studies have excluded frail patients. Consequently, there is little evidence of ICI efficacy in special populations, including ECOG PS 2 and elderly patients. In the real world, they have poor therapeutic chances, so it is necessary to strengthen the research [68-71].

Novel immune-based agents address the treatment landscape of NSCLC, and several clinical trials are exploring immunotherapy at various stages [72-74]. ICI indications are likely to expand in the future, but some challenges remain. Immunotherapy confers long-term durable response, but most patients do not respond or develop progressive disease during treatment. Then, understanding of primary and secondary resistance mechanisms to these agents is the key for the future development of cancer immunotherapy. The identification of reliable predictive and prognostic biomarkers remains the crucial item for patient selection and guiding therapeutic choices.

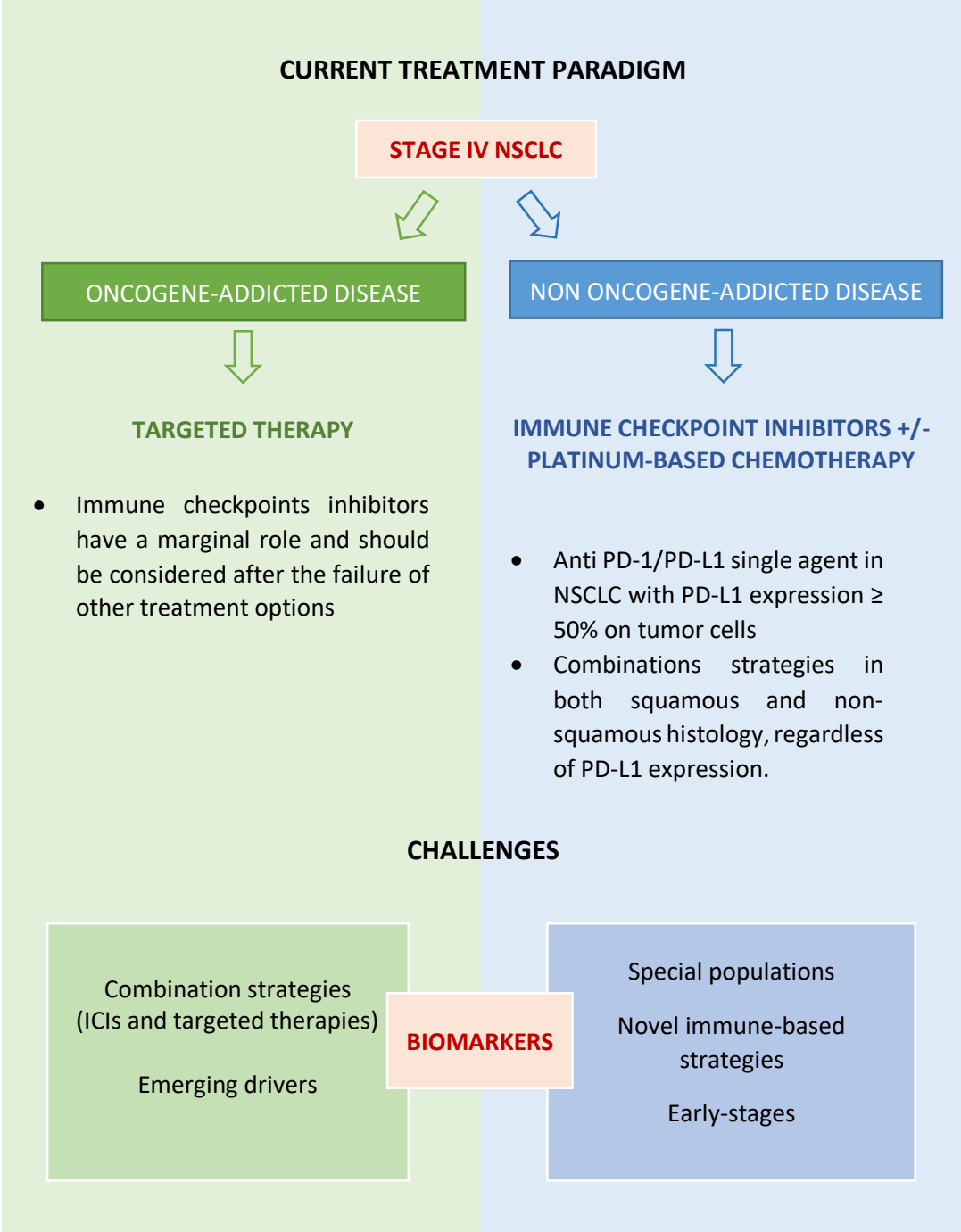


Figure 6. Immunotherapy for NSCLC: the current treatment paradigm and challenges

Targeted therapies represent the first choice of care for patients with actionable mutations (oncogene-addicted diseases). Immune checkpoint inhibitors are the standard front-line treatment for metastatic non oncogene-addicted disease. Combinations of ICIs with platinum-based doublet chemotherapy received regulatory approval in both squamous and non-squamous histology, regardless of PD-L1 expression. Anti PD-1/PD-L1 monotherapy remains a therapeutic option in patients with PD-L1 expression ≥ 50% in tumor cells. ICI indications are likely to expand in the future, but some challenges remain. The identification of reliable predictive and prognostic biomarkers remains the crucial item for patient selection and guiding therapeutic choices.

6. IMMUNOTHERAPY BIOMARKERS

Predictive and prognostic biomarkers have been the main field of research in Immuno-oncology for years. A large number of cells and particles participate in the tumor-immune interaction. Therefore, several molecules and parameters could correlate with clinical outcomes and predict response to ICI treatment. There are broad categories hosting potential biomarkers, including tumor microenvironment (TME), immune checkpoints, cancer neoantigens, tumor mutational burden, inflammation, and multiple tumor-associated components circulating in the bloodstream [75,76]. At present, only a few of tissue-based biomarkers have been shown to predict the efficacy of ICIs in NSCLC patients, mainly including the PD-L1 expression and tumor mutation burden (TMB). Many other potential biomarkers are under investigation, both from tissue samples and liquid biopsies.

6.1. Tissue biomarkers

The main biomarker widespread used in clinical practice is the PD-L1 expression on TCs or infiltrating immune cells, assessed by immunohistochemistry (IHC) staining. Several prospective trials demonstrated a correlation with efficacy of anti-PD-1/PD-L1 treatment. The first strong evidence comes from the Keynote 010 and Keynote 024 trials which led to the approval of Pembrolizumab in the second-line setting for PD-L1 positive patients and as frontline treatment for patients with PD-L1 expression ≥ 50%, respectively [77]. Later, Atezolizumab also showed improved survival compared to platinum-based chemotherapy among NSCLC patients with high PD-L1 expression [78]. Multiple exploratory analyses support these findings. Among these, in the PACIFIC trial, an exploratory post-hoc analysis requested by EMA revealed no survival benefit from Durvalumab after chemoradiotherapy for unresectable NSCLC in patients with PD-L1 expression <1% [79]. In contrast, a proportion of PD-L1 negative tumors can also respond to anti PD-1/PD-L1 treatments, including Nivolumab and Atezolizumab as single agents after platinum-chemotherapy failure [80, 81]. Additionally, the CheckMate 227 trial reported OS benefit from Nivolumab plus Ipilimumab compared to chemotherapy in both PD-L1 positive and PD-L1 negative tumors [82]. In general, most evidence shows that PD-L1 expression may be

a predictor of the efficacy of anti-PD-1/PD-L1 antibodies when used as single-agent [83]. However, with the advent of combination strategies, its predictive value is significantly diminished. ICI plus platinum-based chemotherapy reported survival improvement regardless PD-L1 expression. Although the efficacy appears to increase with higher PD-L1 levels, it has also been proven in PD-L1 negative tumors [84].

Whether PD-L1 expression is the best biomarker for lung cancer immunotherapy is still a matter of debate as several controversies limit its reliability [85-87]. Studies have shown the heterogeneous nature of PD-L1 expression which differ within tumors, may be inconsistent in sections of the same tumor sample, and can vary during treatment [88,89]. Finally, clinical trials used various cutoff points and detection methods resulting the need for standardization [90].

In last years, tumor mutational burden (TMB) has arisen as another biomarker for immunotherapy. It is defined as the total number of somatic mutations in a tumor sample. A high number of mutations results in a greater number of tumors neoantigens and increase activation of immune cells. Thus, TMB reflects neoantigen load and represent a surrogate for cancer immunogenicity [91,92]. The evidence for TMB as immune-related biomarker in NSCLC origins from retrospective or subgroup analysis of RCTs. The CheckMate 026 trial tested first-line Nivolumab in patients with a PD-L1 expression of 5% or more. Nivolumab did not show significantly longer PFS and OS than platinum-based chemotherapy. An exploratory analysis revealed that among patients with a high burden of tumor mutations (243 or more mutations), the response rate and PFS were higher in the nivolumab group, but no OS differences were observed [93]. Similarly, in the CheckMate 227, high TMB (≥ 10 mutations per megabase) was associated with longer PFS in patients receiving combination of nivolumab plus ipilimumab compared to chemotherapy, but failed to predict OS [94]. Despite many studies have suggested that the TMB correlates with the ICIs efficacy, a clear association with the OS benefit has not yet been demonstrated and its role as a biomarker for immunotherapy remains uncertain. In addition, no correlation with PD-L1 expression have been showed [95] and other issues limit its utility and validation. With the advent of new molecular diagnostic tools such as NGS, TMB will be increasingly available in clinical practice. However, at present, there is no

standardization across the testing platforms, and there is no consensus to define "high" TMB as different cut-off have been used in clinical trials [96,97].

Mismatch repair deficiency (MMRD) and microsatellite instability (MSI) have been associated with an increased TMB and may be determinants of tumor immunogenicity [98]. Consequently, some clinical trials have studied their potential role as predictive biomarker for ICIs. Recent evidence reported high response rate and increased survival in several MSI-high and MMRD solid cancers, leading to the first tissue/site-agnostic approval by the FDA [99-100]. KEYNOTE-177 trial found that first-line pembrolizumab significantly improved PFS than chemotherapy for MSI-H–dMMR metastatic colorectal cancer [101]. Nevertheless, MMRD and MSI are rare in lung cancers and are poorly studied as potential biomarkers for NSCLC ICIs [102].

Emerging biomarkers arise from the characterization of TME cell populations. TME is highly heterogeneous and dynamic, composed of immune cells, fibroblasts, vascular and lymphatic vessels surrounding the tumor cells. Among these, Tumor-infiltrating lymphocytes (TILs) have been correlated with ICI efficacy in several cancer types, including NSCLC (103,104). High levels of TILs, particularly CD8 + T cells, reflect an increased immune response against cancer and characterize the inflamed phenotype, the so-called "hot" tumor. Categorizing tumors into "hot" and "cold" tumors through the identification of tissue biomarkers could help the selection of patients who are more likely to benefit from ICIs [105,106]. A methodology named "Immunoscore" has been defined to measure intra- and peritumoral T cell infiltrate [107]. It has a validated prognostic value and provides a reliable estimate of the recurrence risk in colon cancer [108,109]. An immunoscore for NSCLC patients is currently under development [110].

6.2. Liquid biopsy and emerging blood-based biomarkers

Tissue biopsy is the standard method for cancer diagnosis, histological classification, and molecular diagnostics. However, invasiveness limits its feasibility and repeatability and tumor lesions are sometimes difficult to biopsy. Furthermore, it can fail to monitor disease evolution and capture tumor heterogeneity. To overcome these limitations, liquid biopsy has become an attractive opportunity for cancer diagnostics. It is a non-invasive tool and consists of analyzing cancer-associated elements in

biological fluids, such as blood, urine and saliva. Primarily, blood-based liquid biopsy in one of the main fields in ICI biomarker search. It allows the isolation of multiple tumor-derived or tumor-associated components circulating in the bloodstream, including circulating tumor cells (CTCs), immune cells, extracellular vesicles (EVs), cell-free DNA (cfDNA) and microRNA (miRNA) [111].

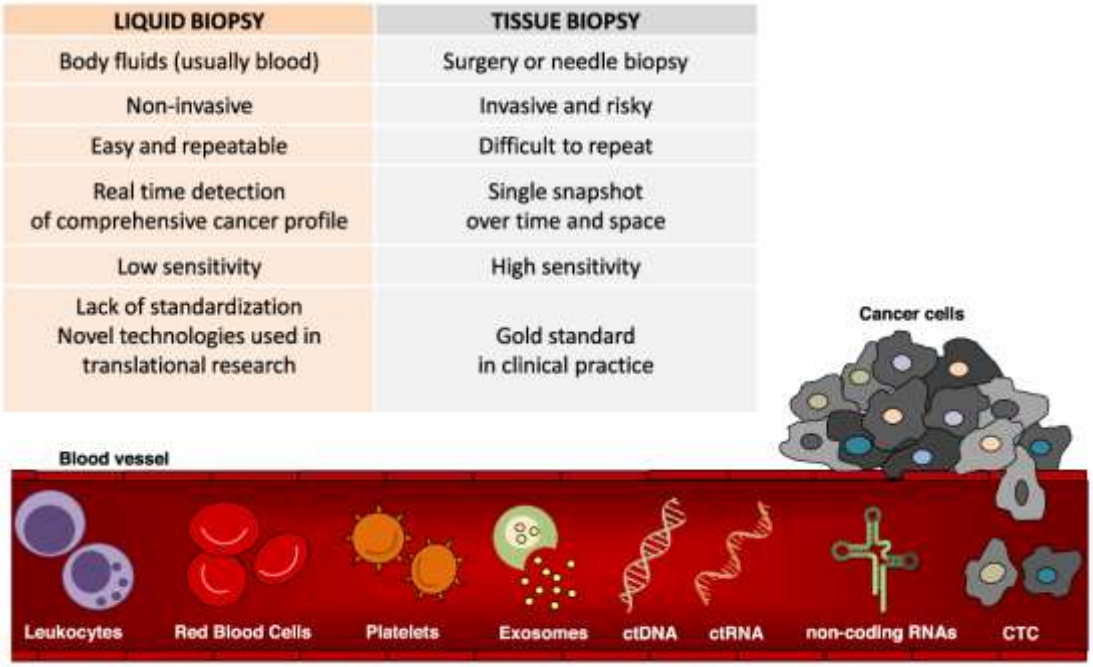


Figure 7. Liquid biopsy: Pros and Cons

Peripheral immune cells populations could reflect the interaction between immune system and cancer. Peripheral blood mononuclear cells (PBMC), absolute lymphocyte count, absolute neutrophil count (ANC), absolute eosinophil count, absolute monocytic count, platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio, and neutrophil-to-lymphocyte ratio (NLR), have all been investigated as predictor of response to immunotherapy in different solid cancers [112]. In particular, several studies have reported that a high NLR has a negative predictive and prognostic value. Two recent metanalyses found that elevated blood NLR was associated with poor PFS and OS in NSCLC patients treated with PD-1/PD-L1 antibodies [113,114]. Researchers also explored the baseline and dynamic changes in T lymphocytes, especially PD1 + CD 8 T cells, as a straightforward marker for distinguishing responders and non-responders [115,116]. Nevertheless, data remain controversial and

none of above-mentioned parameters has a certain role as biomarker for NSCLC immunotherapy.

CTC and cfDNA have been used for assessing PD-L1 expression, TMB and other immune-related signatures. Lower expression of soluble PD-L1 and high blood-TMB appear to correlate with good response to ICIs [117-119]. However, main evidence come from retrospective exploratory analysis or small prospective series [120,121]. Additionally, the studies evaluating concordance between tissue and liquid-biopsy have produced conflicting results [122]. Moreover, multiple detection diagnostics have been used, as workflow and protocols are not harmonized [123].

In general, despite the potential advantages, due to the lack of standardized methodologies, the low accuracy rates and controversial findings, blood-based biomarkers are not yet validated in the clinical setting.

6.3. Focus on MiRNA

Among novel blood-biomarkers, microRNAs are attracting great interest.

TME hosts many nanosized particles, named extracellular vesicles (EV), which are produced and released into the bloodstream by various cell types, including cancer cells and immune cells [124]. EVs represent a heterogeneous group of membranous structure, classified according to cellular origin and biological functions. Two subtypes, exosomes and plasma membrane-derived microparticles (microvesicles) are primarily considered mediators of intercellular communication, and there is evidence of their involvement in tumor growth, metastatization and immunomodulation [125,126]. EVs-derived microRNAs, single-stranded non-coding RNA, are believed to be the main regulators of these processes. They act at the post-transcriptional and translational level for various cellular functions. NSCLC-derived exosomal miRNAs affect TME components, such as macrophages, endothelial cells, and immune cells, promoting metastasis through modulation of angiogenesis and inhibition of tumor suppressors. Furthermore, growing evidence demonstrates that they are involved in drug resistance for both ALK- and EGFR-TKIs. These findings prompted to investigate the potential therapeutic application of EVs and miRNAs in NSCLC. To date there is no sufficient data about the efficiency and safety but it represents a fascinating topic of research [127].

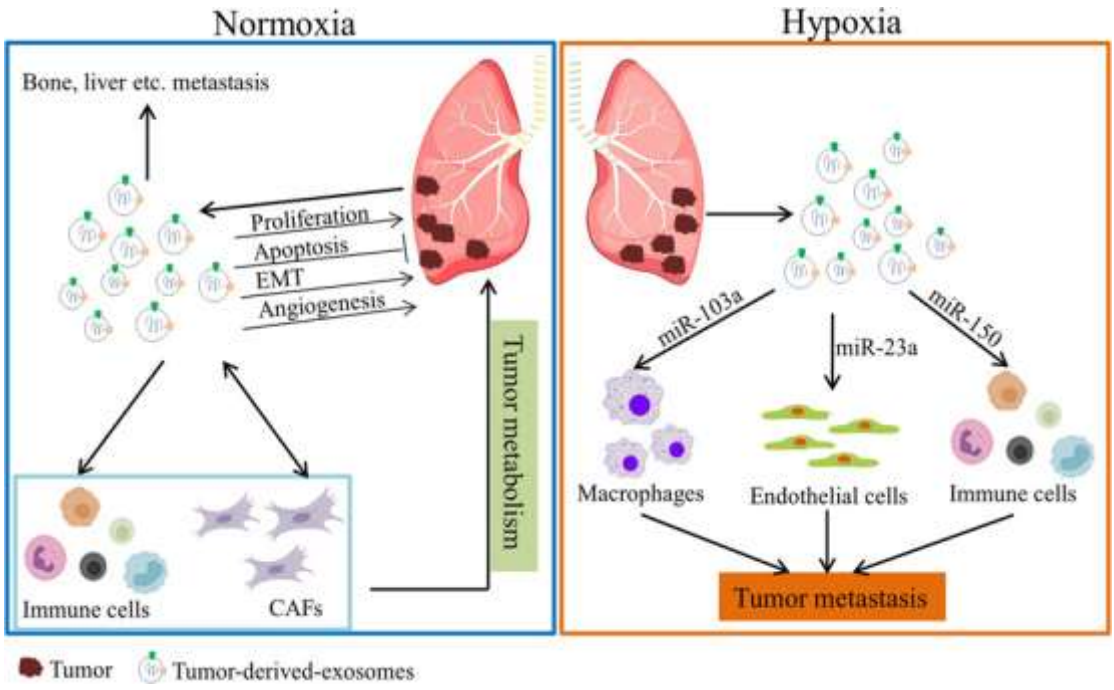


Figure 8. Tumor microenvironment regulation by EVs and miRNAs [127]

More and more evidences indicate a crucial role of miRNAs in the development, maturation and activation of immune cells [128-131]. Therefore, they have emerged as key players in tumor immunity and represent potential biomarkers for immunotherapy.

Recent studies have explored the role of plasma-derived miRNA signatures in NSCLC patients treated with ICIs. In a cohort of patients (n=80) treated with Nivolumab, a 10-high expressed miRNA pattern was associated with statistically significant improvement in PFS and OS [132]. A plasma exosomal miRNA profile (Hsa-miR-320d, Hsa-miR-320c and Hsa-miR-320b) was identified as potential predictor of efficacy for anti-PD-1 treatment [133]. In a consecutive series of 140 patients receiving ICI treatment, a plasma immune-related miRNA-signature classifier (MSC) composed of 24 miRNAs was correlated with ORR, PFS and OS. Significance was greater if combining MSC with PD-L1 expression. The author concluded that MSC could supplement PD-L1 to identify NSCLC patients with worse response and survival [134]. Moreover, Cortez et al found that PD-L1 was regulated by p53 via miR-34 which directly binds to the PDL1 3' untranslated region in models of NSCLC [135].

Undoubtedly, further studies are needed to clarify the role of microRNAs and identify which signatures can better predict responses to ICI. Detection methods also need to

be refined and standardized. However, the preliminary data available in the literature are promising and pose a new challenge for clinical and translational research: microRNAs as novel biomarkers for NSCLC immunotherapy

7. CONCLUSIONS

Immunotherapy has revolutionized the treatment paradigm of NSCLC. Until a few years ago, patients with metastatic disease received chemotherapy alone. Immune checkpoint inhibitors broadened therapeutic opportunities and significantly improved prognosis. However, the initial enthusiasm was later dwarfed when it was discovered that only a minority of patients have lasting responses. Despite combination strategies have further increased response rates, it remains unclear why most patients develop progressive disease. Further studies should focus on identifying biomarkers for prediction treatment responses and resistances. MicroRNAs are arousing growing interest and represent one of the main targets of ongoing research.

The Study

Large-scale profiling of extracellular vesicles identified miR-625-5p as a novel biomarker of immunotherapy response in advanced non-small cell lung cancer patients

1. INTRODUCTION

Immune Check Point Inhibitors (ICIs) have dramatically changed the therapeutic landscape for patients with non-small cell lung cancer (NSCLC) [1]. Anti- PD-1 / PD-L1 antibodies as single agents or in combination to platinum-based chemotherapy have become the standard of care as front-line treatment for advanced disease lacking targetable oncogenic drivers. Tumour cell PD-L1 expression represents the main predictor of response [2]. However, only a subset of patients benefits from ICIs and most them develop progressive disease [3]. Hence, novel biomarkers need to be identified for a more personalized approach to immunotherapy selection.

Cancer cells and their microenvironment produce heterogeneous mixtures of extracellular vesicles (EVs) namely exosomes and microvesicles (MVs), that can be detected in body fluids including blood [4]. EVs released by cancer cells can suppress the immune system response inactivating T lymphocytes or natural killer cells, as well as promoting differentiation of regulatory T lymphocytes and tumor growth [5]. An important breakthrough was the discovery of the presence of nucleic acids such as mRNA and miRNA in EVs. In particular, miRNAs, a class of small, single-stranded non-coding RNAs, have emerged as key players in modulating cancer cell phenotype and, more recently, as crucial regulators of innate and adaptive immune responses by negatively regulating the expression of key regulators of developmental checkpoints [6, 7].

From this perspective, circulating EV-associated miRNAs (EV-miRNAs) could provide relevant information regarding not only cancer cell biology, but also tumor microenvironment including tumor-immune system interactions. Here, we screened EV-miRNAs profiles in plasma of 88 advanced NSCLC patients undergone to anti-PD-1/PD-L1 therapy as single agent to identify potential novel biomarkers of response to ICIs.

2. MATERIALS AND METHODS

Sample Size

Objective Response (OR) is a direct measure of a drug antitumor activity that can be evaluated in single-arm studies. It represents a reliable clinical endpoint to identify candidate biomarkers of biological significance as it is less influenced than time-dependent outcomes from other clinical variables and can hence be attributable directly to the drug, not the natural history of the disease. However, OR is considered a relatively poor surrogate of Overall Survival (OS) in patients treated with ICIs [8].

To identify EV-miRNAs of both biological and clinical interest, we designed our study in two steps. In the first step, we considered two groups (responder; non-responder according RECIST 1.1 criteria) design to identify EV-miRNAs significantly associated after False Discovery Rate (FDR) correction to OR during treatment with ICIs. The following parameters were used: G_0 (estimating a number of undifferentially detectable EV-miRNAs) = 250; $E(R_0)$ (mean number of false positives) = 2; expected differential expression between case and control conditions of $|\mu_1|$ = 0.5 on a log-2 scale; anticipated experimental error standard deviation (σ) = 0.70 on a log-2 scale; standard deviation of the difference in log-expression between treatment and control conditions (σ_d) = $\sqrt{2}\sigma$ = $\sqrt{2}(0.70)$= 0.9899; ratio $|\mu_1|/\sigma_d$ = 1.000/0.9899 = 1.010. For these specifications, 24 samples for each group were needed, with a non-centrality parameter $\psi_1 = 48(1.010)^2 = 48.9648$ [9].

Secondly, expression levels of miRNA(s) successfully identified in the first step were dichotomized according to an optimized cutoff value (based on distribution and sensitivity/specificity method). With an Hazard Ratio (HR) threshold of 0.50/2.00, a value for alpha of 0.05 (one-sided), a desired power of 80% and estimating an allocation ratio from the optimal dichotomization process from 1:1 to 1:3 (i.e. with the group with the lower number of patients between 50% and 25% of the total sample size), the total number of required events (deaths)ranged from 51 to 69 [10].

Following the most conservative estimates, we therefore planned to continue enrollment and follow-up until obtaining a cohort of informative patients with at least 24 OR and 69 OS events.

Study design and patient characteristics

A consecutive series of 218 NSCLC patients was administered with anti–PD1 from 01-2018 to 02-2020 and followed up until 05-2021 at Campus Bio Medico of Rome University Hospital. Forty-eight patients were excluded from the study because they did not meet inclusion criteria and 6 because they declined to participate.

The study was conducted in accordance with the principles of the Helsinki Declaration. All experimental protocols were approved by the Internal Review and Ethics Boards of the Campus Bio Medico University Hospital of Rome (Prot. N. 48.17OSS) and all patients provided informed consent.

The inclusion criteria were patients who were at least 18 years old, with a performance status of 0 or with no signs of active autoimmune disease and treated with anti–PD-1 (pembrolizumab or nivolumab) as monotherapy as first, second or third line of treatment for advanced disease. Plasma samples were all collected prior the first cycle of ICI. 35 out 164 patient's plasma were excluded for low yield or presence of hemolysis. The patients' disease had to be measurable per RECIST 1.1 at baseline and had to be periodically evaluated for response to treatment by radiological evaluation (CT scan or PET/CT scan). All patients' disease progression (PD) had to be demonstrated by radiological evaluation. According to the RECIST 1.1 best response criteria, patients were classified as responders (R), patients with stable disease (SD), and PD. The OR was defined as complete response (CR) or a partial response (PR). Tumor burden calculated as the sum of diameters of all target lesions (unidimensional measurements) for all patients included in the study at every radiological evaluation Progression Free Survival (PFS) was defined as the time from the first infusion of anti–PD1 to the first documented tumor progression. OS was defined as the time from the first infusion of anti–PD1 to death or last news. Twenty-one out of 129 patients with adequate plasma were excluded because they died before first radiological evaluation or were lost to follow up. Twenty out of 108 patients included for miRNA expression analysis did not passed Ncounter Quality Check leading to 88 patients that were included in the final study analysis. Clinical and biological data were collected through a dedicated patient file database. The redaction of the manuscript followed the TRIPOD guidelines for prognostic/predictive studies [11].

EV miRNA profiling of plasma samples

Whole blood was collected the same day of the first ICI infusion in four 3 mL K2EDTA Vacutainer tubes and the plasma was collected after two centrifugation steps at 1,258 × g for 10 minutes. EVs were isolated using membrane affinity spin methods with exoRNeasy Serum/Plasma Kit (Qiagen) according to manufacturers' instructions. EV total RNA isolation was performed using exoRNeasy Serum/Plasma Kit (Qiagen) according to manufacturers' protocol. miRNA expression analysis was assessed by nCounter Analysis System (NanoString Technologies).

Data Normalization

Reporter Code Count (RCC) files generated by nCounter instrument were imported to nSolver 4.0 software. Quality Check (QC) on Binding Density, Image Quality and Positive Control Linearity as well as Positive Control Limit of Detection and Ligation was performed using the default QC settings. Samples (n=20) that did not pass QC were excluded from the analysis.

In order to identify the pool of miRNAs considered detectable from nCounter platform, raw expression of endogenous miRNAs was compared with that of negative controls. Kruskal-Wallis test followed Dunn's post test was used to select miRNAs with mean ranks significantly higher compared to the mean ranks of negative controls. 196 miRNAs out 799 were selected as expressed above background (**Supplementary Figure 1 Panel A**).

Several normalization methods were hence explored using NormalyzerDE R package. Normalyzer is implemented in R using Bioconductor packages. Briefly such as Global Intensity (GI), Median Intensity (Median), Mean Intensity (Mean), Quantile (preprocessCore package), Variance Stabilizing Normalization (VSN, vsn package), Robust Linear Regression (RLR), and CycLoess (limma package). Selection of the optimal normalization method was carried out considering different quantitative and qualitative statistical measures. CycLoess resulted normalization method with the lowest Pooled intragroup Coefficient of Variation (PCV), Pooled intragroup Median Absolute Deviation (PMAD) and Pooled intragroup estimate of variance (PEV) (**Supplementary Figure 1 Panel B**). Presence of bias introduced during normalization was hence excluded using qualitative plots checking for skewness (Density Plot), for

improvement in whisker alignment and lengths. among replicates (RLE Box Plot), checking if variance is independent of mean (MeanSDplots) and checking for replicate clustering and outliers presence (MDS Plots) (**Supplementary Figure 1 Panel C**).

Statistical Analysis

Limma R package was used to identify differentially expressed miRNAs that can discriminate between patients who achieved OR (Responders) vs patients who experienced SD or PD as best response (Not Responders) to ICI treatment. FDR correction for multiple hypothesis testing was applied. OptimalCutpoints R package was used to identify the optimal cutoff to dichotomize patients according hsa-miR-625-5p (miR-625-5p) expression and OR) (**Supplementary Figure 2 Panel A**). Optimal cutoff was selected for its ability to best separate density curves of responders and non-responders. CutoffFinder R package was used to plot respectively HRs for OS and PFS and Odd Ratios for OR of all possible cutoffs) (**Supplementary Figure 2 Panel B**). Univariable and Multivariable Logistic Regression models was used to determine Odd Ratios and 95% confidence intervals (CIs) for OR. Survival curves were estimated by the Kaplan–Meier method and compared with the Log-rank test (univariate analysis). Univariate HRs were calculated using log-rank method. Multivariable Cox regression model was used to determine HRs and 95% confidence intervals (CIs) for OS and PFS. Variables found to be statistically significant at the $P < 0.05$ level were entered into a multivariate models. Differences between Tumor Change at best response and miR-625-5p/PD-L1 status were evaluated using Kruskall Wallis followed by Dunn test for pairwise comparison with Bonferroni Adjustement. The Spearman Rank and Pearson correlation test were employed to examine relationships between Tumor Change at best response and miR-625-5p expression as continuous variable.

3. RESULTS

Clinicopathological findings of the patient population

From 2018 to 2020, a consecutive series of 218 advanced NSCLC patients treated with anti–PD1 therapy was assessed for eligibility. Of these, 88 were analyzed for pre-

treatment EV-miRNAs. A flow chart with the reasons for exclusion is provided as (**Supplementary Figure 3).** In the final cohort of patients, the median follow up was 38 months (95% CI 33–not reached).

The median PFS and OS were 6.45 (95% CI 4.70-12.0) and 11.5 months (95% CI 8–16.5), respectively. Among these patients, 35 (39.8%) were treated with nivolumab and 55 (60.2%) with pembrolizumab. Thirty-seven patients (42.0%) received an anti–PD1 therapy as first line therapy, while 51 (58.0%) in second or third line. Female patients were 27 (30.7%) and 46 patients (52.3%) less than 70 year old. Sixty-three patients (71.6%) had an adenocarcinoma histology while the remaining 25 (28.4%) had a squamous cell histology. Forty-four patients (50.0%) had PD-L1 immunohistochemical staining < 50%.

The complete patient characteristics are summarized in (**Supplementary Table 1)**.

MiR-625-5p is associated to OR during treatment with ICIs

The isolated-EV were characterized through electron microscopy confirming the presence of microvesicles, whose diameter ranges from 130 to 350 nm and exosomes (50-100 nm) **(Supplementary Figure 4)** [12]. Differentially expressed miRNAs extracted from EVs were normalized and evaluated based on OR during treatment with ICIs (responder vs non-responder groups).

Ten EV-miRNAs showed a significant difference in expression between groups ($p < 0.05$) before FDR correction. After FDR correction, the only significant differentially expressed miRNA was miR-625-5p (FDR: 0.0366) (**Figure 1 Panel A**). Notably, miR-625-5p levels significantly correlated with tumor size change at best response (Spearman's $\rho = 0.35$, $p = 0.001$; Pearson's $r = 0.33$, $p = 0.002$) (**Figure 1 Panel B**).

Next, the optimal cutoff value of miR-625-5p to discriminate between groups was evaluated with sensitivity and specificity analyses. A cutoff of 5.47 was selected (**Supplementary Figure 1 Panel A**). Based on this threshold, 55 patients were classified as miR-625-5p High and 33 patients were classified as miR-625-5p Low. The miR-625-5p Low class showed a median tumor size reduction of 30% compared to a median tumor size increase of 18% in the miR-625-5p High class. This difference was highly significant ($p < 0.001$) (**Figure 2**).

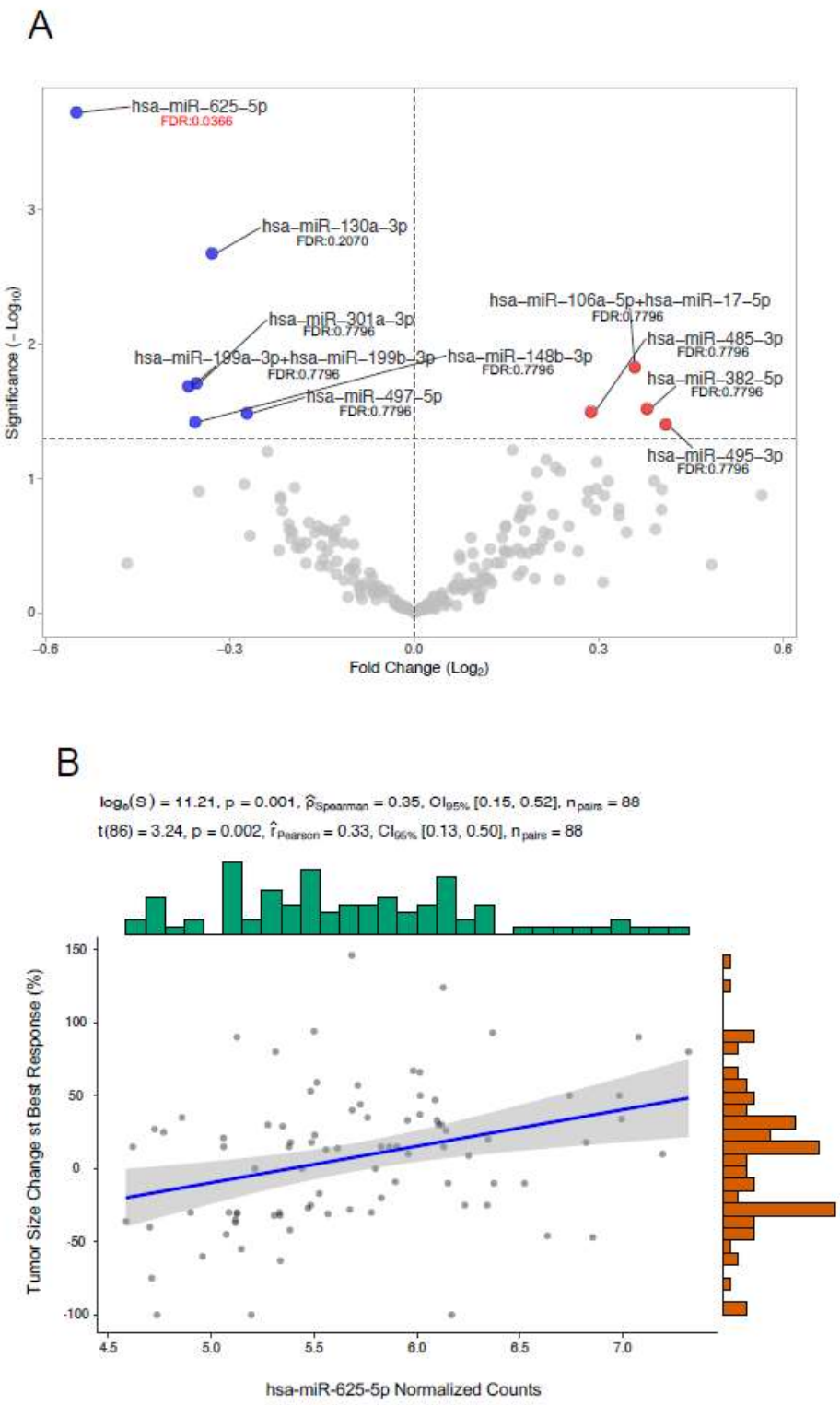


Figure 1 Volcano Plot for differential EV-miRNAs expression based on the Objective Response. miRNAs significantly overerexpressed are on the right (red dots), while miRNAs significantly downregulated are on the left (blue dots) (**Panel A**).Scatter Plot representing the correlation between miR-625-5p levels and tumor size change at best response (**Panel B**)

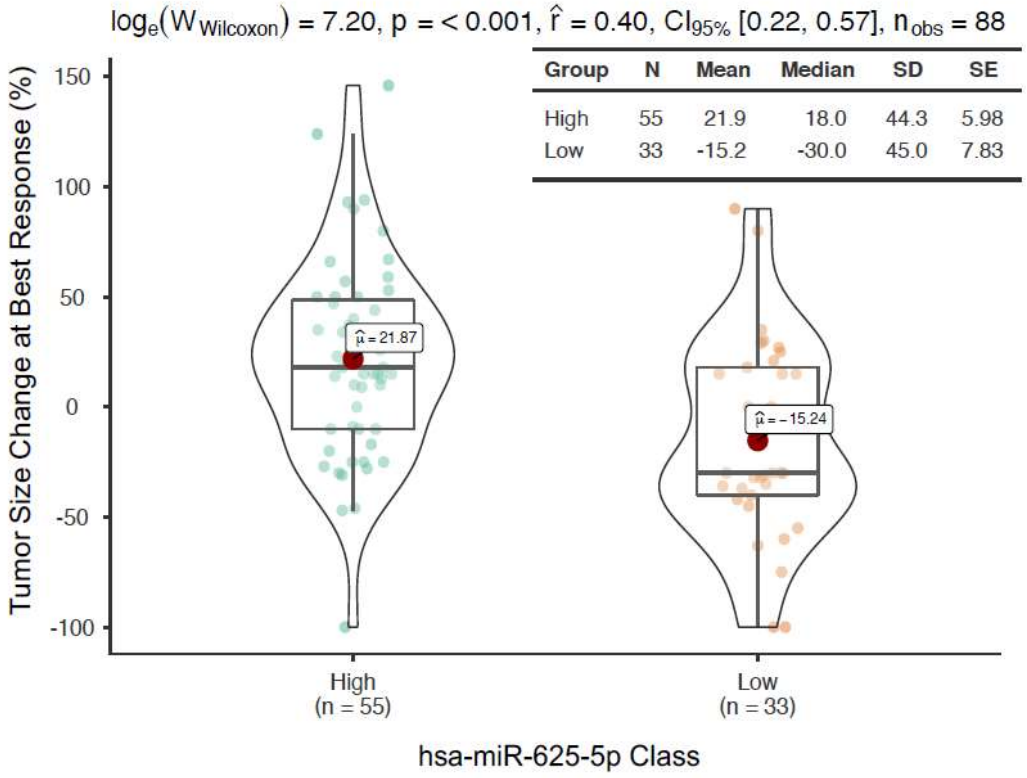


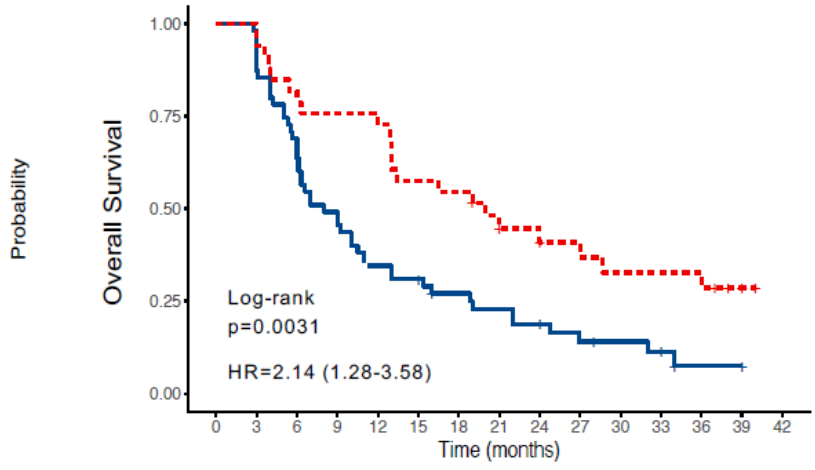
Figure 2 Box/Violino Plot representing tumor size change at best response in miR-625-5p High and Low class

MiR-625-5p class is associated to survival in NSCLC patients treated with ICIs

First, OS and PFS were evaluated in our cohort based on miR-625-5p classes. The median OS in the miR-625-5p Low and High class were respectively 20.0 months (95% CI 13.0–not reached) and 8.0 (95% CI 6.1–11.0) (HR 2.14, 95% CI 1.28–3.58, p = 0.0031) (**Figure 3 Panel A**). Similarly, the median PFS in the miR-625-5p Low and High class were respectively 13.2 months (95% CI 6.9–27.0) and 4.7 (95% CI 3.1–7.3) (HR 2.04, 95% CI 1.24–3.35, p = 0.0046) (**Figure 3 Panel B**).

Next, univariate analyses for OR, PFS and OS with all clinicopathological variables, described in Supplementary Table 1, were conducted. Briefly, the variables significantly associated to OR were setting of treatment (p < 0.001), type of treatment (p = 0.002), PD-L1 staining (p < 0.001) and miR-625-5p class (p < 0.001) (**Supplementary Table 2**). Those significantly associated to OS were setting of treatment (p = 0.025), type of treatment (p = 0.005), ECOG (p = 0.044), tumor burden (p = 0.005), presence of liver metastases (p < 0.001), PD-L1 staining (p < 0.001) and miR-625-5p class (p = 0.004) (**Supplementary Table 3**).

A

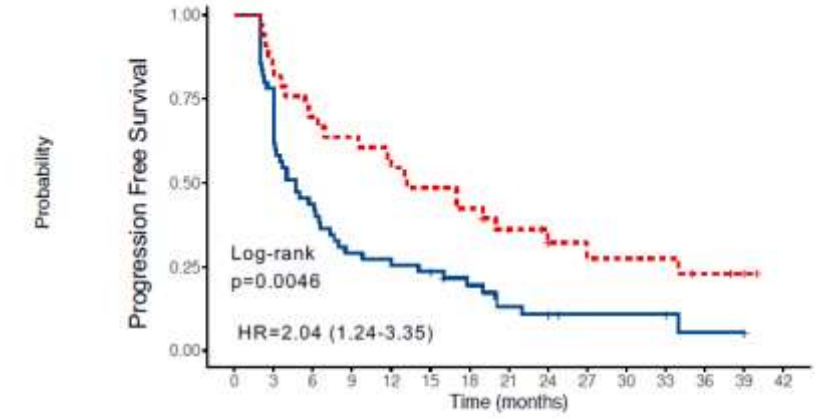


Number at risk

hsa.miR.625.5p.Class=High	55	54	38	27	19	17	13	11	9	6	5	4	1	1	0
hsa.miR.625.5p.Class=Low	33	33	27	25	25	19	18	14	12	10	8	8	8	4	0

						95% Confidence Interval	
Levels	Records	Events	rmean	se_rmean	Median	Lower	Upper
hsa.miR.625.5p.Class=High	55	48	13.1	1.55	8.00	6.10	11.0
hsa.miR.625.5p.Class=Low	33	22	21.9	2.44	20.00	13.00	NaN

B



Number at risk

hsa.miR.625.5p.Class=High	55	43	24	16	15	13	9	6	5	3	3	3	1	1	0
hsa.miR.625.5p.Class=Low	33	28	23	21	19	16	14	11	9	7	6	6	4	3	0

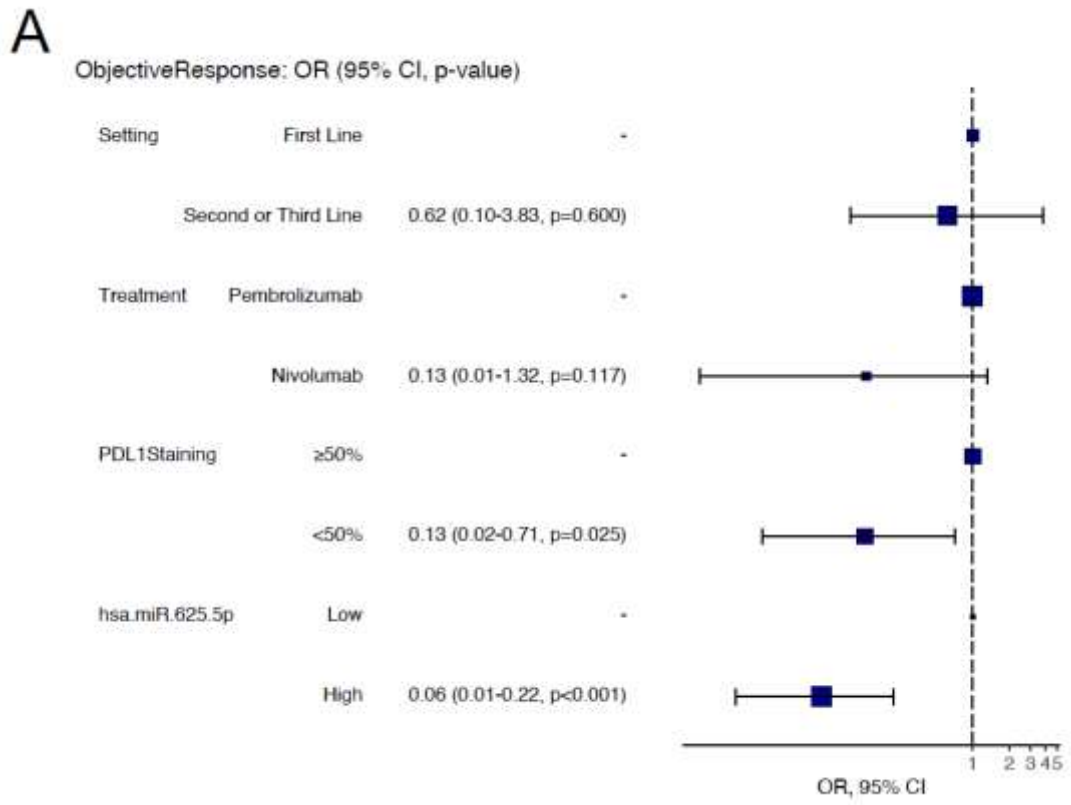
						95% Confidence Interval	
Levels	Records	Events	rmean	se_rmean	Median	Lower	Upper
hsa.miR.625.5p.Class=High	55	49	9.83	1.50	4.70	3.10	7.30
hsa.miR.625.5p.Class=Low	33	24	18.30	2.51	13.20	6.90	27.00

Fig. 3 Kaplan-Meier curves reporting the OS (**Panel A** and PFS (**Panel B)** of patients dichotomized in miR-625-5p High and Low class

Lastly, the variables significantly associated to PFS were setting of treatment (p = 0.016), type of treatment (p = 0.031), tumor burden (p = 0.003), presence of liver metastases (p = 0.002), PD-L1 staining (p < 0.001) and miR-625-5p class (p = 0.005) (**Supplementary Table 4**).

Finally, multivariate analyses with all the variables significant in the respective univariate models were conducted. For OR, the variables that retained their significance were PD-L1 staining (p = 0.025) and miR-625-5p class (p < 0.001) (**Figure 4 Panel A**).

For OS, the variables that retained their significance were tumor burden (p=0.002), PD-L1 staining (p = 0.028) and miR-625-5p class (p = 0.048) (**Figure 4 Panel B**). Variables that retained their significance in the multivariate PFS model were tumor burden (p=0.001), PD-L1 staining (p = 0.004) and miR-625-5p class (p = 0.039) (**Figure 4 Panel C**).



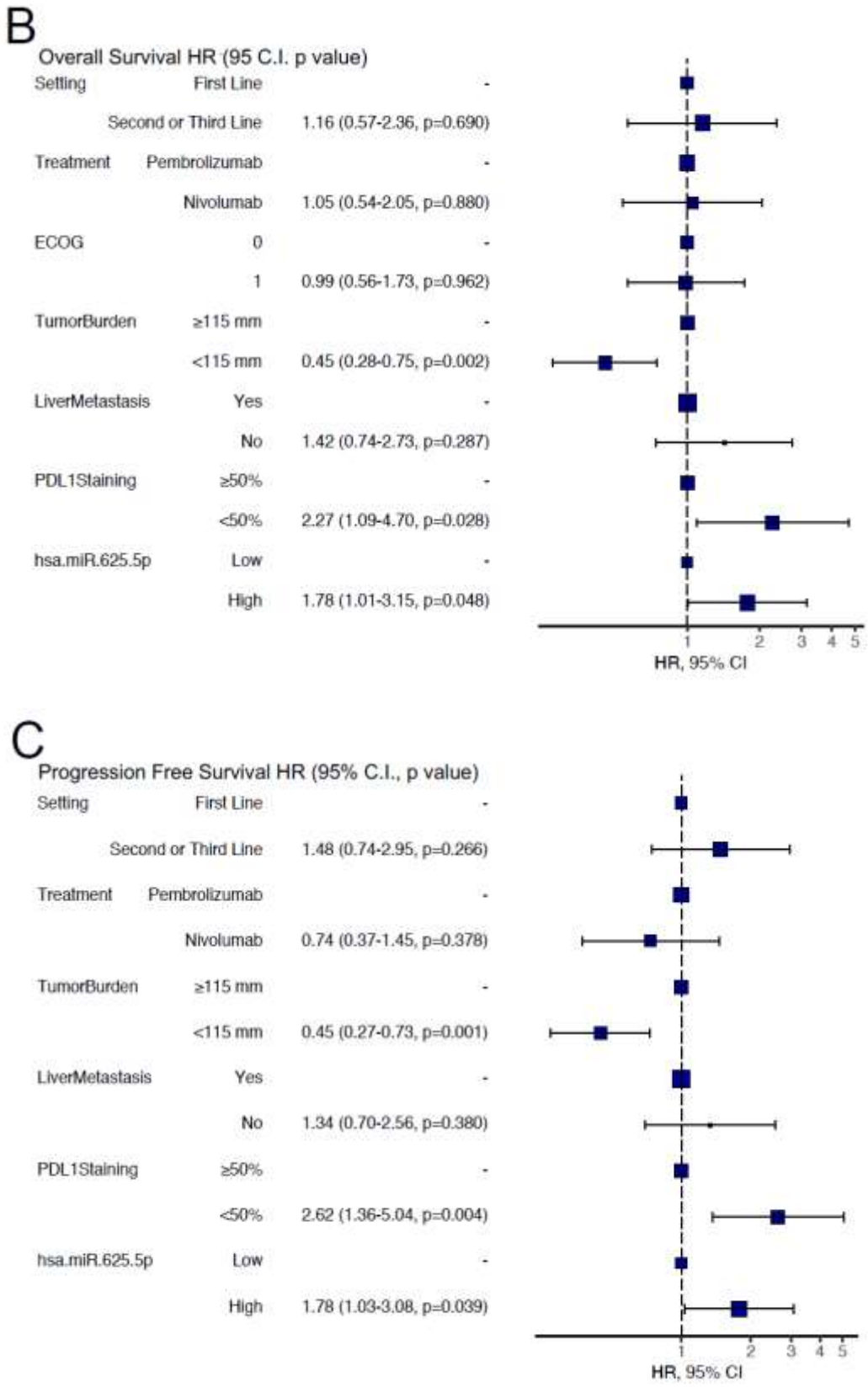


Figure 4 Forest Plot representing odd ratios and 95% C.I. for OR (**Panel A**) hazard ratio and 95% C.I. for OS (**Panel B**) and PFS (**Panel C**) in multivariate analysis

Prognostic classes based on miR-625-5p and PD-L1

In the multivariate analyses, PD-L1 staining and miR-625-5p class were independently associated to patient outcomes. In order to further refine the prognostic classification, four groups based derived by the expression of miR-625-5p and PD-L1 (i.e. miR-625-5p High / PD-L1 < 50%, n = 32; miR-625-5p Low / PD-L1 ≥ 50%, n = 20; miR-625-5p High / PD-L1 ≥ 50%, n = 24; miR-625-5p Low / PD-L1 < 50%, n = 13) were compared.

Considering tumor size change at best response, the miR-625-5p Low / PD-L1 ≥ 50% group showed a mean reduction in tumor size of 34.45% (median 33.5%), whereas the other three groups showed a mean increase in tumor size ranging from 8.62% (median 13.5%) to 32.13% (median 30%) ($p < 0.001$) (**Figure 5**). Pairwise comparisons reveal that miR-625-5p expression (high vs low) was significantly associated with tumor shrinkage in patients with PD-L1 ≥ 50%, but not in PD-L1 < 50% groups.

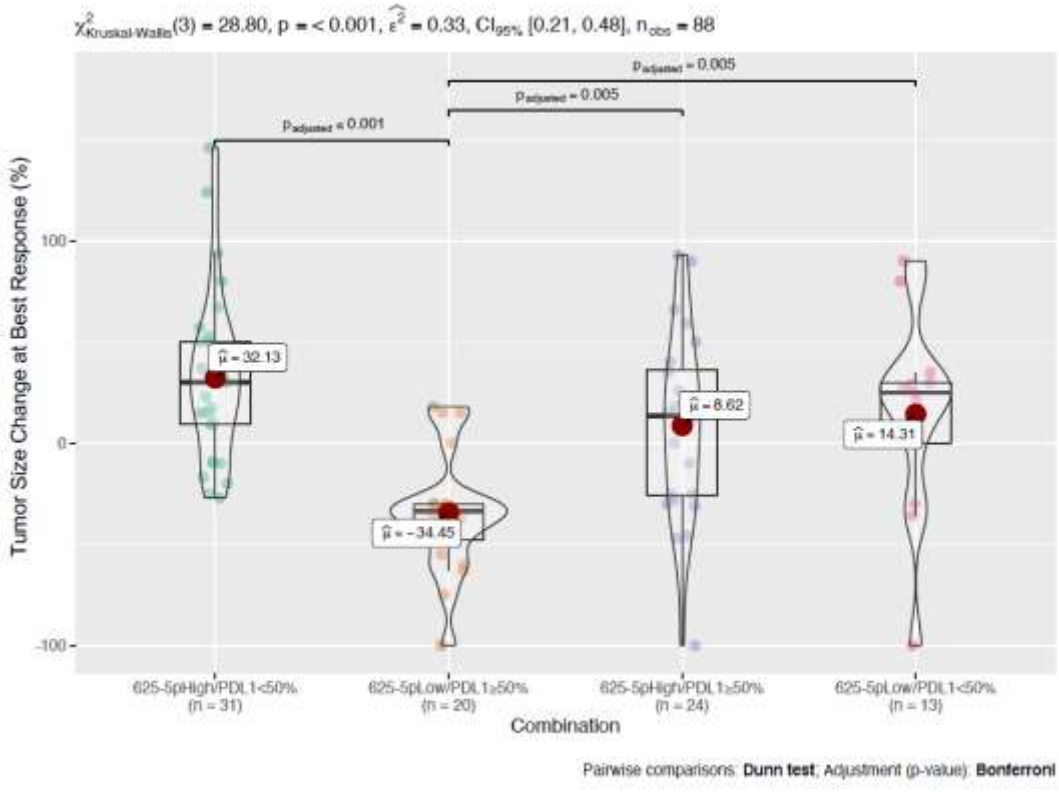


Fig. 5 Box/Violino Plot representing the tumor size change at best response in the four classes of patients: miR-625-5p High/ PDL1 < 50%, miR-625-5p Low / PDL1 ≥50%, miR-625-5p High/ PDL1 ≥50% and miR-625-5p Low/ PDL1 < 50%

In the OS analysis, the miR-625-5p Low / PD-L1 ≥ 50% group showed the longest median survival (27.0 months), the miR-625-5p High / PD-L1 ≥ 50% had an intermediate survival (10.75 months), whereas the groups with PD-L1< 50% had the shortest one (6.3 months) independently from miR-625-5p status. These differences were statistically significant (**Figure 6 Panel A**). Similarly, in the PFS analysis, the miR-625-5p Low / PD-L1 ≥ 50% group showed the longest median survival (24.0 months), the miR-625-5p High / PD-L1 ≥ 50% had an intermediate survival (7.45 months), whereas the groups with PD-L1 < 50% had the shortest one (< 4 months) both in High and Low miR-625-5p. Also, these differences were statistically significant (**Figure 6 Panel B**). These data suggested that miR-625-5p status could significantly influence both OS and PFS in patients with PD-L1 ≥ 50%.

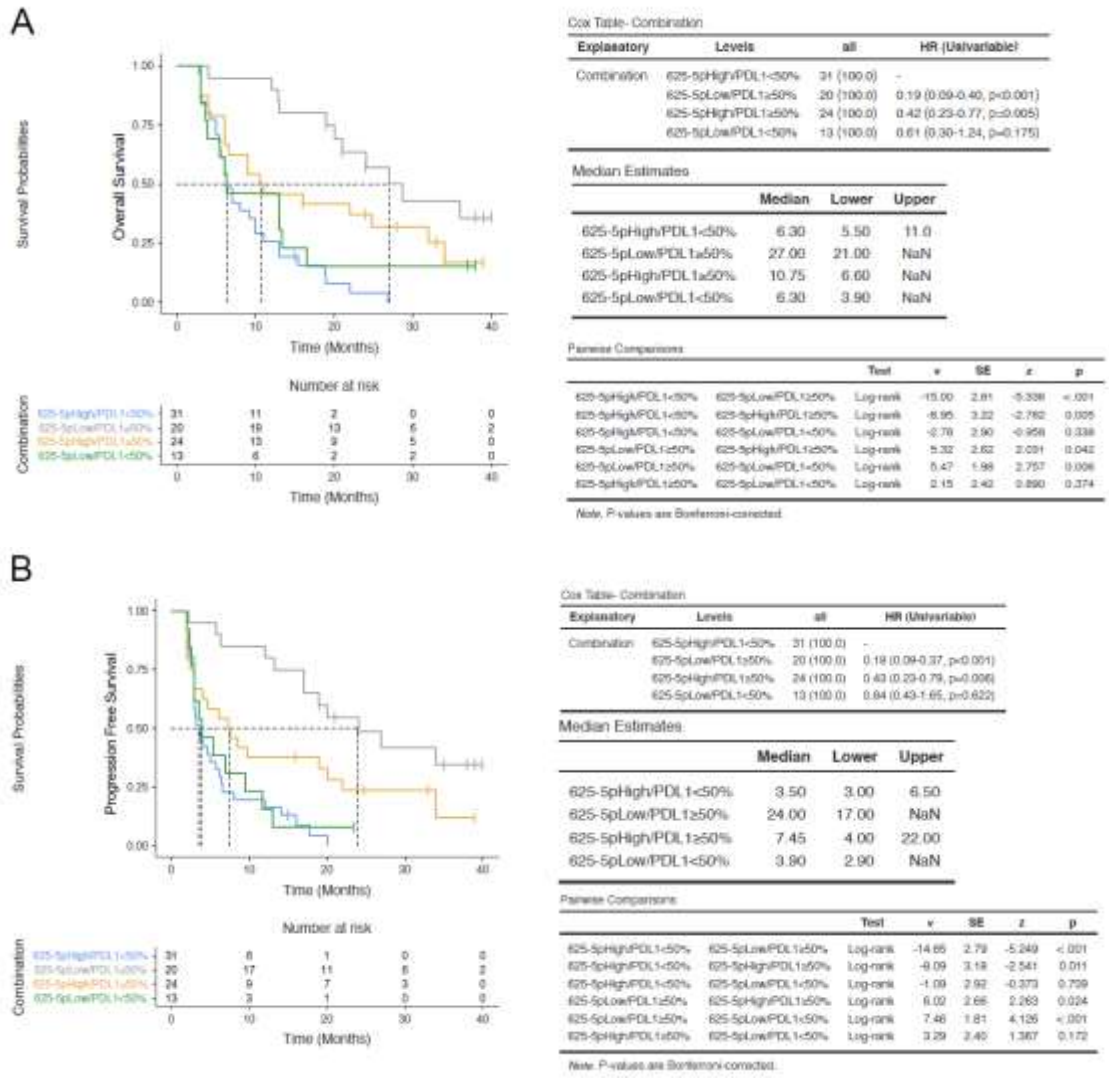


Fig. 6 Kaplan-Meier curves reporting the OS (**Panel A**) and PFS (**Panel B**) in the four classes of patients: miR-625-5p High/ PDL1 < 50%, miR-625-5p Low / PDL1 ≥50%, miR-625-5p High/ PDL1 ≥50% and miR-625-5p Low/ PDL1 < 50%

***In silico* evaluation of potentially relevant miR-625-5p targets**

In order to identify mRNA putative targets of miR-625-5p, three software were used (TargetScan 7.2, miRDB, DIANA-microT-CDS) [13-15]. TargetScan7.21, a sequence-based tool, retrieved 5107 predicted mRNA targets ranked according a specific score namely Cumulative weighted context++ score. DIANA-microT-CDS2, an energy-based tools, retrieved 830 predicted mRNA targets ranked according a specific score namely miTG score. miRDB3, a machine learning–based tool, retrieved 937 predicted mRNA targets ranked according a specific score namely Target score. More than 400 mRNAs were identified as potential targets by all software, as showed by the Venn diagram (**Supplementary Figure 5 Panel A)**. Targets mRNAs were ranked by each software and a cumulative ranking score was then generated using geometric mean. The top 50 genes are shown in (**Supplementary Figure 5 Panel B)**.

4. DISCUSSION

Accumulating evidences showed the clinical relevance of circulating free and EV-miRNAs as prognostic/predictive biomarkers of response to anti-cancer treatments in different types of solid tumors [16-22]. However, EV represents a better source of miRNAs for biomarker studies in terms of quantity, quality and stability of EV encapsulated miRNAs compared to circulating free miRNA [23-24]. Although exosomal miRNAs have been more investigated than MV-associated miRNAs, we decided to not exclude MVs from our analysis since it has been demonstrated their role in regulating inflammation processes and immune responses [25-26].

Our work identified pre-treatment levels of EVs miR-625-5p as a specific biomarker associated to OR, OS and PFS in ICI-treated NSCLC patients. Moreover, miR-625-5p refined the prognostic value of PD-L1 expression, identifying a subgroup of PD-L1 high patients with poorer outcomes. The objective and design of this study were specifically chosen to identify potential EV-miRNA candidates of both biological and clinical interest. As one of the aims was to explore novel potential mechanisms of resistance to ICIs in NSCLC patients, this study is exploratory in nature, and therefore does not include a confirmatory validation cohort [27].

Similar to the current study, Peng *et al.* also analyzed EV-miRNAs in NSCLC cancer patients treated with ICIs [28]. This study was limited by the number of patients used to identify candidate miRNAs (five patients with PR and four patients with PD), by the methodological approach to isolate exosomes (differential centrifugation) and by the fact that only tumor response was analyzed with no information on survival outcomes. Compared to this, our study used a larger sample size to identify candidate miRNAs, exosome isolation was performed with ExoRNeasy –which has been recently reported as the optimal method for exosome isolation from plasma and serum samples [29] and survival endpoints were also evaluated in multivariate analyses. Differently from other studies, miRNA detection was here performed with the nCounter platform. Compared to other platforms, nCounter is known to yield a smaller fraction of miRNAs detected above background [30]. This reduced sensitivity was likely at least in part responsible for the identification of few EVs miRNA candidates in this study. On the other hand, nCounter is provided with the higher specificity in term of miRNA cross-detection bias compared to sequencing and PCR based methods [30]. Moreover this platform has the advantage to eliminates potential bias associated with amplification, is widely available in clinical laboratories and has a short turnaround time, making it a platform with high potential clinical applicability.

Recently, signatures based on free circulating miRNAs have also been developed to stratify NSCLC patient treatment with ICIs. In particular, a 24-miR signature classifier originally developed to predict lung cancer development and prognosis was independently associated to OR, PFS and OS in NSCLC patients treated with ICIs [31]. Contrarily to this study, the one here presented used an unsupervised approach to identify the best candidate EV-miRNAs with a sample size specifically calculated to consider both the radiological and the survival endpoints. A different 7-miR signature generated following unsupervised microRNA profiling was recently associated to OS only in NSCLC patients treated exclusively with nivolumab [32]. The different patients' populations and in particular the different source of miRNA (serum vs exosomes) might explain why miR-625-5p was not identified in this study.

In fact, due to their structure, EV-miRNAs are more likely to be taken up by neighboring or distant cells, and they therefore are more likely to mechanistically modulate biological processes in target cells [12]. Data from The Cancer Genome

Atlas showed that miR-625-5p is significantly more expressed in NSCLC samples (both squamous cell carcinoma and adenocarcinoma) compared to normal lung tissues [33]. Moreover, miR-625-5p has been shown to suppress inflammatory responses in human bronchial epithelial cells [34]. Intriguingly, the top predicted target *in silico* of miR-625-5p is *GIMAP1* (GTPase of the immunity-associated protein 1), a known regulator of cellular and humoral immunity. GIMAP1 is intrinsically required to prevent mature T cells apoptosis in the periphery and it is critical for peripheral B cells survival [35,36]. Besides its independent prognostic role in ICI-treated NSCLC patients, the association of miR-625-5p levels also to OR suggest a potential role also as a predictive biomarker. However, this cannot be confirmed in absence of a group of patients not treated with ICIs. As the large majority of NSCLC patients were receiving ICIs either in first or subsequent lines at the time of accrual, renouncing to this control group allowed a larger sample size necessary for the current explorative analyses.

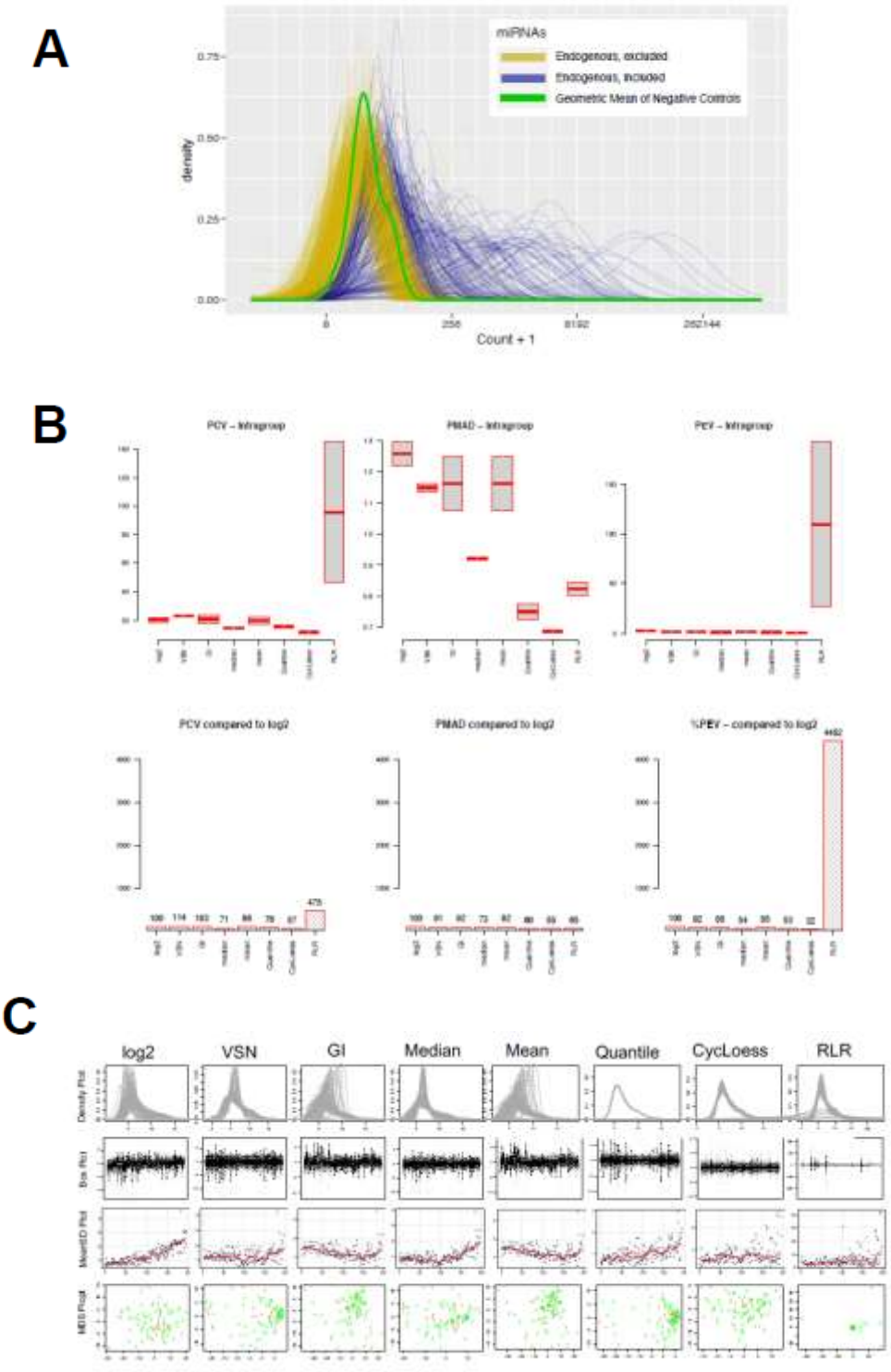
The population enrolled in this study included patients with PD-L1 expression < 50%, who would not necessarily receive ICI monotherapy at the present time, but rather combination regimens containing ICIs plus histology-selected platinum-doublet chemotherapies [2,37]. Although our findings do not directly translate to this subgroup of patients, ongoing studies are being conducted to assess the predictive and prognostic role of miR-625-5p also in this subgroup of patients. Notably, miR-625-5p identified patients with different outcomes in terms of OR and survival particularly in patients with PD-L1 expression ≥ 50% who are routinely offered ICI monotherapy.

Of the 218 patients originally assessed for eligibility, 35 were excluded because of inadequate plasma samples and 8 died before the first CT scan restaging. The results of this study are therefore at least in part limited by this detection and immortal time biases. Moreover, EV-miRNAs were only evaluated at the baseline and there is no longitudinal assessment of EV-miRNAs dynamics during treatment and upon progression. Finally, the limited EV characterization do not allow us to identify the major source of miR-625-5p (exosome vs microvescivescicles). This goes beyond our scope that was to capture all EV-associated miRNas excluding circulating free miRNA. For this purpose, we used a spin column-based method for the isolation of total RNA from EVs that showed a high specificity for vesicular over non-vesicular RNA [38].

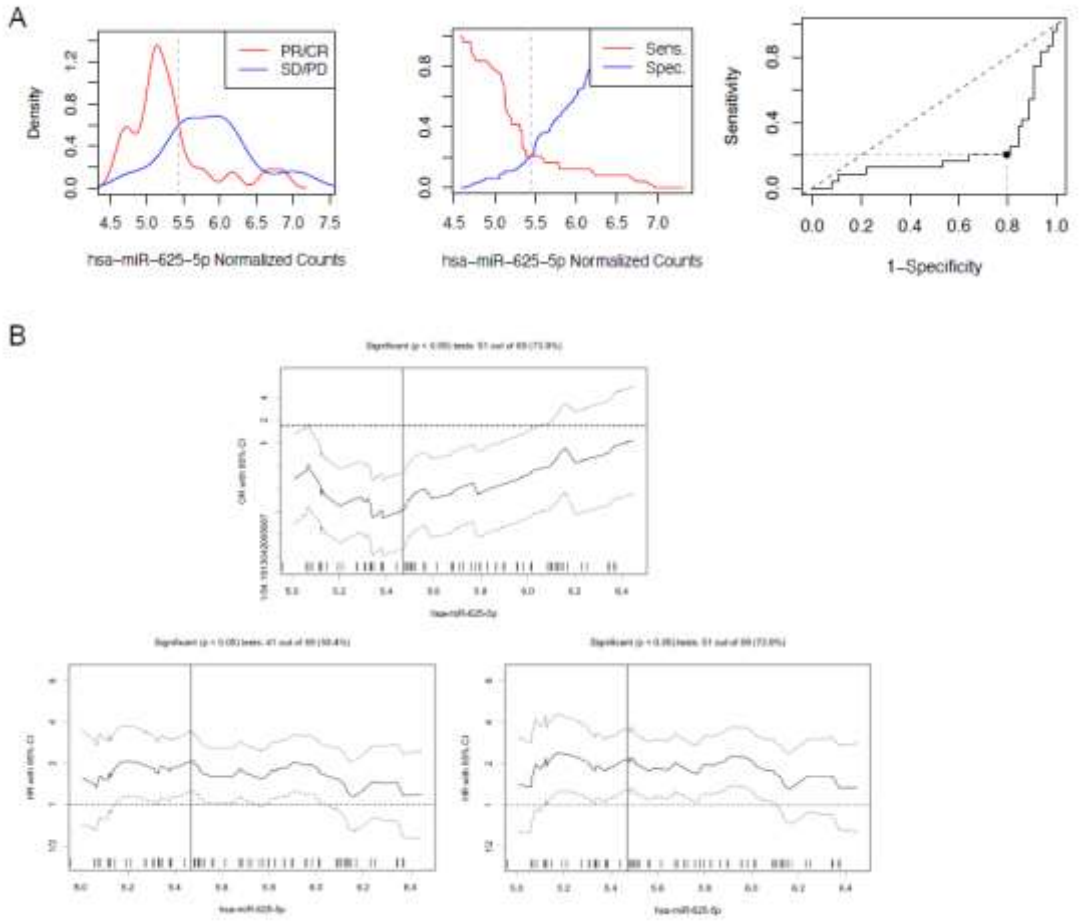
5. CONCLUSIONS

This study suggests that EVs-miR-625-5p might represent a novel biomarker to stratify patients affected by NSCLC treated with ICIs, in particular those with PD-L1 expression ≥ 50%. The association with OR and the biological association of miR-625-5p to immune-related processes also suggest a potential predictive role related to modulation of tumor-associated immunity. Confirmatory results in prospective validation studies involving PDL1 ≥ 50% NSCLC patients treated with ICIs as first line of treatment could help translate this biomarker in clinical practice.

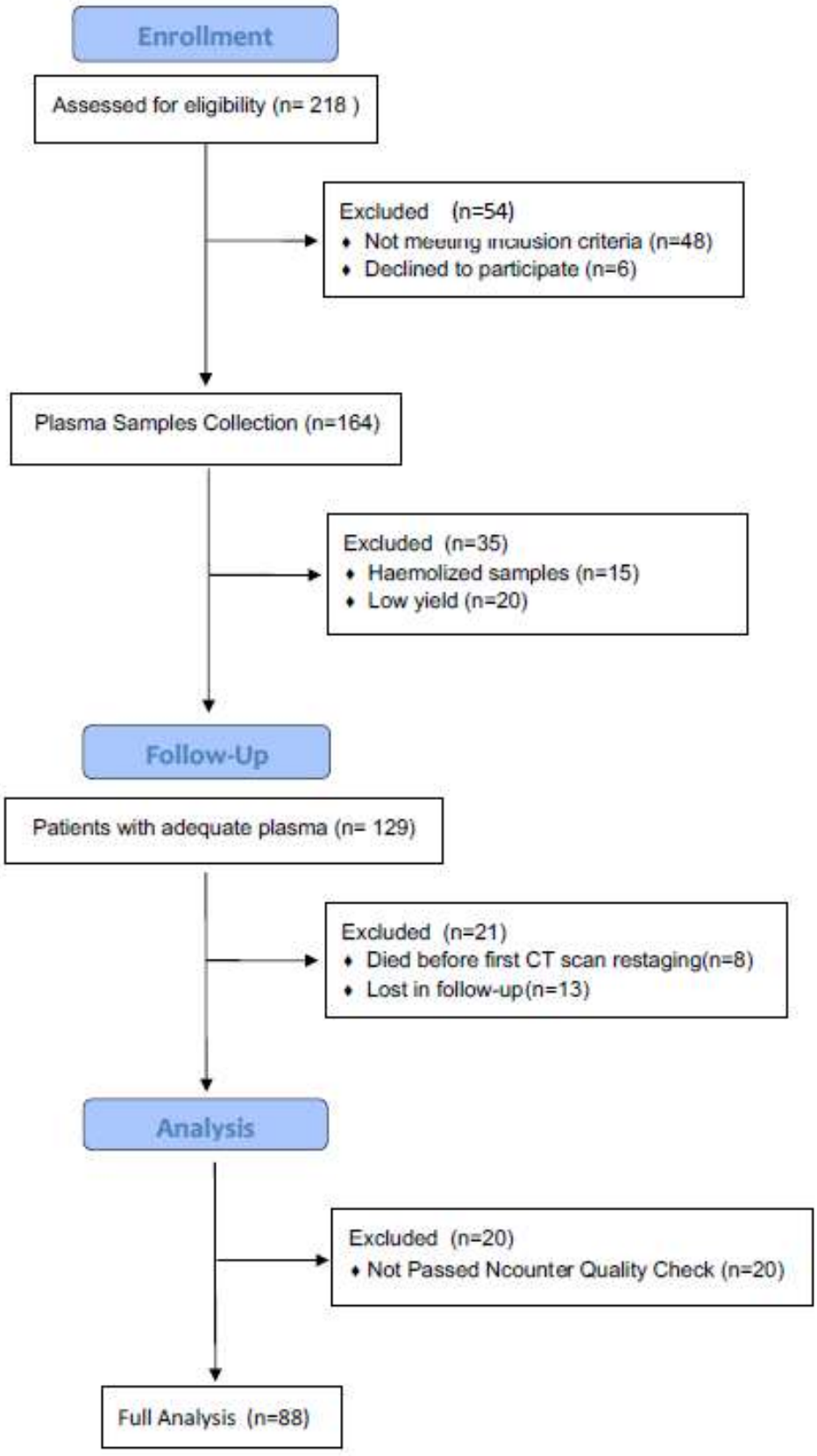
SUPPLEMENTARY FIGURES



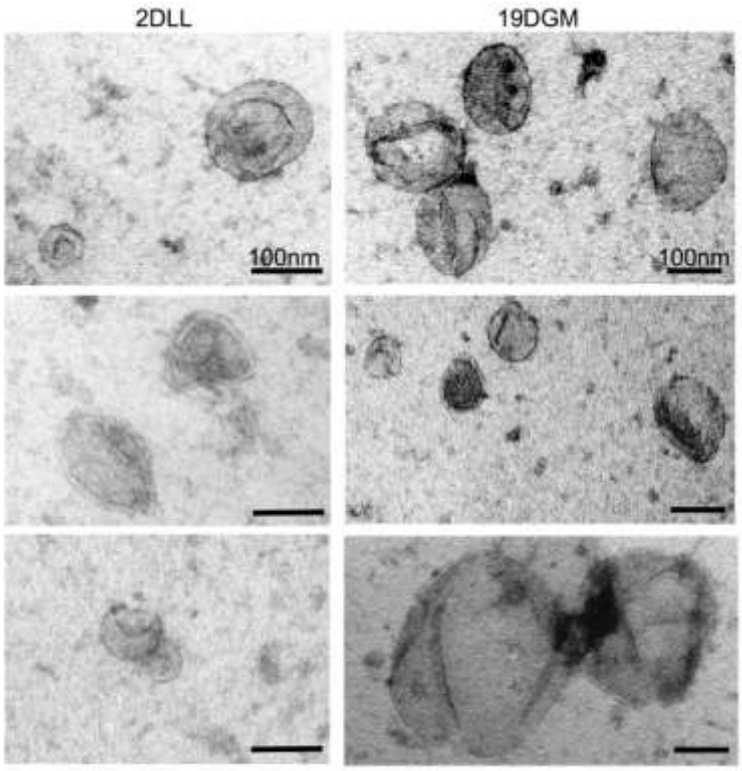
Supplementary Fig. 1 miRNAs with mean ranks significantly higher (blue) compared to the mean ranks of negative controls (green) **(Panel A)**. Pooled intragroup coefficient of variation (PCV), pooled intragroup median absolute deviation (PMAD) and pooled intragroup estimate of variance (PEV) calculated for different normalization methods **(Panel B)**. Density Plot, RLE Box Plot, Mean SD plots, MDS Plots of different normalization methods. Green (Not Responder); Red (Responder) **(Panel C)**



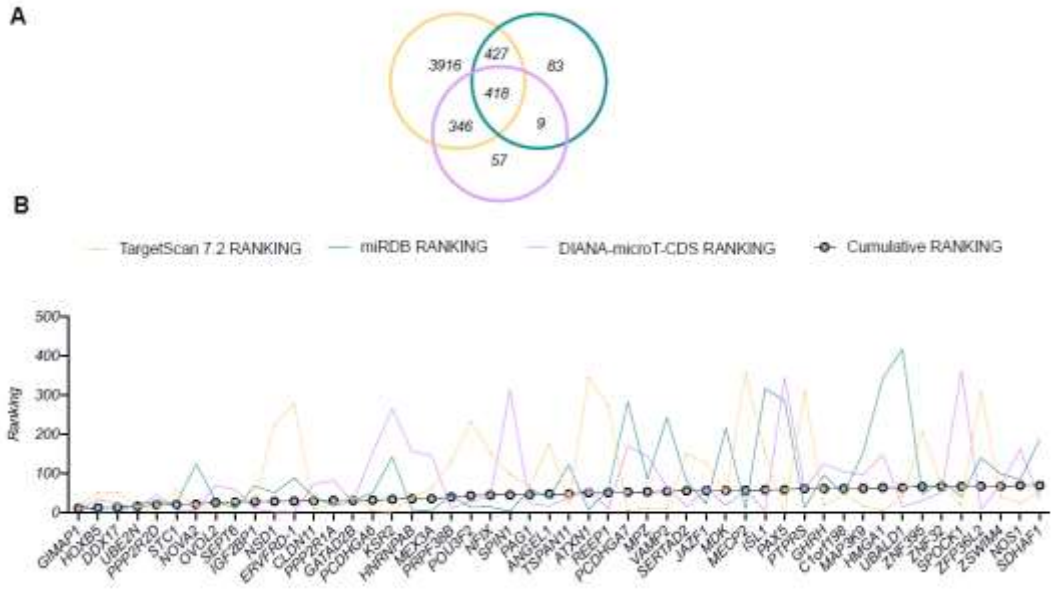
Supplementary Fig. 2 Density Plot for optimal cutoff value of miR-625-5p in PR/CR (Responder) and SD/PD (Not Responder) (*left plot*); sensitivity and specificity analysis for optimal cutoff value (*middle plot*); Optimal cutoff point plotted on ROC Curve (*right curve*) **(Panel A).** Cutoff exploration by correlation with odd ratio (Responders vs Not Responder), hazard ratio for OS (below left) and PFS below right). The odd ratio (OR) and hazard ratio (HR) including 95% CI are plotted in dependence of the cutoff. A vertical line designates the selected cutoff value of miR-625-5p **(Panel B)**



Supplementary Fig. 3 Flow chart of patient inclusion and exclusion



Supplementary Fig. 4 Representative images of EV morphology and size by electron microscopy



Supplementary Fig. 5 Venn Diagram representing the potential targets of miR-625-5p identified by all software **(Panel A)**. Plot representing ranking of mRNAs targets of each software and cumulative ranking score **(Panel B)**

SUPPLEMENTARY TABLES

	Overall (N=88)
Sex	
Female	27 (30.7%)
Male	61 (69.3%)
Age	
<70yy	46 (52.3%)
≥70yy	42 (47.7%)
NeverSmokers	
Yes	6 (6.8%)
No	82 (93.2%)
ECOG	
0	40 (45.5%)
1	48 (54.5%)
Treatment	
Nivolumab	35 (39.8%)
Pembrolizumab	53 (60.2%)
Setting	
Second or Third Line	51 (58.0%)
First Line	37 (42.0%)
Histology	
Adenocarcinoma	63 (71.6%)
Squamous Cell Carcinoma	25 (28.4%)
EGFR	
Mutated	4 (4.5%)
Wild Type	84 (95.5%)
PDL1Staining	
<50%	44 (50.0%)
≥50%	44 (50.0%)
PrimaryTumor	
Not Resected	63 (71.6%)
Resected	25 (28.4%)
TumorBurder	
≥115 mm	45 (51.1%)
<115 mm	43 (48.9%)
BrainMetastasis	
Yes	19 (21.6%)
No	69 (78.4%)
LiverMetastasis	
Yes	17 (19.3%)
No	71 (80.7%)
LungMetastasis	
Yes	54 (61.4%)
No	34 (38.6%)
BoneMetastasis	
Yes	52 (59.1%)
No	36 (40.9%)
PleuralEffusion	
Yes	17 (19.3%)
No	71 (80.7%)
AdrenalMetastasis	
Yes	72 (81.8%)
No	16 (18.2%)
SoftTissueMetastasis	
Yes	6 (6.8%)
No	82 (93.2%)
NodelMetastasis	
Yes	69 (78.4%)
No	19 (21.6%)
OtherSiteMetastasis	
Yes	11 (12.5%)
No	77 (87.5%)

Supplementary Table 1 Descriptive statistics of clinic-pathological variables of study population

Dependent : Objective Response		Not Responder	Responder	OR (univariable)
Setting	First Line	19 (51.4%)	18 (48.6%)	-
	Second or Third Line	45 (88.2%)	6 (11.8%)	0.14 (0.04-0.39, p= < 0.001)
Treatment	Pembrolizumab	30 (56.6%)	23 (43.4%)	-
	Nivolumab	34 (97.1%)	1 (2.9%)	0.04 (0.00-0.20, p= 0.002)
PDL1 Staning	≥50%	23 (52.3%)	21 (47.7%)	-
	<50%	41 (93.2%)	3 (6.8%)	0.08 (0.02-0.26, p < 0.001)
hsa.miR.625.5p	Low	14 (42.4%)	19 (57.6%)	-
	High	50 (90.9%)	5 (9.1%)	0.07 (0.02-0.22, p < 0.001)

Supplementary Table 2 Table representing clinicopathological variables significantly associated with OR

Dependent : Overall Survival		All	HR (univariable)
Setting	First Line	37 (42.0%)	-
	Second or Third Line	51 (58.0%)	1.75 (1.07-2.85, p= 0.025)
Treatment	Pembrolizumab	53 (60.2%)	-
	Nivolumab	35 (39.8%)	1.97 (1.22-3.16, p= 0.005)
ECOG	0	40 (45.5%)	-
	1	48 (54.5%)	1.65 (1.01-2.69, p= 0.044)
Tumor Burden	≥ 115 mm	46 (52.3%)	-
	< 115 mm	42 (47.7%)	0.51 (0.31-0.82, p= 0.005)
Liver Metastases	Yes	71 (80.7%)	-
	No	17 (19.3%)	2.69 (1.50-4.80, p= 0.001)
PDL1 Staning	≥50%	44 (50.0%)	-
	<50%	44 (50.0%)	2.87 (1.75-4.72, p < 0.001)
hsa.miR.625.5p	Low	33 (37.5%)	-
	High	55 (62.5%)	2.14 (1.28-3.58, p= 0.004)

Supplementary Table 3 Table representing clinicopathological variables significantly associated with OS

Dependent : Progression Free Survival		All	HR (univariable)
Setting	First Line	37 (42.0%)	-
	Second or Third Line	51 (58.0%)	1.80 (1.11-2.91, p= 0.016)
Treatment	Pembrolizumab	53 (60.2%)	-
	Nivolumab	35 (39.8%)	1.68 (1.05-2.69, p= 0.031)
Tumor Burden	≥ 115 mm	46 (52.3%)	-
	< 115 mm	42 (47.7%)	0.49 (0.31-0.78, p= 0.003)
Liver Metastases	Yes	71 (80.7%)	-
	No	17 (19.3%)	2.45 (1.38-4.37, p= 0.002)
PDL1 Staning	≥50%	44 (50.0%)	-
	<50%	44 (50.0%)	3.29 (1.96-5.50, p < 0.001)
hsa.miR.625.5p	Low	33 (37.5%)	-
	High	55 (62.5%)	2.04 (1.24-3.35, p= 0.005)

Supplementary Table 4 Table representing clinicopathological variables significantly associated with PFS

List of abbreviations

CIs: Confidence Intervals

CR: Complete Response

EVs: Extracellular Vesicles

EV-miRNAs: EV-associated miRNAs

FDR: False Discovery Rate

GI: Global Intensity

GIMAP1: GTPase of the immunity-associated protein 1

HR: Hazard Ratio

ICIs: Immune Check Point Inhibitors

Mean: Mean Intensity

Median: Median Intensity

MVs: Microvesicles

NSCLC: Non-Small Cell Lung Cancer

OR: Objective Response

OS: Overall Survival

PCV: Coefficient of Variation

PD: Disease progression

PDL-1: Programmed Cell Death Ligand-1

PEV: Pooled intragroup Estimate of Variance

PFS: Progression Free Survival

PMAD: Pooled intragroup Median Absolute Deviation

PR: Partial Response

QC: Quality Check

R: Responders

RCC: Reporter Code Count

RLR: Robust Linear Regression

SD: Stable Disease

TMB: Tumor Mutation Burden

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CHAPERT 2. THE STUDY

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