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Exosomal PD-L1 expression and dynamic changes of immune response associated cytokines as predictive biomarkers for immune checkpoint blockage with PD-1/PD-L1 inhibitors in advanced/metastatic non-small cell lung cancer

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INTRODUCTION

During the last two decades the therapeutic landscape of advanced non-small cell lung cancer (NSCLC) has profoundly changed due to an improved knowledge of the molecular mechanisms underlying lung carcinogenesis and the subsequent development of novel effective systemic therapies. This led to a dramatic therapeutic shift, moving from the old concept "*one size fits all*" to a histology-based approach and finally to the advent of personalized medicine. Recently, the development of immune checkpoint inhibitors (ICIs) further revolutionized the therapeutic algorithm of advanced NSCLC with unprecedented results in terms of overall survival in multiple clinical settings. Four different ICIs have been approved to date for the treatment of advanced/metastatic NSCLC either as monotherapy in previously treated patients (nivolumab, pembrolizumab, and atezolizumab) or in chemotherapy-naïve patients with strong PD-L1 (programed death ligand 1) expression (tumor proportion score ≥50%) (pembrolizumab) or as consolidation therapy in inoperable locally advanced NSCLC following chemo-radiotherapy (durvalumab). Furthermore, different chemo-immunotherapy combinations have proved significant survival benefit in chemo-naïve NSCLC, regardless of PD-L1 status, and their approval/reimbursement in Italy is eagerly awaited.

Despite these positive results, to date, the only reliable predictive biomarker in clinical practice is the immunohistochemical (IHC) expression of PD-L1. Unfortunately, this is an imperfect biomarker and responses to ICIs can be across all PD-L1 IHC levels, although a tumor proportion score (TPS) ≥50% seems to enrich patients with higher probability of response. Furthermore, despite a more favorable safety profile compared with chemotherapy, some patients can experience treatment-related adverse events that are intrinsically connected to the peculiar mechanism of action of these agents and are therefore known as immune-related adverse events (irAEs). In the vast majority of the cases, irAEs are of low/mild intensity, especially using ICIs targeting the PD-1/PD-L1 axis, but occasionally can be severe or even life threatening. Moreover, another not entirely negligible aspect is the increasing financial burden associated with these novel effective drugs.

The identification of new predictive biomarkers is a largely unmet medical need and it is a highly active research field in lung cancer. Recently, liquid biopsy has emerged as a novel powerful tool for molecular profiling of NSCLC and cell free DNA (cfDNA) genotyping has already entered clinical practice for biomarker selection and identification of resistance

mutations in oncogene-addicted patients. This minimally invasive diagnostic assay holds promise as a viable alternative to tissue biopsy and has been evaluated for multiple potential clinical uses, including early cancer detection, biomarker identification, patient selection for treatment, and drug resistance monitoring. Besides cfDNA, the liquid biopsy family comprises many other components that can be evaluated in body fluids, as circulating tumor cells (CTCs), exosomes, platelets, microRNA and long non-coding RNA.

The aim of the research project was to evaluate the potential role of minimally invasive biomarkes, such as cytokines and exosomal PD-L1 expression in patients with advanced/metastatic NSCLC treated with ICIs targeting the PD-1/PD-L1 axis. In addition we evaluated the role of some systemic markers of inflammation, such as the neutrophil-to-lymphocyte ratio (NLR) that we and other groups demonstrated to be a strong independent prognostic factor. In the following chapter we will provide a comprehensive overview of the rapidly evolving therapeutic scenario of advanced NSCLC, focusing on the immunotherapy revolution. Then we will evaluate the new therapeutic and diagnostic challenges posed by this new class of anticancer agents, such as the concomitant use of medication that can affect the efficacy and safety of ICIs as well as the treatment of special populations, including HIV-infected patients, and finally the potential impact of liquid biopsy and systemic markers of inflammation. In the last chapter we will provide the preliminary data of a retrospective study evaluating the role of exosomal PD-L1 expression and the dynamic changes of some cytokines associated with anticancer immune response and PD-L1 expression, such as interferon-γ (IFN-γ), interleukin-6 (IL-6) and transforming growth factor β (TGF-β) in predicting the outcome of ICI-treated advanced/metastatic NSCLC patients from two different cohorts of patients. In addition, we will evaluate also the role of some known prognostic markers, such as NLR levels, and the potential existence of racial differences in the immune response to ICIs between Caucasian (Italian cohort) and Latin American (Colombian cohort) patients. The project is still ongoing and will further explore the potential of exosomes in the prediction of ICB efficacy through the evaluation of additional components of these tumor vesicles, such exosomal microRNAs (exo miRNAs) and proteomics.

CHAPTER 1

The evolution of the therapeutic landscape of advanced non-small cell lung cancer in the immunotherapy era

Extracted from:

"**The changing scenario of 1st line therapy in non-oncogene addicted NSCLCs in the era of immunotherapy**"
Alessandro Russo, Tindara Franchina, Giuseppina Ricciardi, Giuseppe Toscano, Silvia Schifano, Giuseppe Lo Certo, Alessandra Battaglia, Elvira Pantò, Mariangela Scaffidi Fonti, Vincenzo Adamo.

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"**Immunotherapy in Lung Cancer: From a minor God to the Olympus**"
Alessandro Russo, Michael G. McCusker, Katherine A. Scilla, Katherine E. Arensmeyer, Ranee Mehra, Vincenzo Adamo, Christian Rolfo.

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1.1 Introduction

Since early 90s platinum-based chemotherapy represented the mainstay of treatment for advanced/metastatic non-small cell lung cancer (NSCLC) [1, 2]. However, during the last two decades the therapeutic algorithm of metastatic NSCLC has profoundly changed moving from the old "*one size fits all*" concept to a "*histology-based*" approach and then, for a small subgroup of patients (i.e. EGFR mutated and ALK-rearranged NSCLCs), to a "*molecularly-selected"* one [3]. The advent of novel therapeutic approaches that target or manipulate the immune system (immunotherapy) [4] has further revolutionized the therapeutic armamentarium of NSCLC with unprecedented results [5] (**Fig. 1**).

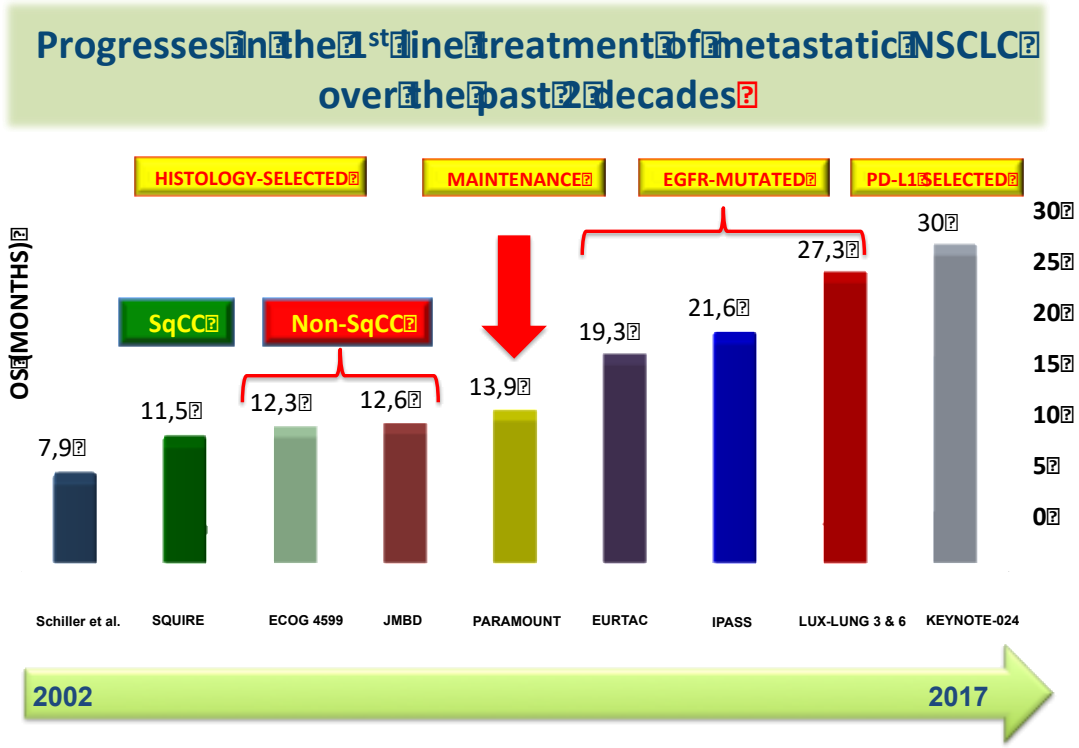

Figure 1 Evolution of the therapeutic strategy of first-line NSCLC and OS gain over the last two decades in major phase III clinical trials (*From Russo A, et al. Crit Rev Oncol Hematol 2018*) [5].

The cancer-immunity cycle refers to a complex series of interaction between the immune system and cancer cells though a delicate balance between the recognition of self and prevention of autoimmunity [6]. Immunotherapy has been extensively studied in oncology and traditionally this strategy has not been effective in lung tumors that were therefore considered relatively immune-resistant. Recently, a renewed interest on this therapeutic approach emerged with the identification of immune checkpoints that exert inhibitory actions in the cancer-immunity cycle [6]. Among these, recently, two immune checkpoints

have emerged as promising therapeutic targets, CTLA-4 (cytotoxic T-lymphocyte antigen-4) and PD-1 (programed death 1) (**Fig. 2**).

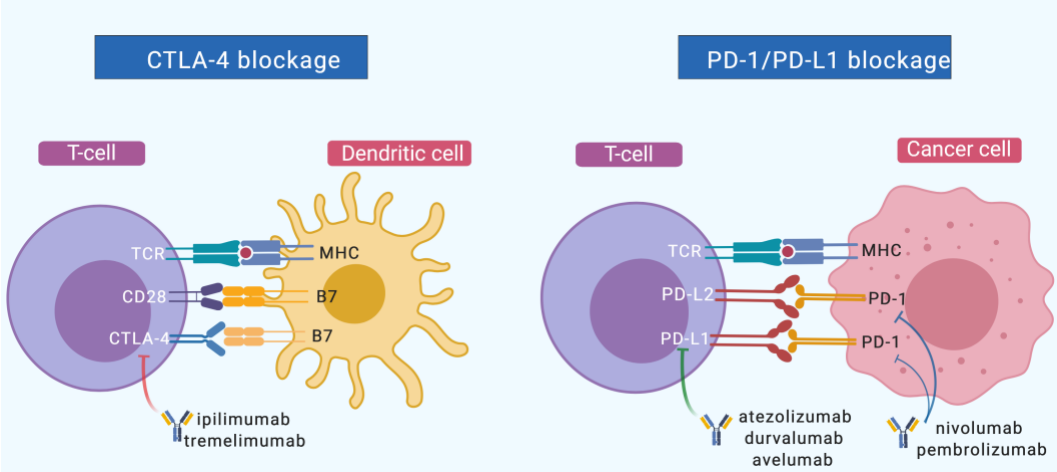

Figure 2. Mechanism of action of CTLA-4 and PD-1/PD-L1 inhibitors (*Credit: created with BioRender)* (*From Russo A, et al. Adv Exp Med Biol. 2019*) [7]

The discovery of these proteins and the potential use of their blocking as an anticancer treatment earned the 2018 Nobel prize in Physiology or Medicine to James P. Allison and Tasuku Honjo. CTLA4 is expressed exclusively on T cells and inhibits the development of an active immune response, by counteracting the activity of the T cell co-stimulatory receptor CD28 through competing for the binding of the same ligands (CD80 also known as B7.1 and CD86 also known as B7.2) [6, 8]. In contrast to CTLA-4 that is involved in early steps of the cancer-immunity cycle (priming and activation), PD-1 and its ligands have a crucial role in last steps (cancer cells killing). Physiologically PD-1/PD-L1 limit the activity of T cells in peripheral tissues limiting autoimmunity [6, 8]. PD-1 is expressed on activated T cells and inhibits T cell responses by interfering with T cell receptor (TCR) signaling. PD-1 has two ligands, PD-L1 (B7-H1) that is expressed in antigen presenting cells (APCs), macrophages, fibroblasts, and T cells and PD-L2 (B7-DC) that is predominantly expressed on APCs. PD-L1 is also overexpressed in several solid tumors, while PD-L2 is expressed relatively rarely [9, 10]. The role of CTLA-4 and PD-1/PD-L1 in immune suppression and their expression in solid tumors provided the rationale for their therapeutic exploitation. Given their non-overlapping activities, simultaneous targeting of both pathways has also been evaluated to restore antitumor immunity [11].

Since the first demonstration of activity of PD(L)-1 agents in lung cancer in early clinical trials in 2012 [12, 13], immune checkpoint blockade (ICB) has emerged as a novel effective

therapeutic strategy in different clinical settings, determining a dramatic shift in the therapeutic landscape of both NSCLC and SCLC (**Fig. 3**). To date, the only widely applicable predictive biomarker for efficacy to these agents in clinical practice is PD-L1 IHC expression [14]. However, PD-L1 is an imperfect biomarker and its use is hampered by several biological and technical limitations. Novel prognostic and predictive biomarkers to these agents are eagerly awaited and data on the most promising candidates will be discussed in the subsequent sections. Herein we summarize the major breakthroughs in the immunotherapy journey in lung cancer and how it is changing our clinical practice.

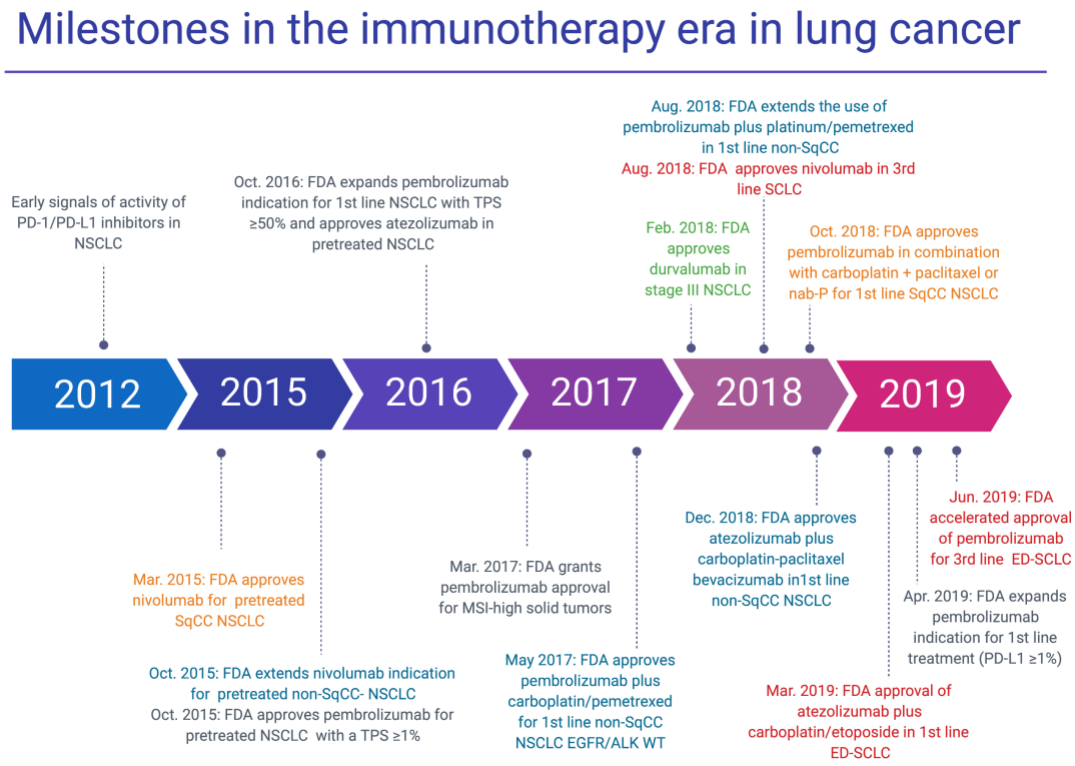

Figure 3. Timeline of major breakthroughs in the immunotherapy era in lung cancer
Legend: In orange and in blue FDA approvals in squamous and non-squamous in metastatic NSCLC, respectively; in black data and FDA approvals in metastatic NSCLC independently of histology; in green FDA approval in locally-advanced NSCLC; in red FDA approvals in extensive disease SCLC (*Credit: created with BioRender)* (*From Russo A, et al. Adv Exp Med Biol. 2019*) [7].

1.2 ICB in early stage and locally advanced NSCLC

In contrast with advanced disease, medical treatment of early stage and locally advanced (LA) NSCLC has little changed over the last two decades and platinum-based chemotherapy remained the cornerstone of treatment either as adjuvant/neo-adjuvant therapy or in association with radiotherapy in inoperable patients. Its use in this setting is

supported by the results of several meta-analyses of randomized phase III trials conducted in 1990s and early 2000s that reported an absolute survival benefit at 5 years of 5% from adjuvant/neo-adjuvant approaches in stage IB-IIIA NSCLC compared with surgery alone [15, 16] and 4.5% with concurrent versus sequential chemo-radiotherapy in inoperable stage III NSCLC [17]. However, major breakthroughs in molecular biology translated little in early stage NSCLC and no targeted therapies have been approved to date in both early stage and locally advanced NSCLC. ALCHEMIST (Adjuvant Lung Cancer Enrichment Marker Identification and Sequencing Trials) is a clinical trial platform to facilitate identification, enrollment and treatment of genotype-selected patients with resected non-squamous NSCLC in trials of genotype-directed adjuvant therapy. It comprises a group of randomized clinical trials that are testing whether adding a targeted therapy based on tumor molecular profile in addition to and after the patient completes the usual standard of care treatment [18]. The results of these studies will provide definitive evidence on the role of targeted therapies in the adjuvant setting in molecularly selected patients.

Recently, ICB has emerged as a new effective and relatively safe therapeutic modality in advanced NSCLC [5], prompting the evaluation of ICIs targeting the PD-1/PD-L1 axis in earlier lines of treatment, leading to the approval of durvalumab as the first in class PD-L1 inhibitor approved as maintenance therapy after concurrent chemo-radiotherapy [19]. The role of ICIs as neo-adjuvant therapy (NAT) has been evaluated in small non-randomized studies with promising results. Single agent PD-1/PD-L1 inhibitors for 2-3 courses followed by surgery have resulted in a major pathological response rate (MPR) (10% or less residual viable tumor after neoadjuvant chemotherapy) of 17-45% in stage I-IIIA NSCLC [20-22]. In contrast, combinations with platinum-based doublets have been associated with higher ORR (70-73%) and MPR (64-80%) [23, 24], suggesting that this might represent a more effective NAT strategy and compare favorably with historical controls reporting a MPR of 19-27% [25, 26] and an ORR of approximately 35-50% with platinum-based chemotherapy alone [27, 28]. Several randomized phase III trials (CheckMate 816, KEYNOTE-617, IMpower030, and AEGEAN) are investigating the role of different chemo-immunotherapy combinations for 3-4 courses as NAT compared with chemotherapy alone. The results of these trials will provide definitive conclusions on the potential role of ICIs in this setting. In order to further improve the activity of ICIs, other studies are evaluating alternative approaches such as the concurrent use with radiotherapy (NCT03237377).

The role of ICIs in the adjuvant setting is unclear and no data have been reported to date. Multiple phase III clinical trials (NCT02273375, PEARLS, ANVIL, IMpower010, and

ALCHEMIST) are ongoing.

The role of ICIs in inoperable stage III NSCLC is firmly established with the recent approval of durvalumab as maintenance therapy in non-progressing patients after concomitant chemo-radiotherapy. Multiple lines of evidence support the combination of radiotherapy and immunotherapy either concomitantly or immediately after radiation therapy in order to augment the antitumor responses typically observed with either modality alone. This allows exploiting the synergistic effect observed with both modalities through multiple mechanisms [29, 30]. After a decade of failures with different alternative strategies to concurrent chemoradiation with platinum-based chemotherapy [31-33], the PACIFIC trial changed our standard of care, adding durvalumab in the therapeutic armamentarium of inoperable LA-NSCLC [19, 34]. This randomized phase III trial evaluated durvalumab at the dosage of 10 mg/m$_2$ i.v. every 2 weeks versus placebo (2:1 randomization) as consolidative therapy in patients with inoperable stage III NSCLC who did not have disease progression after two or more cycles of platinum-based concurrent chemo-radiotherapy [34]. The trial met its two co-primary endpoints, demonstrating a statistically significant improvement in both PFS (17.2 vs. 5.6 months; HR 0.51) and OS (not reached vs. 28.7 months; HR 0.68; p=0.0025) compared with placebo. Furthermore, consolidation therapy with durvalumab was associated with higher ORR (28.4% vs. 16.0%; p<0.001) and longer time to death or distant metastasis (28.3 vs. 16.2 months; HR 0.53) [19, 34]. These data are noteworthy, since consolidation strategies with chemotherapy had not produced any survival benefit after concomitant chemo-radiotherapy [35]. Furthermore, treatment with durvalumab was well tolerated with an incidence of grade 3/4 adverse events (AEs) of 30.5% in the durvalumab group vs. 26.1% in the placebo group. An unplanned post-hoc analysis requested by a health authority evaluated the role of pre-treatment PD-L1 status (unknown in 37% of patients) and showed no benefit in terms of OS in patients with negative PD-L1 expression (tumor proportion score <1%, assessed with the Ventana SP263 immunohistochemical assay) (HR 1.36) [19]. Based on this analysis, EMA restricted durvalumab use in PD-L1 ≥1% patients only. However, this data should be considered only exploratory and no firm conclusions can be made due to the sample size (only 60 patients).

Several phase II/III studies are evaluating the role of nivolumab and pembrolizumab as consolidative therapy after chemo-radiotherapy, including the Italian MP-LALC study. PACIFIC evaluated durvalumab after concomitant chemo-radiotherapy. However, sequential chemoradiation still represents a valid alternative in patients who are not candidate for concurrent treatment because of clinical conditions or medical

contraindications [36]. The role of consolidative immunotherapy in this setting is not known and no experiences have been reported to date. The phase II study PACIFIC-6 will address this specific issue. In addition, the expanded access program of durvalumab allowed the enrollment of patients treated with sequential chemo-radiotherapy. Efficacy data in this subgroup of patients will provide further evidence on the role of consolidative durvalumab therapy.

Finally, several studies (PACIFIC-2, RATIONALE001, NICOLAS, DETERRED, and KEYNOTE 799) are evaluating an alternative strategy with the addition of PD-1/PD-L1 inhibition during concurrent chemo-radiotherapy followed by consolidation with immunotherapy alone. An early interim safety analysis of the phase II NICOLAS showed that the addition of nivolumab to concurrent chemo-radiotherapy is safe and tolerable [37]. Whether this strategy will increase the benefit of ICB in this setting is unclear and the results of these studies are eagerly awaited.

1.3 ICB in chemotherapy-naïve metastatic NSCLC

The development of PD-1/PD-L1 inhibitors and the unprecedented results reported in pretreated NSCLC [38-41] prompted the evaluation of these novel therapeutic agents in 1st line either alone or in combination with platinum-based chemotherapy [5]. The positive results of the KEYNOTE-024, demonstrating the superiority of pembrolizumab compared with platinum-based chemotherapy in strong PD-L1 positive patients (TPS ≥50%) EGFR/ALK wild type [42, 43], represented a major improvement in non-oncogene addicted NSCLCs, which were minimally influenced by major therapeutic innovations in the last two decades [5]. The trial reported an impressive median OS of 30 months with a statistically significant advantage over chemotherapy despite extensive crossover (64.2%) [43] and represented a major shift in the therapeutic landscape of NSCLC, adding a new molecularly defined subgroup of patients with improved outcome after a chemotherapy-free regimen. Subsequent studies tried to extend the benefit of ICB to a higher patient population with different therapeutic strategies, including patients with low PD-L1 expression (TPS ≥1%), chemo-immunotherapy combinations and dual blockade with anti-CTLA4 agents. The results of these trials are summarized in **Tab. 1** and expanded the use of ICIs in chemotherapy-naïve patients.

The KEYNOTE-042 trial aimed to evaluate the role of pembrolizumab in patients with weak and strong PD-L1 expression (TPS ≥1%) compared with platinum-based chemotherapy. The trial met its primary endpoints, reporting a statistically significant

advantage in terms of OS in patients with different TPS threshold: ≥50% (HR 0.69; p=0.0003), ≥20% (HR 0.77; p=0.0020), and ≥1% (HR 0.81; p=0.0018) [44]. However, an exploratory analysis limited to the subgroup of patients with a TPS 1-49% showed no differences in OS (HR 0.92), suggesting that strong PD-L1 expressors mostly drove the benefit observed in the study population. Furthermore, crossover to pembrolizumab was not allowed in the chemotherapy arm and as consequence most of the patients did not received the current standard of care in second line. Surprisingly, based on these results, the US FDA extended the approval of pembrolizumab in chemotherapy-naïve EGFR/ALK wild type NSCLC patients with a TPS ≥1%. The relatively favorable safety profile and activity seen in this trial make the regimen particularly useful in patients who are not candidates or refuse platinum-based chemotherapy, albeit patients enrolled in this study were all in good clinical condition (ECOG PS 0-1), eligible for a platinum-based doublet chemotherapy and median age was 63 years. This issue should be addressed in a randomized clinical trial. In contrast the CheckMate-026, evaluating nivolumab in a similar patient population, failed to meet its primary endpoint showing no statistically significant difference in the ITT population (PD-L1 ≥5%) compared with platinum-based chemotherapy in terms of PFS (HR 1.15; p=0.25). Furthermore, nivolumab was not associated with any differences in terms of OS (HR 1.02) and ORR compared with platinum-based chemotherapy (26% vs. 33%, odds ratio 0.70) [45]. Moreover, an exploratory subgroup analysis involving patients with a PD-L1 expression level ≥50% showed no differences between the two treatment arms in both PFS (HR 1.07) and OS (HR 0.90) [45]. Differences in the study design and population included might have contributed to the differences seen with trials evaluating pembrolizumab monotherapy. Similarly, durvalumab monotherapy failed to prolong both PFS (HR 0.87; p=0.324) and OS (HR 0.76; p=0.036) in the ITT population (PD-L1 ≥25% with SP263 IHC assay) compared with chemotherapy in the phase III MYSTIC trial (arm A vs. B) [46]. However, subgroup analyses of both studies evaluated the predictive role of tumor mutation burden (TMB) with ICIs. In the CheckMate-026 trial TMB was evaluated in the tissue using a whole exome sequencing (WES) assay, dividing patients in three tertiles (<100, 100-242, or ≥243 total missense mutations) [45].

Table 1. Phase III studies with PD-1/PD-L1 inhibitors in 1st line NSCLC (*Adapted from Russo A, et al. Adv Exp Med Biol. 2019*) [7].

Study	n	Arms	Population	PD-L1	Crossover rate	ORR	PFS (mos)	PFS (HR)	OS (mos)	OS (HR)
KEYNOTE-024	154 vs. 151	pembrolizumab vs. platinum-CHT	NSCLC EGFR/ALK WT	≥50%	64.2%	44.8% vs. 27.8%	10.3 vs. 6.0	0.50	30.0 vs. 14.2	0.63
KEYNOTE-042	637 vs. 637	pembrolizumab vs. CP or carbo-pem	NSCLC EGFR/ALK WT	≥1%	N.A.	27.2% vs. 26.5%	5.4 vs. 6.6	1.05	16.4 vs. 12.1	0.82)
CheckMate 026	271 vs. 270	nivolumab vs. platinum-CHT	NSCLC EGFR/ALK WT	≥1%	60%	26% vs. 33%	4.2† vs. 5.9†	1.15†	14.4† vs. 13.2†	1.02†
KEYNOTE 407	278 vs. 281	CP or carbo/nab-P + pembro vs. CP or carbo/nab-P	SqCC	All comers	31.7%	57.9% vs. 38.4%	6.4 vs. 4.8	0.56	15.9 vs. 11.3	0.64
KEYNOTE 189	410 vs. 206	cis/carbo-pem + Pembro vs. cis/carbo-pem	Non-SqCC EGFR/ALK WT	All comers	53.9%	48.0% vs. 19.4%	9.0 vs. 4.9	0.48	22.0 vs. 10.7	0.56
Impower 150 (ARM B vs. C)	400 vs. 400	ABCP vs. BCP	Non-SqCC (EGFR/ALK allowed)	All comers	N.A.	63.5%* vs. 48%*	8.3* vs. 6.8*	0.59**	19.2* vs. 14.7*	0.78*
Impower 150 (ARM A vs. C)	402 vs. 400	ACP vs. BCP	Non-SqCC (EGFR/ALK allowed)	All comers	N.A.	40.6% vs. 40.2%	N.A.	0.91	19.4* vs. 14.7*	0.88*
IMpower 130	451 vs. 228	atezo + carbo/nab-P vs. carbo/nab-P	Non-SqCC (EGFR/ALK allowed)	All comers	19.3%	49.2%* vs. 31.9%*	7.0* vs. 5.5*	0.64*	18.6* vs. 13.9*	0.79*
IMpower 131 (ARM B vs. C)	343 vs. 340	atezo + carbo/nab-P vs. carbo/nab-P	SqCC	All comers	43%	49% vs. 41%	6.5 vs. 5.6	0.74	14.6 vs. 14.3	0.92
IMpower 132	292 vs. 286	atezo + cis/carbo + pem vs. cis/carbo+ pem	Non-SqCC EGFR/ALK WT	All comers	37.1%	47% vs. 32%	7.6 vs. 5.2	0.60	18.1 vs. 13.6	0.81
CheckMate 227 (TMB ≥10)	139 vs. 160	nivolumab-ipilumumab vs. platinum-CHT	NSCLC EGFR/ALK WT	All comers	N.A.	45.3% vs. 26.9%	7.2 vs. 5.5	0.58	23.03 vs. 16.72	0.77
MYSTIC (ARM A vs. C)	374 vs. 372	durvalumab vs. platinum-CHT	NSCLC EGFR/ALK WT	All comers	39.5%	35.6%** vs. 37.7%**	4.7** vs. 5.4**	0.87**	16.3** vs. 12.9**	0.76**
MYSTIC (ARM B vs. C)	372 vs. 372	durvalumab-tremelimumab vs. platinum-CHT	NSCLC EGFR/ALK WT	All comers	39.5%	34.4%** vs. 37.7%**	3.9** vs. 5.4**	1.05**	11.9** vs. 12.9**	0.85**

**EGFR/ALK WT intention-to-treat (ITT) population; **ITT population (PD-L1 ≥25%); †ITT population (PD-L1 ≥5%);*
N.A. Not Available. ABCP, atezolizumab/bevacizumab/carboplatin/paclitaxel; ACP, atezolizumab/carboplatin/paclitaxel; BCP, bevacizumab/carboplatin/paclitaxel.

Nivolumab in TMB high (≥243 total missense mutations) patients was associated with improved ORR (47% vs. 28%) and PFS (HR 0.62), but not OS (HR 1.10), likely due to extensive crossover in the control arm (68%). Interestingly, there was no association between TMB and PD-L1 expression, albeit patients with both PD-L1 ≥50% and high TMB seemed to derive the greatest benefit [45]. However, the use of WES in clinical practice is hampered by several technical and economic issues. Therefore smaller targeted-gene NGS panels have been used to evaluate this potential biomarker with comparable results [47], albeit it is still unclear the role of the mutational study of different genes on TMB calculation with these panels [48]. In the MYSTIC trial a TMB analysis was conducted in both tissue (Foundation One CDx 325-gene panel) (41% of the ITT population) and plasma (GuardantOMNI 500-gene panel). High tissue TMB (≥10 mutations/Mb) predicted a better OS with durvalumab (HR 0.70) and correlated well with plasma results (Spearman's rho = 0.6; Pearson's r = 0.7). Blood TMB ≥20 mutations/Mb was associated with improved OS (HR 0.72) and PFS (HR 0.77) with durvalumab [49]. As reported previously [45], TMB and PD-L1 were independent predictive factors, suggesting that these biomarkers can be used as complementary tools when selecting patients for immunotherapy treatment. However, standardization of methods used and robust analytical/clinical validation are needed before extensive clinical implementation of this biomarker is implemented [48].

ICB has been proved to be associated with durable responses in fraction of patients with NSCLC, including treatment-naïve patients. However, combinatorial approaches may allow to achieve higher response rates and deeper tumor responses, expanding the role of ICIs in the upfront setting, where platinum-doublet chemotherapy achieves ORRs of ~30-40% with a median PFS of ~4-6 months [50-52]. Several lines of evidence support the combination of immunotherapy with chemotherapy [53-57], also combined with antiangiogenetic agents [58]. Several studies have evaluated the safety and efficacy of multiple chemo-immunotherapy regimens. Most of these trials excluded EGFR-mutated and ALK rearranged NSCLCs, due to the lower activity seen in previous studies in pretreated patients with PD(L)-1 inhibitors in these molecular subgroups [38-41] and included PD-L1 all comers patients.

KEYNOTE-021 was a multicohort phase 1/2 study evaluating different chemotherapy regimens in addition to pembrolizumab. One of most promising chemotherapy combination was pembrolizumab plus carboplatin-pemetrexed that was further evaluated in the phase II part of the study in a randomized cohort (cohort G). Preliminary efficacy data showed a significant increase in both ORR (55% vs. 29%, p=0.0016) and PFS (13.0 vs. 8.9 months, HR 0.53), but there were no differences in OS (HR 0.90, at a median follow-

up of 10.6 months), likely to the extensive use of PD-1/PD-L1 inhibitors as salvage therapy in the chemotherapy arm (74%) [59]. Based on these preliminary results, FDA granted accelerated approval to this regimen. Final results of the study (median follow-up of 23.9 months) further confirmed the advantage in terms of ORR (56.7% vs. 30.2%, p=0.0016) and PFS (24.0 vs. 9.3 months; HR 0.53; p=0.0049), but also a statistically significant advantage in terms of OS (median OS not reached in the chemo-immunotherapy arm vs. 21.1 months; HR 0.56, p=0.0151), despite an extensive crossover (73.3%), with a relatively favorable safety profile (AEs G3-5 41% vs. 27%) [60]. The subsequent phase III randomized trial KEYNOTE-189 evaluated pembrolizumab in association with platinum-pemetrexed chemotherapy in non-squamous NSCLC EGFR/ALK wild type, PD-L1 all comers. At the first interim analysis (median follow-up 10.5 months) the trial met both the two co-primary endpoints, showing an advantage in OS (N.R. vs. 11.3 months, HR 0.49; p<0.001) and PFS (8.8 vs. 4.9 months, HR 0.52, p<0.001), regardless of PD-L1 expression. The combination increase also the ORR (47.6% vs. 18.9%, p<0.001) with higher response rates among PD-L1 strongly positive patients (61.4% vs. 22.9%) [61]. The updated survival data of the trial at a median follow-up of 18.7 months continued to show a statistically significant advantage in both OS (22.0 vs. 10.7 months; HR 0.56; p<0.00001) and PFS (9.0 vs. 4.9 months; HR 0.48; p<0.00001) across all PD-L1 TPS groups. Furthermore, a significant prolongation of PFS2 was observed (17.0 vs. 9.0 months; HR 0.49; p<0.00001) [62], suggesting that the combinatorial approach is superior to the sequential use of chemotherapy and ICB (crossover rate 53.9%). Whether this combination is better than pembrolizumab alone in PD-L1 strong positive patients is still unknown and the results of ongoing phase III trial EA5163/S1709 INSIGNA will provide definitive conclusions on the best therapeutic approach in this subgroup of patients. This combination gained FDA approval in August 2018, but it is not yet reimbursed in Italy.

Three randomized phase III trials evaluated atezolizumab in non-squamous NSCLC in association with different platinum-based chemotherapy regimens (**Tab. 2**). IMpower 150 evaluated atezolizumab in association with carboplatin-paclitaxel (ACP – arm A) versus atezolizumab plus bevacizumab/carboplatin/paclitaxel (ABCP – arm B) vs. bevacizumab/carboplatin/paclitaxel (BCP – arm C) in non-squamous NSCLCs. The trial also enrolled EGFR mutated and ALK rearranged tumors TKI-pretreated, although the ITT population included only EGFR/ALK wild type patients. The two primary end points of the study were PFS in the ITT population and in Teff-high WT population (high expression of an effector T-cell gene signature in the tumor), and overall survival in the WT population. ABCP was associated with longer PFS than BCP in the entire study population (8.3 vs. 6.8

months; HR 0.62; $p<0.001$), in the ITT population (WT) (8.3 vs. 6.8 months; HR 0.61; $p<0.001$), and in the Teff-high WT population (11.3 vs. 6.8 months; HR 0.51, $p<0.001$) [63]. At first interim analysis (median follow-up ~20 months), OS was significantly longer in the WT population with ABCP than with BCP (19.2 vs. 14.7 months; HR 0.78, $p=0.02$) [63]. Interestingly, improved OS with ABCP vs. BCP was observed in the small subgroup of patients with sensitizing EGFR mutations (Not estimable vs. 17.5 months; HR 0.31) and in those with baseline liver metastases (13.3 vs. 9.4 months; HR 0.52). The benefit was independent of PD-L1 expression. A synergistic effect between bevacizumab and atezolizumab can be hypothesized, since no OS benefit was observed with the addition of atezolizumab to carboplatin/paclitaxel in both EGFR-positive patients (21.4 vs. 18.7 months; HR 0.93) and in patients with liver metastases (8.9 vs. 9.4 months; HR 0.87) [64]. These data suggest that ABCP can be a novel treatment option in first line non-squamous NSCLC. The use in EGFR-mutated patients progressing after an EGFR TKI is promising, but these data should be confirmed prospectively in a larger cohort of patients. In December 2018, the FDA granted approval for ABCP combination as 1st line therapy in EGFR/ALK wild type NSCLC patients.

IMpower130 studied the addition of atezolizumab to carboplatin/nab-paclitaxel (nab-P) in chemotherapy-naïve non-squamous NSCLC patients. The trial met its co-primary endpoints, showing a statistically significant improvement in both OS (18.6 vs. 13.9 months; HR 0.79; $p=0{\cdot}033$) and PFS (7.0 vs. 5.5 months; HR 0.64; $p<0{\cdot}0001$) in the ITT WT population. The benefit was observed across all PD-L1 subgroups, but no benefit was observed in the EGFR/ALK positive cohort (HR 0.98 for OS and 0.75 for PFS) [65].

KEYNOTE-407 and IMpower131 evaluated the addition of a PD(L)-1 agent to platinum-based chemotherapy in patients with squamous cell carcinoma of the lung. The addition of pembrolizumab to carboplatin/nab-P or paclitaxel was associated with a statistically significant improvement of both PFS (6.4 vs. 4.8 months; HR 0.56; $p<0.001$) and OS (15.9 vs. 11.3 months; HR 0.64; $p<0.001$), primary endpoints of the study, independent of PD-L1 status and taxane used [66]. Based on these results, in October 2018 FDA extended 1st line pembrolizumab approval in combination with carboplatin/nab-paclitaxel or paclitaxel in chemotherapy-naïve NSCLC with squamous histology. This represented a major improvement in the upfront treatment of squamous NSCLC that had little changed in the last two decades with marginal incremental benefits with the addition of anti-EGFR monoclonal antibodies [67] or the use of novel chemotherapy agents [52].

Table 2 Phase III studies with chemo-immunotherapy combinations in 1st line PD-L1 all comers NSCLC. Legend: *WT population; IO, immuno-oncology; BPD, beyond progression disease; FU, follow-up; SqCC, squamous cell carcinoma; C, carboplatin; P, paclitaxel; Pem, pemetrexed; mos, months.

Name	n	Arms	Histology	Treatment BPD	Duration of IO	Crossover rate	Median FU	ORR	PFS (mos)	OS (mos)
KEYNOTE407 [66]	559	C + P or nab-P ± Pembro	SqCC	Allowed	Up to 35 cyles	31.7%	7.8 mos	57.9% vs. 38.4%	6.4 vs. 4.8 (HR 0.56)	15.9 vs. 11.3 (HR 0.64)
KEYNOTE189 [61, 62]	616	Cis/Carbo-Pem ± Pembro	Non-SqCC EGFR/ALK WT	Allowed	Up to 35 cyles	41.3%	10.5 mos	47.6% vs. 18.9%	8.8 vs. 4.9 (HR 0.52)	N.R. vs. 11.3 (HR 0.49)
Impower 150 (ARM B vs. C) [63, 64]	400 vs. 400	ABCP vs. BCP	Non-SqCC (EGFR/ALK allowed)	Allowed	Until PD	N.R.	~20 mos	63.5% vs. 48%*	8.3 vs. 6.8 (HR 0.59)*	19.2 vs. 14.7 (HR 0.78)*
Impower 150 (ARM A vs. C) [63, 64]	402 vs. 400	ACP vs. BCP	Non-SqCC (EGFR/ALK allowed)	Allowed	Until PD	N.R.	~20 mos	N.R.	N.R.	19.4 vs. 14.7 (HR 0.88)*
IMpower 130 [65]	451 vs. 228	Atezo + C + nab-P vs. C + nab-P	Non-SqCC (EGFR/ALK allowed)	Allowed	Until PD	19.3%	19 mos	49.2% vs. 31.9%	7.0 vs. 5.5 (HR 0.64)*	18.6 vs. 13.9 (HR 0.79)*
IMpower 131 (ARM B vs. C) [68]	343 vs. 340	Atezo + C + nab-P vs. C + nab-P	SqCC	Allowed	Until PD	43%	17.1 mos	49% vs. 41%	6.5 vs. 5.6 (HR 0.74)	14.6 vs. 14.3 (HR 0.92)
IMpower 132 [69]	292 vs. 286	Atezo + Cis/C + Pem vs. Cis/C + Pem	Non-SqCC EGFR/ALK WT	Allowed	Until PD	37.1%	14.8 mos	47% vs. 32%	7.6 vs. 5.2 (HR 0.60)	18.1 vs. 13.6 (HR 0.81)

The IMpower131 trial evaluated the addition of atezolizumab to either carboplatin/paclitaxel (arm A) or carboplatin/nab-P (arm B) versus carboplatin/nab-paclitaxel alone (arm C). Preliminary data of arm B vs. C were presented (median follow-up of 17.1 months). The addition of atezolizumab to carboplatin/nab-paclitaxel was associated with a statistically significant improvement in PFS (6.3 vs. 5.6 months; HR 0.71; p=0.0001), but failed to meet the other co-primary endpoint, with no statistically significant differences in terms of OS (14.0 vs. 13.9 months; HR 0.96; p=0.6931) [68]. Finally, IMpower132 evaluated atezolizumab in combination with platinum-pemetrexed in chemotherapy-naïve non-squamous NSCLC without EGFR or ALK genetic alterations. The study met one of its two co-primary endpoints with a significant advantage in terms of PFS (7.6 vs. 5.2; HR 0.60; p<0.0001), but did not show any statistically significant advantage in terms of OS (18.1 vs. 13.6 months; HR 0.81; p=0.0797) at the first interim analysis (median follow up 14.8 months) despite a 4.5 months survival gain [69]. A longer follow-up can provide definitive conclusions on the efficacy of these combinations.

Another potential strategy is to combine PD(L)-1 inhibitors with other ICIs to provide a more comprehensive blockage of immune suppressive signals. The most extensively studied combination is with CTLA-4 inhibitors, which has already shown efficacy in metastatic melanoma [70] and renal cell carcinoma [71]. The multi-cohort phase 1 CheckMate-012 study showed that nivolumab 3 mg/kg every 2 weeks in combination with ipilimumab 1 mg/kg every 12 weeks or every 6 weeks was associated with an ORR of 47% and 38% and median PFS of 8.1 months and 3.9 months, respectively. High PD-L1 expression (≥1%) was associated with higher ORR (57% in both treatment arms). The combination was associated with high frequency of serious adverse events (33-37% irAEs G3-4) [72]. Evaluation of tissue TMB through WES showed that this biomarker strongly predicted efficacy of the combination, regardless of PD-L1 expression [73]. The schedule nivolumab 3 mg/kg every 2 weeks plus ipilimumab 1 mg/kg every 6 weeks was further evaluated in the phase II CheckMate-568 study, with ORR in PD-L1 ≥1% patients as primary endpoint. The combination was associated with increased activity among PD-L1 positive patients (ORR was 41% in PD-L1 ≥1% vs. 15% in PD-L1 <1%). Efficacy on the basis of TMB, evaluated with the FoundationOne CDx assay, was included as a secondary endpoint. TMB ≥10 mut/Mb was identified as the optimal cut-off value for efficacy and was associated with improved ORR (43.7% vs. 23.5% for TMB high and low, respectively) and PFS (7.1 vs. 2.6 months for TMB high and low, respectively) independently of PD-L1 IHC expression. Safety profile was in line with previous studies (29% irAEs G3-4) [74]. These results were confirmed in the randomized phase III CheckMate-227 study, which

met its co-primary endpoints of PFS with the nivolumab-ipilimumab combination versus chemotherapy in first-line advanced NSCLC with high TMB (≥10 mutations/Mb), using the FoundationOne CDx assay, regardless of PD-L1 expression. Among patients with TMB ≥10%, dual blockage was associated with higher ORR (45.3% vs. 26.9%) and longer PFS (7.2 months vs. 5.5 months, HR 0.58; p<0.001) compared with platinum-based chemotherapy. Reponses were durable (43% 1-year PFS rate) and the advantage in PFS was independent of PD-L1 expression (≥1% vs. <1%). No differences were observed in terms of PFS in patients with low TBM (<10 Mb) (HR 1.07) [75]. Based on this promising efficacy data, nivolumab-ipilimumab was submitted for FDA approval in July 2018. Unfortunately, in October 2018 updated OS data, the other co-primary endpoint of the trial, for the combination showed no difference in OS between patients whose tumors had TMB ≥10 mut/Mb or <10 mut/Mb compared with chemotherapy (23.03 vs. 16.72 months; HR, 0.77). In January 2019 BMS withdrew the application for FDA approval while awaiting the final data from part 1a of the study (nivolumab-ipilimumab vs. chemotherapy in PD-L1 ≥1% patients). A second press release in July 2019 announced that Part 1a of the CheckMate-227 trial met the co-primary endpoint of OS, demonstrating a statistically significant benefit for the combination versus chemotherapy in patients with PD-L1 ≥1% expression, while in the Part 2 of the study (chemotherapy plus nivolumab vs. chemotherapy alone, regardless of PD-L1 expression) nivolumab plus chemotherapy did not meet its primary endpoint of OS vs. chemotherapy (18.83 vs. 15.57 months; HR 0.86). These results are quite surprising if compared with those reported with other chemo-immunotherapy combinations in similar patient populations. The final results of this study are expected at the end of this year and could provide more evidence on this discrepancy.

The role of TMB as predictive biomarkers for dual immune checkpoint blockage was also explored in the randomized phase III MYSTIC trial. This was a three arm randomized phase III trial comparing durvalumab (arm A) or durvalumab-tremelimumab (arm B) with chemotherapy in stage IV NSCLC EGFR/ALK wild type, irrespective of PD-L1. Primary endpoints were PFS and OS with durvalumab-tremelimumab vs. chemotherapy in PD-L1≥25% patients (ITT population). The trial failed to meet its co-primary endpoints with no statistically significant differences in both PFS (3.9 vs. 5.4 months; HR 1.05, 97.54% CI 0.722-1.534; p=0.705) and OS (11.9 vs. 12.9; HR 0.85, 98.77% CI 0.611-1.173; p=0.202) in PD-L1 ≥25% patients. However, an exploratory analysis evaluated TMB in tissue (using FoundationOne CDx assay) and in the blood (using the 500-gene GuardantOMNI panel). Interestingly, increasing blood TMB (bTMB) values correlated with increased OS HR and a bTMB ≥20 mut/Mb was selected as optimal cut-off value.

Indeed, patients with high bTMB experienced longer OS (21.9 vs. 10 months; HR 0.49, 95% CI 0.32-074) with durvalumab-tremelimumab compared with chemotherapy, but not in those with low bTMB (≤20 mut/Mb) (median OS 8.5 vs. 11.6 months; HR 1.16) [46, 49]. In contrast, in August 2019 a press release of AstraZeneca announced that a second phase III trial (NEPTUNE) evaluating the combination durvalumab-tremelimumab versus chemotherapy in treatment-naïve patients, regardless of PD-L1 expression, did not meet its primary endpoint of improving OS compared to standard of care chemotherapy in patients with bTMB ≥20 mut/Mb. The full data will be presented in the next future and will help to identify the potential causes of this apparent discrepancy. The role of predictive biomarkers to ICIs will be discussed more extensively in the following chapters. Based on the results of these studies, the therapeutic algorithm of non oncogene-addicted advanced/metastatic NSCLC has been revolutionized. These changes are depicted in **Fig. 4**.

1.4 Pretreated NSCLC

ICIs targeting PD-1/PD-L1 dramatically changed the therapeutic landscape of pretreated NSCLC. In 2012 the first in human trial of nivolumab in heavily pretreated solid tumors (CA209-003), including NSCLC, showed promising activity for this agent with a response rate of 18% and durable responses, exceeding results with historical controls using conventional therapeutic agents [13], proving that the activity of ICB in a disease not traditionally considered to be immunogenic disease. The five-year OS data were recently presented showing an impressive survival rate of 34.2% among patients with melanoma, 27.7% among patients with RCC, and 15.6% among patients with NSCLC [76]. Since the initial study, several PD(L)-1 compounds were tested in 2nd/3rd line NSCLC, demonstrating superiority over the standard of care at that time (docetaxel) and now nivolumab, pembrolizumab, and atezolizumab are approved in this setting. Development of these drugs followed different pathways, since some of them were tested in unselected patient populations (nivolumab, atezolizumab, and avelumab), whereas others followed biomarker-driven development (pembrolizumab). Nivolumab was evaluated in two large randomized phase III studies with similar designs using docetaxel as the control arm. CheckMate-017 evaluated nivolumab in 2nd line squamous NSCLC [38], whereas CheckMate-057 addressed 2nd/3rd line non-squamous NSCLC [39].

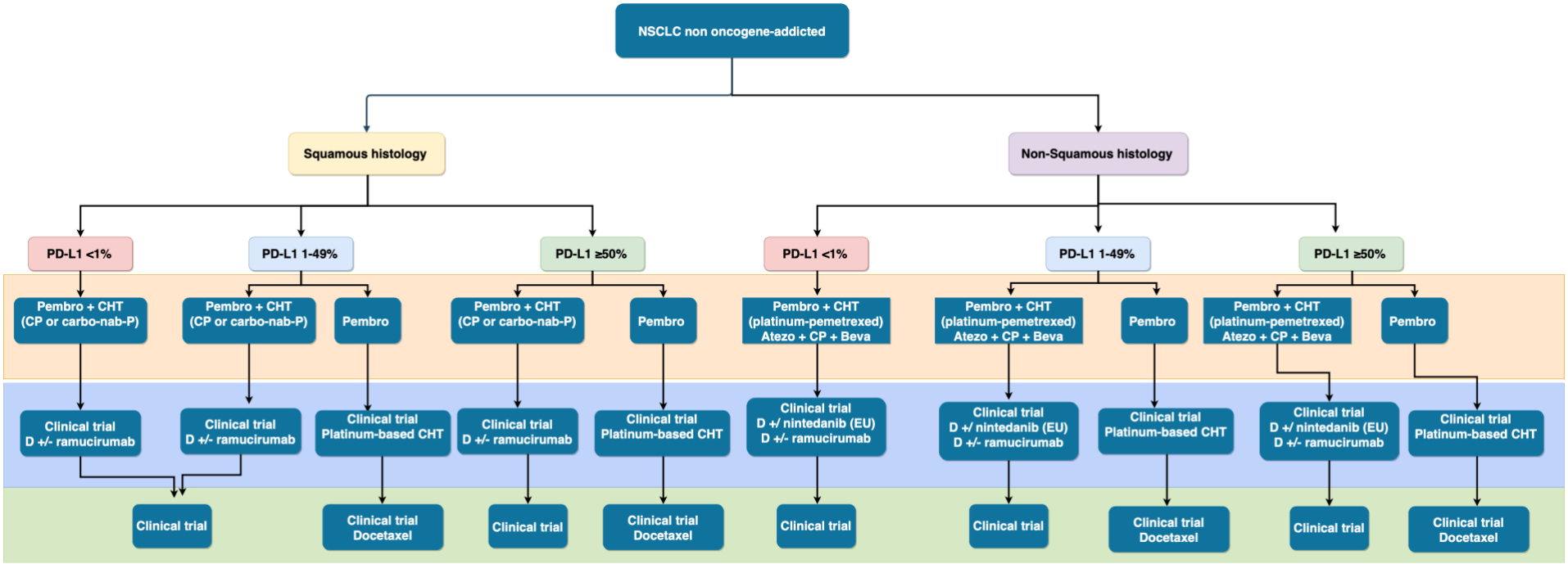

Figure 4. New therapeutic algorithm in advanced/metastatic NSCLC with available therapeutic options (*From Russo A, et al. Adv Exp Med Biol. 2019*) [7]

Legend: Pembro, pembrolizumab; Atezo, atezolizumab; D, docetaxel; CP, carboplatin/paclitaxel; nab-P, nab-paclitaxel; Beva, bevacizumab; EU, approved only by European Medicine Agency; CHT, chemotherapy.

Both studies met the primary endpoints, showing a statistically significant advantage in terms of OS compared with docetaxel in both squamous (9.2 vs. 6.0 months; HR 0.59; $p<0.001$) and non-squamous NSCLC (12.2 vs. 9.4 months; HR 0.73; $p=0.002$) [38, 39]. Nivolumab was also superior to docetaxel in terms of ORR (19-20% vs. 9-12%) and safety profile (treatment-related AEs G3-4 in 7-10% vs. 54-55%) in both studies, as well as in PFS in squamous histology only (3.5 vs. 2.8 months; HR 0.62; $p<0.001$) [38, 39]. Interestingly, PD-L1 expression as a predictive biomarker produced contrasting results between the two trials, despite similar study designs and the same assessment methods (IHC clone 28-8). Biological differences (mutational frequency, smoking-related damages, frequency of oncogene drivers aberrations) between squamous and non-squamous NSCLC has been proposed to explain this different behavior. Moreover, a landmark analysis of the CheckMate-057 demonstrated that, excluding patients who had died within the first 3 months of treatment, nivolumab was superior to docetaxel in both PD-L1 positive and negative patients [77]. For this reason nivolumab was approved in both squamous and non-squamous pretreated NSCLC patients irrespective of PD-L1 status. Recently, a pooled analysis of both studies showed an encouraging 3-year OS of 17% [78]. These results are noteworthy when compared to conventional chemotherapy. Only 8% of the patients in the docetaxel arm were alive at 3 years, and the plateau in the survival curves suggests a potential long-term benefit.

Atezolizumab was compared with docetaxel in pretreated NSCLC in phase II (POPLAR) and phase III randomized studies (OAK), showing improved OS across all PD-L1 expression levels with incremental efficacy results at the increase of PD-L1 IHC expression in tumor cells (TC) or tumor-infiltrating immune cells (IC) using the SP142 assay [41, 79]. However, this IHC assay reported in some harmonization study lower tumor cell staining than other tests [80, 81] and is not FDA approved for lung cancer patients. An exploratory analysis was conducted in plasma samples collected in both trials to evaluated bTMB, showing that the cut-off value of $\geq$ 16 mut/Mb was clearly predictive of improved PFS, with a good correlation with tissue TMB values and no association with strong PD-L1 expression [82]. Based on the results of the OAK trial, in October 2016, FDA granted atezolizumab approval for pretreated NSCLC irrespective of PD-L1 status.

Pembrolizumab was first evaluated in the phase 1 multi-cohort study KEYNOTE-001, which evaluated the safety and activity of this compound and validated the companion diagnostic 22C3 IHC assay for PD-L1 expression. Pembrolizumab was well tolerated with few treatment-related AEs of grade 3 or more (9.5% of the patients) and showed good clinical activity (ORR 19.4%, median PFS 3.7 months, and median OS 12.0 months), with

no significant differences between the different schedules used (2mg/kg or 10 mg/kg every 3 weeks). Interestingly, a PD-L1 TPS ≥50% was associated with a higher response rate and longer PFS and OS [83]. In October 2015, the U.S. FDA granted accelerated approval for pembrolizumab for pretreated NSCLC patients with tumor expression of PD-L1, assessed with the companion diagnostic test. The subsequent randomized phase II/III study KEYNOTE-010 compared pembrolizumab at two different dosages (2 mg/kg or 10 mg/kg every 3 weeks) to docetaxel in pretreated NSCLC patients with a TPS ≥1%. The trial met its primary endpoint with a statistically significant advantage in OS in both pembrolizumab arms (10.4 vs. 8.5 months and 12.7 vs. 8.5 months, respectively for pembrolizumab 2 mg/kg and 10 mg/kg, with a HR of 0.71 and 0.61). No differences were observed in PFS curves between the three treatment arms. Patients with strong PD-L1 expression (TPS ≥50%) derived the greatest OS benefit with both pembrolizumab 2 mg/kg (14.9 vs. 8.2 months; HR 0.54; p=0.0002) and 10 mg/kg schedules (17.3 vs. 8.2 months; HR 0.50; p<0.0001) [40].

In contrast with the results reported with other PD-1/PD-L1 inhibitors, avelumab failed to demonstrate a survival advantage compared with docetaxel in PD-L1 unselected pretreated patients in the phase III randomized study JAVELIN Lung 200. The trial failed to meet its primary endpoint, showing no statistically significant differences in terms of OS between the two treatment arms in the overall study population (10.5 vs. 9.9 months; HR 0.90; p=0.12) and in PD-L1 positive patients (≥1%) (11.4 vs. 10.3; HR 0.90; p=0.16) [84]. One of the possible explanation for the lack of OS benefit could be the better performance of the control arm than expected on similar randomized trials of anti–PD-1/PD-L1 agents (8.5-9.6 months) [40, 41], likely due to the subsequent use of ICIs. Finally, durvalumab was evaluated as 3rd line option in the single arm phase II study ATLANTIC. The trial included three cohorts of patients: EGFR+/ALK+ NSCLC with PD-L1 expression ≥25% (cohort 1), EGFR/ALK wild type NSCLC with PD-L1 expression ≥25% (cohort 2), or PD-L1 ≥90% (cohort 3). The clinical activity and safety profile of durvalumab was consistent with that of other PD(L)-1 inhibitors. As expected, responses were higher in EGFR/ALK wild type patients and increased with higher PD-L1 expression levels (30.9% in PD-L1 ≥90% and 16.4% in PD-L1 ≥25% among EGFR/ALK wild type patients) [85]. The 12.2% ORR reported among EGFR/ALK positive patients suggests that a subgroup of oncogene-addicted NSCLC can derive benefit from ICB and supports further evaluation of this strategy in these patients. Neither durvalumab nor avelumab are approved in stage IV NSCLC.

1.5 Conclusions

Immunotherapy represented a major breakthrough in lung cancer management and today represents a backbone of treatment in several settings. Although the benefit from this novel therapeutic approach is undeniable, several open questions still remain unanswered. Longer follow-up of clinical trials reported so far and post-approval studies will provide further details on long-term safety of ICIs [**Tab. 4**] either as single agent or in combination with chemotherapy. Future clinical trials should define the optimal treatment duration (elective discontinuation after 2 years? Until progression?), efficacy and safety in special populations that are often excluded (patients with viral chronic infections, autoimmune disease, ECOG performance status ≥2, and active brain metastases) or underrepresented in clinical trials (elderly, racial minorities), and novel predictive biomarkers that can better select candidates for immunotherapy. These emerging challenges will be extensively discussed in the subsequent chapters of this thesis.

Table 4. Long-term results with immune checkpoint inhibitors in advanced NSCLC (*From Russo A, et al. Transl Cancer Res 2019*) [86]

Trial	Phase	ICI arm(s)	Treatment duration	Population (n)	PD-L1 selection	Median FU	Median OS (95% CI)	2-yr OS	3-yr OS	5-yr OS
KEYNOTE-001 (1)	1	Pembrolizumab Pembrolizumab	Until PD*	1st line NSCLC (101)‡ Pretreated NSCLC (449)	≥1% All comers	34.5 mos	22.3 mos (17.1-31.5) 10.5 mos (8.6-13.2)	49% 29.9%	26.4% 19%	-
KEYNOTE-010 (6)	2/3	Pembrolizumab	24 months or until PD	Pretreated NSCLC (690)	≥1%	42.6 mos	11.8 mos (10.4–13.1)	-	23% 11%	-
KEYNOTE-024 (8)	3	Pembrolizumab	24 months	1st line, EGFR/ALK WT NSCLC (154)	≥50%	25.2 mos	30 mos (18.3-NR)	70.3%	51.5%	-
CHECKMATE-017 (5)	3	Nivolumab	Until PD	Pretreated squamous NSCLC (131)	All comers	3-yr minimum	9.23 mos (7.33-12.62)	23%	16%	-
CHECKMATE-057 (5)	3	Nivolumab	Until PD	Pretreated non-squamous NSCLC (287)	All comers	3-yr minimum	12.21 mos (9.66-15.08)	29%	18%	-
CA209-003 (7)	1	Nivolumab	96 weeks	Pretreated NSCLC (129)	All comers	58.28 mos minimum	9.9 mos (7.8-12.4)	25%	18%	16%
POPLAR (3, 4)	2	Atezolizumab	Until PD	Pretreated NSCLC (144)	All comers	38 mos	12·6 mos (9·7–16·4)	32.2%	18.7%	-
OAK (2)	3	Atezolizumab	Until PD	Pretreated NSCLC (425)	All comers	28 mos	13.8 mos (11.8–15.7)	30.9%	-	-
MYSTIC (9)	3	Durvalumab Durvalumab-Tremelimumab	Until PD**	1st line, EGFR/ALK WT NSCLC (374) 1st line, EGFR/ALK WT NSCLC (372)	All comers	30.2 mos†	16.3 mos (12.2–20.8)† 11.9 mos (9.0–17.7) †	38.3%† 35.4%†	-	-

Abbreviations: PD, progressive disease; WT, wild type; mos, months; yr, year; CI, confidence interval; NR, not reached; FU, follow-up.

**after a protocol amendment in April 2016 discontinuation after 24 months of treatment and resume upon disease progression was allowed.*
***in the durvalumab-tremelimumab arm, durvalumab was continued until PD after 4 courses of anti-PD1 + CTLA-4 courses*
‡after a protocol amendment the enrollment in this arm was limited to EGFR/ALK WT patients
† PD-L1 ≥25% subgroups

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CHAPTER 2

Emerging challenges with immunotherapy in clinical practice: concomitant medications and treatment of special populations

Extracted from:

"**Concomitant medications during immune checkpoint blockage in cancer patients: Novel insights in this emerging clinical scenario.**"
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"**Immunotherapy Use in Human Immunodeficiency Virus (HIV)-infected Non-small Cell Lung Cancer Patients**"
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2.1 Introduction

The use of immune checkpoint inhibitors (ICIs) has revolutionized the therapeutic management of several solid tumors and therapeutic indications continue to grow. However, patients enrolled in clinical trials hardly ever correspond to those treated in clinical practice where oncologists have to face unexpected challenges. Indeed, most of the pivotal clinical trials with these drugs have excluded patients using corticosteroids (i.e. prednisone ≥10 mg daily). Additionally, most clinical trials do not report efficacy or safety data regarding the impact of concomitant use of ICIs with commonly used drugs in clinical practice, such as antibiotics and proton pump inhibitors (PPIs) that may affect the activity of these compounds through the alteration of gut microbiota. Moreover, the prophylactic use of vaccines is common in cancer patients in order to prevent infectious complications resulting from the immune suppressive effects associated with conventional cancer treatments, such as chemotherapy and radiotherapy. However, the use of simultaneous medications in cancer patients treated with immunotherapy is not well studied [1]. In addition, HIV-infected patients were excluded from all the pivotal trials with these agents, due to safety concerns and the hypothesis that severe HIV/AIDS with low CD4+ T-cell counts may compromise the efficacy of immune checkpoint blockage (ICB). Many uncertainties still exist regarding use of ICIs for lung cancer in the HIV-infected patient population [2].

In this chapter we will analyze the role of different concomitant medications that may influence the therapeutic efficacy and/or safety of ICIs, including corticosteroids, antibiotics, vaccines, and proton pump inhibitors to address an unmet need in this growing complex clinical scenario. Furthermore, the role of ICIs in HIV-infected lung cancer patients will be also evaluated.

2.2 Steroid use and immune checkpoint inhibitors

Corticosteroid therapy has been traditionally considered as the antidote of possible side effects of immunotherapy, capable of extinguishing immune-related adverse reactions (irAEs). On the hypothesis that corticosteroid use could reduce the efficacy of immunotherapeutic drugs, clinical trials with these agents have considered concomitant treatment with prednisone doses above 10 mg or equivalent as an exclusion criterion. For this reason there is no prospective data from randomized trials to evaluate the impact of corticosteroids on the efficacy of ICIs. Recently several retrospective studies have investigated potential interferences between early corticosteroid use and immunotherapy

in advanced NSCLC patients treated with ICIs [**Tab. 1**].

Table 1 Retrospective studies evaluating the impact of early steroid use on ICIs targeting PD(L)-1 +/- CTLA-4 efficacy compared with non steroid use in NSCLC [*Adapted from Rossi G...Russo A. Crit Rev Oncol Hematol 2019*) [1]

Ref.	Window of steroid use respective to ICI start	Early steroid users (%)	ORR (%)	PFS (mos)	OS (mos)
[4]	Within 1 month	12%	N.A.	N.A.	4.3 vs. 11.0 (p=0.017)
[3]	At the beginning	14%	7 vs. 18 (p<0.005)	N.A. v.s N.A. (p<0.001)	N.A. v.s N.A. (p<0.001)
[5]	Within 1 month	23%	17 vs. 24% (p=0.39)	1.98 vs. 3.94 (p=0.003)	4.86 vs. 15.14 (p<0.001)
[6]	Within 24 hours of the first dose	14.3%	10.8 vs. 19.7 (p=0.04)	2.0 vs. 3.4 (p= 0.01)	4.9 vs. 11.2 (p< 0.001)

** Prednisone ≥10 mg/d for cancer-related palliation; **Prednisone ≥10 mg/d for cancer-unrelated indications.*
Legend: NSCLC, non small cell lung cancer; ORR, overall response rate; PFS, progression free survival; OS, overall survival; ICIs, immune checkpoint inhibitors; N.A. not available; mos, months.

Collectively, these studies [3-6] showed that concomitant use of steroids is associated with impaired outcomes in NSCLC patients treated with ICIs, although starting corticosteroids during immunotherapy for the management of irAEs does not compromise its efficacy [4], as shown in different clinical settings with both anti-CTLA4 [7] and anti–PD(L1) agents [8, 9]. In addition, a modulation of peripheral blood immune cells has been hypothesized, which may have contributed to the lower antitumor response [5] However, the conclusions from these studies are not warranted: It was correctly acknowledged that their studies could not distinguish the prognostic and predictive effects of corticosteroids in these patients. In all of these studies steroid treatment before or within 30 days from starting ICIs was associated with patients' characteristics usually associated with a worse prognosis (higher ECOG PS, baseline brain metastases, chronic obstructive pulmonary disease, higher metastatic sites) [3-5]. For this reason they performed multivariate analyses to minimize confounding effects of these factors.

The prognostic role of steroid use was already studied with chemotherapy and might be deleterious in lung cancer patients [10]. Moreover, the fact that the association between baseline steroids and prognosis persisted after multivariate adjustments for known prognostic factors is not sufficient to prove that there is a true cause-effect relationship, for 2 reasons:

1. When a strong prognostic factor (like as ECOG PS) shows a large difference in distribution between the groups being compared (in this case steroids users and

non-users), adjustment that uses classes of the factor may not be sufficient to entirely remove its confounding effect. This phenomenon is referred to as intraclass (or residual) confounding [11] and may cause spurious associations of considerable size in multivariate analyses in observational studies. Therefore, multivariate analyses could not completely adjust away the differences in the distribution of ECOG PS and in the frequency and clinical impact of brain metastases between steroid users and non-users.

2. It is also plausible that the use of steroids at the time of the initiation of immunotherapy had the effect of improving the clinical condition of several patients, thereby "downgrading" their ECOG PS. As a consequence, these patients had a more aggressive disease and a worse prognosis than patients assigned to the same ECOG PS class who were not taking steroids; independent of any negative effect of steroids.

These potential biases are not proof that the observed association between baseline steroid use and response/prognosis in NSCLC patients receiving ICI is fortuitous rather than due to the fact that steroids are prescribed to patients with a worse prognosis and further evidence is needed to prove either hypothesis. Proof can derive only from properly conducted subgroup analyses of already conducted randomized trials comparing ICIs to chemotherapy in NSCLC (and in other cancers, as well) or from new randomized trials. Meanwhile, the management of NSCLC patients who are candidates for ICIs and are receiving steroids should not be affected by the results of these studies.

2.3 Antibiotics use and ICIs

Antibiotics (ATBs) are another common concomitant medication during cancer treatment that recently gained great attention in patients treated with ICIs, due to their ability to alter gut microbiota leading to dysbiosis and potentially influencing immune responses [12, 13]. Indeed, recent evidence suggests that gut microbiota is able to exert a significant influence on response to ICIs [14-17].

Several *in vitro* and *in vivo* models showed that ATBs affect gut microbiota during ICIs. Antitumor effects of CTLA-4 blockade seem to depend on distinct *Bacteroides* species. *Bacteroides* involvement during therapy with anti-CTLA-4 is partially explained by the T-helper 1 activation against *B. fragilis* capsular polysaccharides [18]. This data suggests that immunotherapy is able to modify intestinal microbiota that in turn influences the response to immunotherapy itself. In addition, *Bifidobacterium* has been reported to mitigate in a mouse colitis model immune-related colitis, a frequent irAE seen in anti-CTLA-4 treatment

[19], likely modulating the metabolic functions of T regulatory cells (T_{regs}). This data supports the use of caution in case of concomitant use of ATB during CTLA-4 blockage, as commonly used antibiotics, such vancomycin, inhibit *Bifidobacterium* species [20] and recently, promising data was reported in a preliminary study of patients with ICI-associated colitis successfully treated with fecal microbiota transplantation, with reconstitution of the gut microbiome and a relative increase in the proportion of T_{regs} within the colonic mucosa [21].

The composition and heterogeneity of gut microbiota plays a pivotal role in providing a robust immune defense [22] and may influence the efficacy of ICIs targeting PD(L)-1 and CTLA-4 as primary resistance to these agents can be in part attributed to abnormal gut microbiome composition. Metagenomics analyses of patient stool samples at diagnosis revealed correlations between clinical responses to ICIs and the relative abundance of *Akkermansia muciniphila* [23]. Interestingly, gut microbiota has been shown to be a key contributor involved in the onset of obesity-related disorders and in a recent paper [24] reported that supplementation with *Akkermansia muciniphila* improves several metabolic parameters. This can provide an explanation to the recent observation that obese patients have a better outcome with ICIs compared with others [25, 26].

Based on this preclinical data, several retrospective studies evaluated the impact of ATB use in patients treated with ICIs [**Tab. 2**]. These studies included different patient populations both in terms of tumor histology (mostly NSCLC, RCC and melanoma) and different ATB use windows. However, the vast majority investigated the role of early ATB use (before 1-2 months and 1 month after the start of immunotherapy) [**Tab. 2.**]. Most of these studies have investigated the impact of ATB use on ICIs targeting PD-1/PD-L1 [23, 27-35] albeit some studies have also included patients treated with anti-CTLA4 agents either alone or in combinations [33-39].

Collectively, these studies suggest that ATB use has a negative impact on outcomes in patients receiving immune checkpoint inhibitors in terms of ORR [37-39], PFS [23, 27, 28, 31, 34, 37-40], and OS [23, 27, 28, 30, 31, 33-35, 38, 40]. The hypothesis emerging from these studies is that the ATB-related dysbiosis might decrease the diversity of gut microbiota thereby eliminating the most immunogenic bacteria [38]. Gut and blood microbiota profiling studies could help to predict the efficacy of ICIs and to evaluate the impact of different bacteria species on the outcome of these patients. Recently, in a preliminary study, it was reported that early ATB use influenced plasma citrulline levels, an amino-acid produced entirely by *Enterocytes*, independently of nutritional status. Citrulline is a validated marker of intestinal barrier and *Enterocytes* function and plasma

levels have been correlated in NSCLC patients treated with nivolumab with clinical benefit, PFS and OS [33].

Prospective studies are needed to better define the optimal ATB window, the differences in ATB classes, the route of administration, the duration of ATB therapy, and the potential impact of other concomitant medications and conditions that might alter the microbiome, such PPI use, steroid use and the diet composition [**Fig. 1**]. Therefore, in the absence of clear evidence, the use of ATBs, especially for long or repeated courses, during immunotherapy should be carefully evaluated. However, the use of ATB still remains mandatory in cases of bacterial infectious diseases. Further, opportunistic infections that may emerge in cases of immune depression, as observed in patients requiring prolonged steroid therapies as for severe irAEs [1].

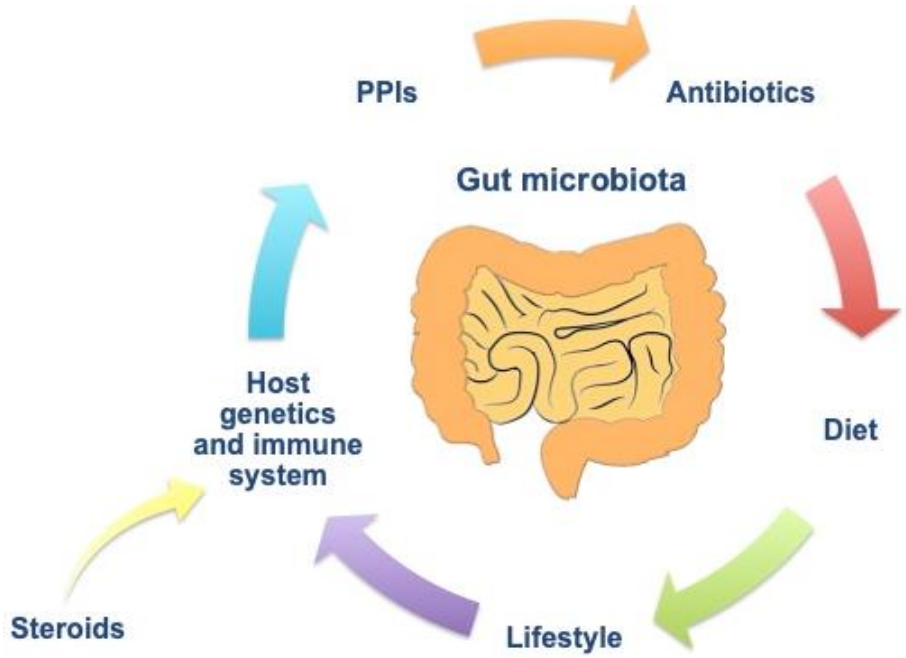

Figure 1 Multiple factors influencing gut microbiota composition (*From Rossi G…Russo A. Crit Rev Oncol Hematol 2019*) [1]

Table 2. Retrospective studies evaluating the impact of antibiotics prescription in patients treated with immune checkpoint inhibitors targeting PD(L)-1 +/- CTLA-4 (*Adapted from Rossi G...Russo A. Crit Rev Oncol Hematol. 2019*) [1].

Ref.	Cancer type(s) (n)	ATB window respective to ICIs start	ATB+ patients	ORR (%) ATB- vs. ATB+	PFS (mos) ATB- vs. ATB+	OS (mos) ATB- vs. ATB+
[23]	NSCLC (140), RCC (67), UC (32)	Within 2 months before and 1 month after	28%	N.A.	4.1 vs. 3.5 (p=0.017)	20.6 vs. 11.5 (p<0.001)
[38]	RCC (121) NSCLC (249)	Within 1 month before	13% (RCC) 20% (NSCLC)	26 vs. 13 (p<0.01) (RCC) 23 vs. 13 (p<0.01) (NSCLC)	7.4 vs. 1.9 (p<0.01) (RCC) 3.8 vs. 1.9 (p=0.03) (NSCLC)	30.6 vs. 7.3 months (p=0.03) (RCC) 24.6 vs. 7.9 (p<0.01) (NSCLC)
[27]	NSCLC (30)	Within 1 month before and 1 month after	36.7%	N.A.	3.1 vs. 2.9 (p=0.031)	15.1 vs. 7.5 (p=0.026).
[29]	NSCLC (74)	Within 3 months before and during ICI therapy	20.3%	22 vs. 26.7 (p=0.75)	*N.A. vs. N.A.* (p=0.72)	N.A.
[37]	Melanoma (74)	Within 1 month before	13.5%	34 vs. 0 (p<0.01)	7.3 vs. 2.4 (p=0.01)	18.3 vs. 10.7 (p=0.17)
[39]	RCC (146)	Within 4 weeks before and 8 weeks after	21%	24.2 vs. 19.3 (p=0.005)	8.1 vs. 2.6 (p=0.008)	*N.A. vs. N.A.* (p=0.257)
[40]	Melanoma (201), NSCLC (58), RCC (46)	Within 2 weeks before and 6 weeks after	31%	N.A.	5.8 vs. 3.2 (p=0.049)	21.4 vs. 10.4 (p=0.001)
[30]	NSCLC (109)	Within 1 month before the first dose and 1 month after the last dose	79.8%	N.A.	N.A.	17.2 vs. 5.4 (p=0.0004)
[31]	NSCLC (90)	Within 1 month before	14.4%	N.A.	4.4 vs. 1.2 (p=0.04)	N.R. vs. 8.8 (p=0.037)
[42]	NSCLC (109)	Within 1 month before and 1 month after	18.3%	22.5 vs. 15 (p=0.092)	9.6 vs. 3.7 (p<0.0001)	21.9 vs. 6.1 (p=0.0021)
[32]	NSCLC (157)	Within 1 month before and 3 months after	17.2%	11.1% vs. 24.6 (p=0.2018)	3.3 vs. 2.2 (p=0.1772)	5.9 vs. 11.9 (p=0.2492)
[33]	NSCLC (72)	Within 2 months before and 1 month after	38.9%	*N.A. vs. N.A.* (p=0.276)	3.3 vs. 2.8 (p=0.249)	13.4 vs. 5.1 (p=0.027)
[34]	NSCLC (757)	Within 1 month before and 1 month after	27%	N.A.	1.76 vs. 2.79 (p=0.08)	8.54 vs. 14.06 (p<0.01)
[35]	NSCLC (119), Melanoma (38), others (39).	Within 1 month before and concurrently	15%	N.A.	N.A.	(p<0.001) (pATB) (p=0.76) (cATB)

Legend: N.R. not reached; N.A. not available; NSCLC, non small cell lung cancer; RCC, renal cell carcinoma; UC, urothelial carcinoma; ATB+, patients treated with antibiotics; ATB-, patients who did not received antibiotics; ORR, overall response rate; PFS, progression free survival; OS, overall survival; ICIs, immune checkpoint inhibitors; pATB, prior antibiotic use; cATB, concurrent antibiotic use.

2.4 Proton pump inhibitors and immunotherapy

Proton pump inhibitors (PPIs) are in widespread use for multiple indications including gastro-esophageal reflux disease and prevention and treatment of peptic ulcer disease. PPIs are now some of the most frequently prescribed drugs throughout the world, with large numbers of patients provided ongoing treatment with PPIs administration for several years [44]. Potent gastric acid suppression using PPIs has important effects on gastrointestinal microbiome [45, 46]. PPIs inhibit gastric acid secretion and cause an increase of the intragastric PH, which may perturb microbial communes, leading to dysbiosis and an increased risk of enteric infection and diarrhea in humans [47-49]. Several retrospectives studies have shown that PPI use increases the risk of enteric infections, community-acquired pneumonia [50, 51] and also small intestinal bacterial overgrowth [51-53]. PPI use has been associated in clinical studies with a decreased diversity of the gut microbiome as compared to non-users [54] and significant changes in the gut microbiome composition during PPI treatment (increase of *Lactobacillus* species and *Streptococcus* species) [55] that might predispose patients to dysbiosis and enteric infections. Given the increasing importance attributed to gut microbiome in the efficacy of ICIs with the enrichment of particular bacteria species in responding patients and a possible detrimental effect of the ATB use, studies have investigated the potential effect of PPI use in cancer patients treated with these inhibitors. However, only a few studies have been reported to date, with conflicting results [**Tab. 3**].

Table 3. Impact of PPI use on the therapeutic efficacy of ICIs in patients with solid tumors (*Adapted from Rossi G...Russo A. Crit Rev Oncol Hematol. 2019*) [1]

Study	Type of PPI	PPI window respective to ICIs start	PPI users patients	ORR (%) PPI users vs. non users	PFS (mos) PPI users vs. non users	OS (mos) PPI users vs. non users
[23]	Not specified	Within 2 months before and 1 month after	N.A.	N.A.	3.8 vs. 4.0 (p=0.431)	13.1 vs. 19.0 (p=0.285)
[31]	Not specified	Within 1 month before	19%	N.A.	N.A.	8.8 vs. NR (p=0.04)
[42]	Not specified	Within 1 month before and 1 month after	36.5%	27.5 vs.17.4 (p=0.213)	9.63 vs. 6.23 (p=0.343)	11.9 vs. 23.7 (p=0.754)
[56]	Not specified	Concomitant use	46.2%	N.A.	4.9 vs. 3.4 (p=0.77)	NE vs. NE (p=0.77)
[34]	Not specified	Within 1 month before and 1 month after	31%	N.A.	1.89 vs. 2.83 (p<0.01)	9.63 vs. 14.52 (p<0.01)

**included also patients receiving H_2Bs (histamine H_2-blockers)*

Legend: N.R. not reached; N.A. not available; PPI, proton pump inhibitor; ORR, overall response rate; PFS, progression free survival; OS, overall survival; ICIs, immune checkpoint inhibitors.

Some retrospective studies have reported no statistically significant differences in the clinical activity of ICIs in different solid tumors both in terms of PFS and OS [23, 28, 56], and irAEs frequency [56] between PPI users and non-users. In contrast, a pooled analysis (1512 patients) of the phase II/III trials OAK and POPLAR reported shorter PFS and OS in PPI users compared to non-users treated with atezolizumab in both univariate ($p<0.01$ for both PFS and OS) and multivariate analyses ($p=0.02$ and $p<0.01$, respectively) [34]. In addition, a small retrospective Japanese study reported a negative survival impact with the use of PPIs during ICI treatment in NSCLC ($p=0.04$), not confirmed in multivariate analyses ($p=0.15$) [31].

These studies confirm the need to investigate potential roles of PPI use on primary resistance to ICIs and may provide rational for therapeutic strategies exploiting gut microbioma as a driver for immunotherapy activity. However, most of these studies are limited by small sample sizes and were conducted at single institutions. In addition, in all of these studies the type and dose of PPIs used as well as the compliance to PPI treatment were not assessed and might have significantly impacted gastric acid suppression. Moreover, only selected studies evaluated the effects of other concomitant medications that could potentially affect the gut microbiome and might have confounded to the effect of these agents on ICIs activity [1].

2.5 Impact of common vaccination on efficacy and safety of ICIs

Prevention of infection is crucial for individuals with impaired immunity, including cancer patients during anticancer therapies. Viral infections in cancer patients often result in high morbidity and mortality rates that may reach 9% for influenza syndrome (IS) [57, 58]. Therefore, international guidelines recommend that all adult solid tumor patients should receive yearly vaccination with inactivated influenza vaccine regardless of age [59]. Recently, some concerns emerged in patients treated with ICIs, due to the potential risk that vaccine administration might result in exaggerated activation of the immune system [60], resulting in a higher incidence of vaccine-related AEs or serious irAEs. The exact pathophysiology of irAEs onset is not completely understood [61, 62]. Most data is derived from preclinical models and correlative human studies. How the combination of prophylactic vaccination and PD-1 blockade could increase irAEs also remains speculative. The physiological role of the PD-1/PD-L1 pathway is to mediate peripheral tolerance of T cells and inhibition of immune checkpoints could break such tolerance [63]. In a small study including 23 patients with lung cancer patients and 11 age-matched healthy controls using a trivalent inactivated influenza vaccine, Läubli et al. reported an unusual increase in

irAEs (52.2%) and severe irAEs (26.1%). This study included two cases of encephalitis and a single case of autoimmune peripheral neuropathy [64], raising important concerns about the safety of applying the seasonal influenza vaccination to patients undergoing immunotherapy. In contrast, three different studies, evaluating the safety of influenza vaccination in patients with solid tumors receiving ICIs, did not confirm these findings with a rate of irAEs comparable to published trials [60, 65, 66] [**Tab. 4**] and no correlation was reported in a recent study evaluating 101 patients who had developed immune-related myocarditis [68].

Table 4. Retrospective studies evaluating the impact of vaccination on safety and/or therapeutic efficacy in cancer patients treated with ICIs (*Adapted from Rossi G...Russo A. Crit Rev Oncol Hematol. 2019*) [1]

Ref.	ICIs used	Type of vaccine	Vaccinated patients	irAEs (%) Vaccinated vs. non-vaccinated	irAEs G3-4 (%) Vaccinated vs. non-vaccinated	OS (mos) Vaccinated vs. non-vaccinated
[67]	PD(L)-1 inhibitors, CTLA-4 (<1%)	Trivalent or quadrivalent inactivated influenza vaccine	26.3%	N.A.	N.A.	N.A. vs. N.A. (p=0.32)
[60]	PD-1 inhibitor	Trivalent inactivated influenza vaccine	33%	26 vs. 22 (OR 1.20)	7 vs. 4 (OR 2.04)	N.A.
[63]	PD-1 inhibitors	Trivalent inactivated influenza vaccine	100%	52.2*	26.1*	73.5*
[65]	PD-1 inhibitors	Influenza and/or pneumococcal vaccines	27.8%	N.A. vs. N.A. (p=0.265)	N.A.	N.A.
[66]	PD(L)-1 and or CTLA-4 inhibitors	Trivalent or quadrivalent inactivated influenza vaccine	100%	20*	8*	N.A.

**included only vaccinated patients*

Legend: N.R. not reached; N.A. not available; OS, overall survival; ICIs, immune checkpoint inhibitors.

Concurrent administration of influenza vaccination with PD-1 or PD-L1 inhibitors therefore still remains the currently accepted community practice, based on the rationale that patients receiving immune checkpoint inhibitors may be at increased risk of infections due to their underlying malignancy. Therefore patients should receive appropriate vaccines to avoid infectious complications or delay in therapy [59]. To date limited data is available on the concomitant use of CTLA-4 inhibitors and influenza vaccine. Clinical experience suggests that patients on a CTLA-4 inhibitor (such as ipilimumab) are advised to wait 6–8 weeks after the last dose as ipilimumab generally exhibits a worse adverse effect profile than that of PD-1 or PD-L1 inhibitors [59, 69].

Future prospective studies are warranted to better understanding the impact of anti-viral

vaccines in patients treated with ICIs.

2.6 ICIs in special populations: Immunotherapy Use in Human Immunodeficiency Virus (HIV)-infected NSCLC Patients.

(HIV)-infected individuals are at an increased risk of developing lung cancer [70-72], but historically this patient population has been underrepresented in clinical trials with <1% of protocols explicitly included persons with HIV infection in the pre-immunotherapy era [73]. In addition, pivotal trials evaluating ICIs in solid tumors excluded HIV-infected individuals, due to safety concerns and the hypothesis that severe HIV/AIDS with low CD4+ T-cell counts may compromise the efficacy of these agents. As consequence, many uncertainties still exist regarding use of ICIs for lung cancer HIV-infected patients [2].

IHC PD-L1 expression is the most extensively studied predictive biomarker for ICIs targeting PD(L)-1 and few studies evaluated PD-L1 expression in individuals diagnosed with concomitant lung cancer and HIV [74-76]. Collectively evaluating the results of these limited studies, PD-L1 expression does not appear to significantly differ between HIV-infected and HIV-uninfected individuals, and the data appear to support inclusion of HIV-infected lung cancer patients in immunotherapy clinical trials. Other potential biomarkers of interest, including tumor mutational burden, have not been evaluated in this patient population [2]

In chronic viral infections such as HIV, persistent viral replication leads to progressive loss of T-cell functions of proliferation and cytokine secretion [77-79], a phenomenon known as “T-cell exhaustion”. This process results in ineffective immune response and inability to adequately clear the virus. PD-1 is upregulated on both HIV-specific CD4+ and CD8+ T-cells and PD-1 expression correlates with HIV-specific cytotoxic T-cell dysfunction [77-79]. Studies of simian immunodeficiency virus (SIV) in Rhesus macaques, which is thought to closely model HIV disease in humans, have demonstrated that blockade of PD-1 and PD-L1 interaction in SIV results in reversal of functional exhaustion of T-cells (**Fig. 2**) [78]. Some investigators hypothesize that PD-1 or PD-L1 inhibition could theoretically improve outcomes from both an HIV and malignancy standpoint.

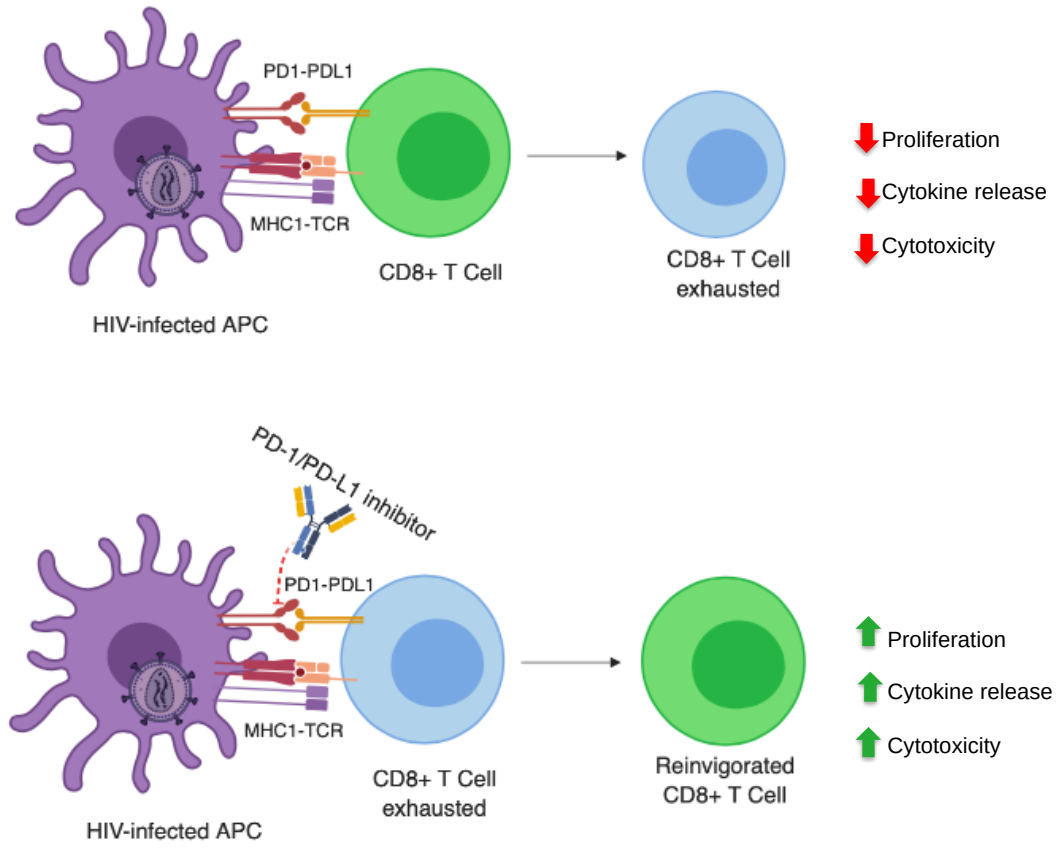

Figure 2. Interactions between PD-1/PD-L1 pathway and HIV
Abbreviations**:** *PD-1, programmed death-1; PD-L1, programmed death ligand-1; MHC-1, major histocompatibility complex class 1; TCR, T-cell receptor; APC, antigen-presenting cell; HIV, human immunodeficiency virus; CD8+ T cells (cytotoxic T lymphocytes, or CTLs). Credit: Created with BioRender (From Scilla KA, Russo A, et al. J Immunother Precis Oncol. 2019*) [2]

Published data evaluating the use of immunotherapy agents in HIV-infected lung cancer patients has mainly been limited to case reports or small case series. Collectively, HIV-infected NSCLC patients treated with ICIs targeting the PD-1/PD-L1 axis do not exhibit significant differences from HIV-uninfected patients in terms of both activity (ORR 27.5% and DCR 55.2%) and safety (irAEs 35% and grade 3/4 irAEs 9.7%) [2]. No cases have been reported to date evaluating chemotherapy-immunotherapy combinations or dual immune checkpoint blockade in the HIV-infected NSCLC population.

Data from a recent systematic review analyzing 73 HIV-infected patients with various advanced solid tumors (including 25 NSCLC patients) treated with ICIs either as single agent or in combination showed similar results, with no new safety signals noted in this population and relatively good efficacy (objective response rate of 30% for NSCLC) [76]. Of the 34 included patients with known paired pre-treatment and post-treatment HIV viral load (VL), HIV VL remained suppressed in 93% of the cases with pre-treatment undetectable HIV VL. CD4+ T-cell counts were noted to increase in 14 of the 25 included

patients with known paired pre-treatment and post-treatment CD4+ T-cell counts; mean [SD] change in CD4+ T-cell count was 12.3 [28.5] /μL) [76]. These results suggest that ICI treatment does not negatively impact HIV VL or CD4+ T-cell counts in HIV-infected cancer patients, including those with NSCLC. Similar results were reported in the phase 1 CITN-12 (Cancer Immunotherapy Trials Network 12) study evaluating pembrolizumab in HIV-infected patients with different advanced cancers, including one NSCLC patient [81]. All the participants were on antiretroviral therapy (ART) and none met the U.S. Department of Health and Human Services criteria for uncontrolled HIV. Pembrolizumab was well tolerated with most irAEs graded as mild/moderate (73.3%); 20% of the irAEs were grade 3. No statistically significant differences were noted in CD4 count in all participants (median increase of 19 cells/μL; p=0.18) or in those with stable disease for ≥24 weeks (median increase 152 cells/μL; p=0.13). HIV remained suppressed in all participants. One treatment-related death was reported in a Kaposi sarcoma (KS) patient from a diffuse KS herpesvirus (KSHV)-associated polyclonal B-cell lymphoproliferation. The patient had a previous history of elevated peripheral blood mononuclear cell-associated KSHV and KSHV-associated inflammatory cytokine syndrome [81]. Therefore, in these patients, treatment with ICIs should be evaluated with caution. ICIs are particularly active in KS patients (ORR 67% with 1 CR in a recent retrospective study) [82] and upfront treatment with pembrolizumab is under evaluation in the phase II KAPKEY study.

Conclusions and future perspectives

Immune checkpoint inhibitors are a new class of anticancer agents with a unique mechanism of action and a peculiar spectrum of side effects. In a relatively small fraction of unselected patients use of these agents lead to long-term disease control, with a more favorable safety profile than that seen with conventional anticancer agents, such as chemotherapy. The potential for long-term exposure to these agents and their unique mechanisms of action, as well as the growing number of patients treated worldwide pose novel therapeutic challenges in clinical practice. Several retrospective studies have therefore evaluated the potential effects on therapeutic efficacy and/or safety of different concomitant medication that might theoretically interfere with the mechanisms of ICI action.

The role of antibiotics use in cancer patients treated with ICIs is one of the most well

studied. Accumulating evidence indicates that the composition of the intestinal microflora has a major impact on patient prognosis, revealing a strong interaction between specific immunogenic bacteria and systemic immune response [83]. Collectively, these studies suggest that ATB use seems to have a negative impact on outcomes in patients receiving ICIs by decreasing the diversity of gut microbiota and eliminating the most immunogenic bacteria [38]. However, several questions still remain unanswered, including the optimal duration and window of antibiotic use respective to ICIs, the class and the route of administration of antibiotics, and the potential impact of other concomitant medications that might contribute to the dysbiosis of cancer patients, such as proton pump inhibitors, steroids, and diet composition. In addition, the survival impact of antibiotics might be influenced in these studies by other poor prognostic factors that may be associated with the use of these medications, such as a poor ECOG PS, hospitalization, and concomitant presence of bacterial infections. Further prospective studies are needed to better clarify the impact of ATBs on the efficacy of ICIs and, in absence of clear evidence, the use of ATBs, especially for long or repeated courses, during immunotherapy should be carefully evaluated, bearing in mind that their use cannot be avoided or delayed in cases of bacterial infections and opportunistic infections present in patients requiring prolonged steroid therapies due to severe irAEs.

The same considerations for ATBs are also valid for the concomitant use of proton pump inhibitors. Widely used PPIs might potentially induce changes in gut microbioma and has been recently porposed to interfere with therapeutic efficacy of ICIs. If confirmed, this data may provide useful information for exploiting gut microbioma as a driver of antitumor immunity instead of only a predictive biomarker of efficacy for ICIs, allowing for overcoming primary resistance to these agents.

Moreover, an adequate evaluation of concomitant medications during ICIs is essential as several medications can affect not only immunotherapy efficacy, but also its safety profile. For instance, the use of PPIs and/or non-steroidal anti-inflammatory drugs (NSAIDs) has recently been advocated as one of the potential causes of acute tubulointerstitial nephritis (ATIN), a rare complication during anti-PD1 treatment. This rare drug-related renal manifestation is associated with drug-specific T-cells. ATIN may be exacerbated by the reactivation of the T-lymphocyte immune response following ICI therapy disrupting long-standing immunological tolerance to drugs that have been used safely previously, leading to the development of drug-induced ATIN [84, 85]. Although corticosteroid therapy is recommended, the recognition and discontinuation of concomitant drugs, especially those known to induce ATIN, is necessary for the management of kidney injury associated with

anti-PD-1 therapy and may allow for reinitiating ICI therapy after complete resolution of renal damage and withdrawn of other potentially offending agents.

The use of corticosteroid is the mainstay treatment for irAEs, due to their immunosuppressive properties and in the hypothesis that this action could reduce the efficacy of immunotherapy. All ICI pivotal trials excluded patients requiring concomitant treatment with prednisone doses above 10 mg daily or equivalent. However, in clinical practice a not negligible portion of cancer patient needs steroid doses of ≥10 mg a day for different clinical conditions, such as brain metastases and COPD control. Therefore, several retrospective studies evaluated the effect of early steroid use in cancer patients treated with ICIs in real world populations. Collectively, these studies suggest that early steroid use is associated with a poor prognosis, even though it was also correlated in some of these studies with other unfavorable prognostic factors, such as high tumor burden, poor ECOG PS, and brain metastases. Therefore in most of these studies multivariate analyses were conducted to minimize the confounding effect of these well-known prognostic factors [3-5]. Whether early steroid use is truly an independent prognostic factor is still debated and it is still unclear whether the duration of treatment (prolonged versus intermittent use), the dosage and the route of administration might have a differential impact. The use of corticosteroids as premedication in phase III randomized trials evaluating different chemo-immunotherapy combinations seemed not to compromise the efficacy of ICIs addition [86-88], suggesting that a prolonged instead of an intermittent use of corticosteroids might have a higher impact. In addition, the use of corticosteroids in patients experiencing irAES was reported not to negatively impact the outcomes of patients treated with either CTLA4 [7] or PD(L)-1 inhibitors [8, 9]. Further prospective studies are needed to clearly define the role of concomitant use of steroids at dosages above 10 mg/daily of prednisone.

Furthermore, the potential effect of vaccination in cancer patients treated with immunotherapy has been recently evaluated. The picture emerging from these studies is that the use of inactivated influenza vaccine in patients undergoing treatment with PD(L)-1 inhibitors is safe. Very limited data has been reported to date for other commonly used vaccines and their usage should be carefully evaluated in these patients.

Finally, as the number of indications for ICB grows, the number of HIV-infected patients potentially treated with these agents is destined to rise. Current evidence, coming mostly from case reports and small case series, suggest that single agent PD-1/PD-L1 inhibitors can be used safely in HIV-infected NSCLC patients with similar efficacy results observed in the overall population. Several ongoing clinical trials are evaluating ICIs in HIV-infected patients with different solid tumors (NCT03094286, NCT02408861) or NSCLC only

(CHIVA-2/NCT03304093).

In conclusion, multiple studies have evaluated the effects of different concomitant medications commonly used in clinical practice on ICI activity and/or safety. Prolonged steroid therapies as well as extensive use of antibiotics, whenever possible, should be limited, given the potential negative impact on outcomes of such patients. However, the use of these agents in case of irAEs as well as other common indications (i.e. bacterial infections and COPD) should not be avoided and further prospective studies are needed. Moreover, the growing role of gut microbioma on the efficacy of ICIs deserves further investigations, not only as a marker of primary resistance to these agents, but also as a potential therapeutic strategy. Accumulating evidence suggest that inactivated influenza vaccine can be safely administrated to cancer patients in treatment with PD(L)-1 inhibitors, albeit the safety of other commonly used vaccine is far less known and deserves further studies. An accurate evaluation of concomitant medications is essential in patients receiving anticancer therapies, including ICIs, in order to prevent unexpected toxicities or compromise therapeutic efficacy. Finally, the role of ICIs in patients with chronic infections, including HIV-infected, is still debated and the results of ongoing clinical trials in this special population are eagerly awaited and will provide further evidence on safety and efficacy of immunotherapy.

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CHAPTER 3

Markers of systemic inflammation and outcome in advanced NSCLC patients treated with immune checkpoint inhibitors

Extracted From:

"Baseline neutrophilia, derived neutrophil-to-lymphocyte ratio (dNLR), platelet-to-lymphocyte ratio (PLR), and outcome in non small cell lung cancer (NSCLC) treated with Nivolumab or Docetaxel"

Alessandro Russo, Tindara Franchina, Giuseppina Ricciardi, Alessandra Battaglia, Antonino Scimone, Rosa Berenato, Antonio Giordano, Vincenzo Adamo.

Published in "Journal of Cell Physiology"

"**Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and lactate dehydrogenase (LDH) levels and outcome with nivolumab in pre-treated Non Small Cell Lung Cancer (NSCLC): a large retrospective multicenter study**"

Alessandro Russo, Marco Russano, Tindara Franchina, Maria Rita Migliorino, Giuseppe Aprile, Giovanni Mansueto, Alfredo Berruti, Alfredo Falcone, Michele Aieta, Alain Gelibter, Antonio Russo, Sandro Barni, Michele Maio, Olga Martelli, Francesco Pantano, Daniela Iacono, Lorenzo Calvetti, Silvia Quadrini, Elisa Roca, Enrico Vasile, Marco Imperatori, Mario Occhipinti, Antonio Galvano, Fausto Petrelli, Luana Calabrò, Giulia Pasquini, Salvatore Intagliata, Giuseppina Ricciardi, Giuseppe Tonini, Daniele Santini, Vincenzo Adamo.

Under revision in "Targeted Oncology"

3.1 Introduction

The therapeutic landscape of advanced/metastatic NSCLC has been recently revolutionized with the clinical introduction of immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 axis with unprecedented results in terms of overall survival in different clinical settings [1, 2]. Nivolumab is a therapeutic option in NSCLC patients progressing after platinum-based chemotherapy in both squamous and non-squamous histology, regardless of PD-L1 expression [3, 4]. Immunotherapy has the notorious ability to induce highly durable tumor responses [5] and NSCLC is not an exception, with reported 3-year survival rates of 17% in the two pivotal trials with nivolumab in pre-treated NSCLCs [6]. Therefore, the identification of predictive biomarkers is crucial for the optimal selection of patients candidate for 2nd line therapy. However, there are no currently approved predictive biomarkers for nivolumab in NSCLC and the role of immunohistochemical (IHC) expression of PD-L1, used as selection criteria for pembrolizumab in both 1st and 2nd line therapy [7, 8] is controversial. The identification of reliable predictive biomarkers to these agents is lacking and multiple clinic-pathological factors have been evaluated to date [9]. Hence, there is still a high-unmet medical need and novel additional clinical and bio-molecular parameters allowing a proper patients selection are eagerly awaited.

Lymphocytes play a central role in the action of anti-PD-1/PD-L1 agents and their activation and intra-tumor invasion are necessary for antitumor immune response reactivation. However, the immune response is the results of multiple interactions between T cells and other regulatory cells, including neutrophils, and they are critical in forming the immune environment. Indeed, neutrophils have recently proved to play pleiotropic actions in the cancer-immunity interactions, generating an immunosuppressive environment through the production of chemokines and cytokines that are involved in complex cross talk with other immune cells [10, 11]. Given their peculiar mechanism of action, alterations in the relative proportion of peripheral blood leukocytes may influence the efficacy of ICIs.

Inflammation is an established hallmark of cancer and plays a central role in tumor promotion and progression [12]. Therefore, it is not surprisingly that multiple markers of systemic inflammation have been correlated with poor outcome in multiple solid tumors, including NSCLC. Neutrophils dominate the immune landscape of NSCLC and, in addition to the well-known role in host defense, have been recently associated with important and significant actions in tumor biology with both anti- (N1 phenotype) and pro-tumor (N2 phenotype) functions, probably in a context-dependent fashion [13-15].

Recently, some Authors, including our group, suggested a possible prognostic role of peripheral markers of inflammation in NSCLC patients treated with ICIs targeting the PD-1/PD-L1 axis. In this chapter we will summarize the latest evidence on this emerging research field.

3.2 Neutrophil-to-lymphocyte ratio (NLR) & ICIs outcome in NSCLC

Neutrophil-to-lymphocyte ratio (NLR) is a marker of chronic inflammation and reflects the alterations in the peripheral blood leukocytes associated with inflammation. This marker has been extensively associated with poor outcomes in NSCLC and other solid tumours in the pre-immunotherapy era and, more recently, it has being associated with poor outcomes in pre-treated NSCLC patients undergoing nivolumab therapy with different cut-off values [10, 16-18]. Moreover, some studies have reported a potential predictive role for changes of NLR levels during treatment with nivolumab [19-21], suggesting that treatment with anti-PD-1/PD-L1 agents may be associated with a broad spectrum of changes in the immune microenvironment of the tumour, leading to decrease in the neutrophil count and increase in the lymphocyte count in responding patients. Other Authors, in order to limit the possible interaction of other confounding factors, developed a predictive model (iSEND) that included sex, ECOG PS, NLR levels (≥5 or <5), and delta NLR (calculated with NLR at baseline and before the second course of nivolumab) [22], showing that

patients within the poor risk group (iSEND poor) were significantly associated with progressive disease. In addition, other Authors have evaluated the derived neutrophil-to-lymphocyte ratio (dNLR), a novel parameter that includes, in addition to absolute neutrophil count, other granulocyte populations, reporting a poorer outcome with nivolumab in patients with high dNLR values (dNLR ≥3) [23, 24].

Recently, we confirmed, in a large multicentre study conducted in 14 Italian oncology centers, the negative predictive role of high baseline NLR (NLR ≥ 5) in patients treated with nivolumab, with a shorter PFS (p=0.03) and OS (p=0.001) and a trend towards a decreased DCR (p=0.06) compared to patients with low baseline NLR levels (NLR < 5) [**Fig. 1**]. High NLR levels may therefore the results of an increase in neutrophil-dependent inflammation as well as reduced lymphocyte activity and infiltration, determining a weaker lymphocyte-mediated immune response and subsequent poor response to ICIs [17]. These data suggest that pre-treatment evaluation of NLR levels may be a useful [17] the decision making of unselected patients candidate to 2nd line therapy in NSCLC.

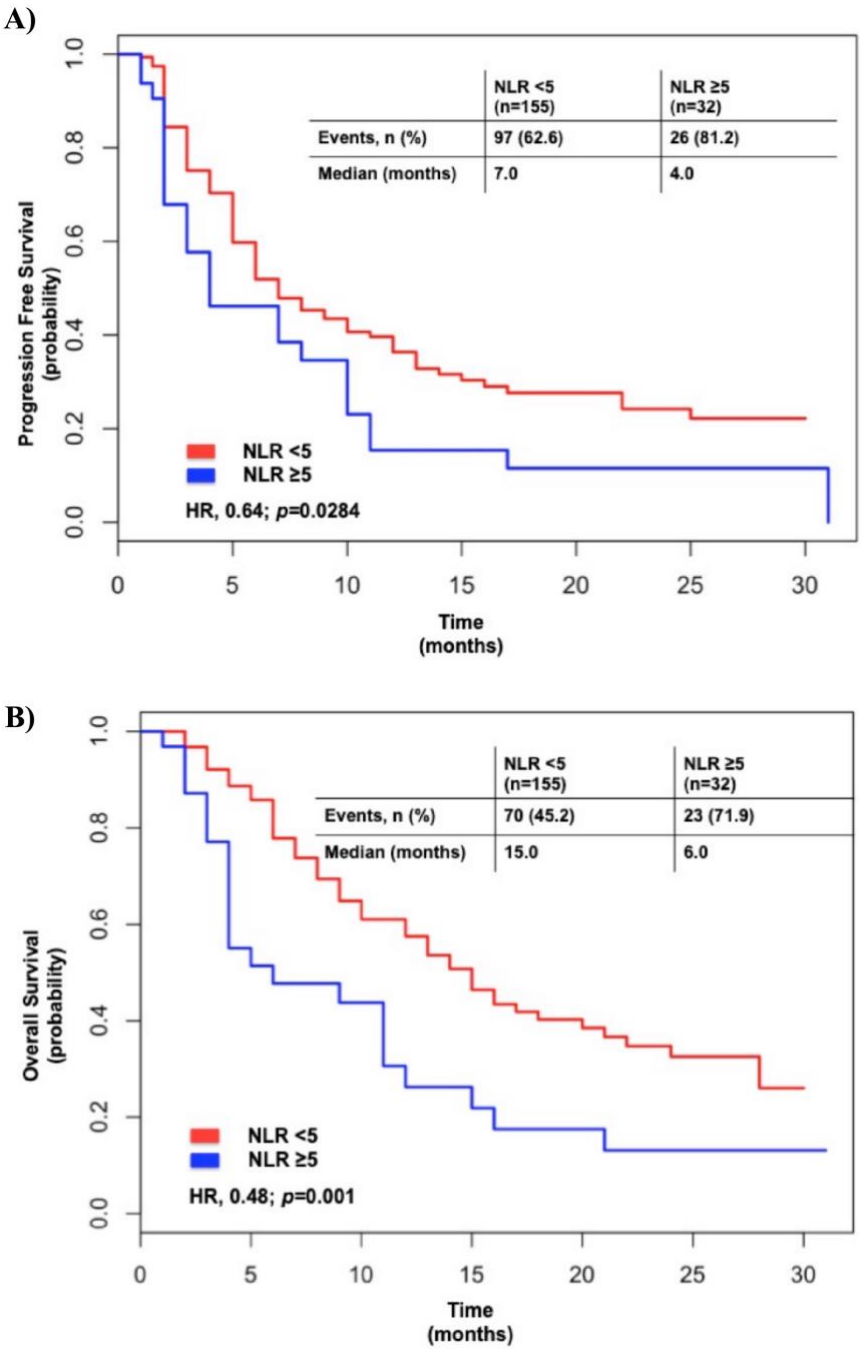

Figure 1 Kaplan-Meier curves for PFS (A) and OS (B) according to NLR levels (*From Russo A, et al. Targeted Oncology – under revision*)

In addition, we recently evaluated in a small retrospective study conducted at the Medical Oncology Unit of the A.O. Papardo (Messina, Italy) [25] the dynamic changes of some markers of inflammation over time, including NLR, and the outcome of NSCLC patients treated with nivolumab or pembrolizumab. Interestingly, NLR ≥5 was associated with

lower PFS and OS, with an increased predictive value over time (p=0.01 and p=0.009 at baseline; p=0.007 and p<0.001 at 6 weeks; p<0.001 and p<0.001 at 12 weeks, respectively) [**Fig. 2**].

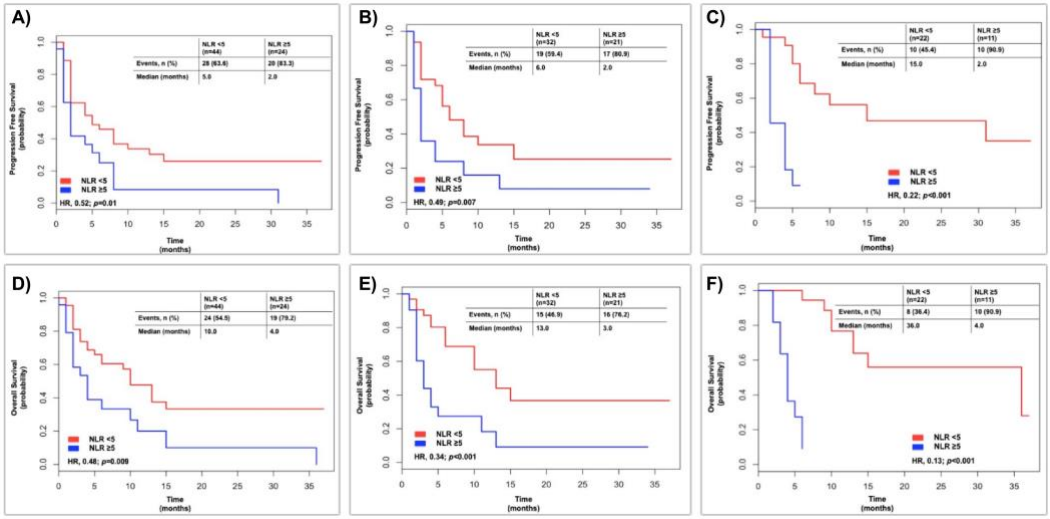

Figure 2. PFS and OS according to NLR levels changes over time (A-D baseline, B-E at 6 weeks, C-F at 12 weeks) (*From Russo A, et al. ASCO annual meeting 2019*) [25]

3.3 Platelet-to-Lymphocyte ratio (PLR) and immunotherapy

Baseline platelet-to-lymphocyte ratio (PLR) levels have been correlated with poor prognosis in several solid tumours, including NSCLC [26]. Recently this hematologic parameter has been evaluated in small retrospective studies also in NSCLC patients treated with nivolumab. Using different cut-off values (PLR ≥160 and ≥200, respectively), some Authors did not find any statistically significant difference in terms of OS or ORR between NSCLC patients treated with nivolumab with high pre-treatment levels compared with those with low PLR values [23, 17]. In particular, in a small monocentric retrospective study evaluating the role of PLR in patients treated with nivolumab or chemotherapy, we reported that patients with high PLR levels tended to have a shorter OS in the overall population (4.0 vs. 12.0 months, p=0.085) [**Fig. 3**] and with both Nivolumab (6.0 vs. 10.0

months, p=0.756) and Docetaxel (4.0 vs. 8.5 months, p=0.352) compared to those with PLR levels <160, albeit these differences were not statistically significant [23].

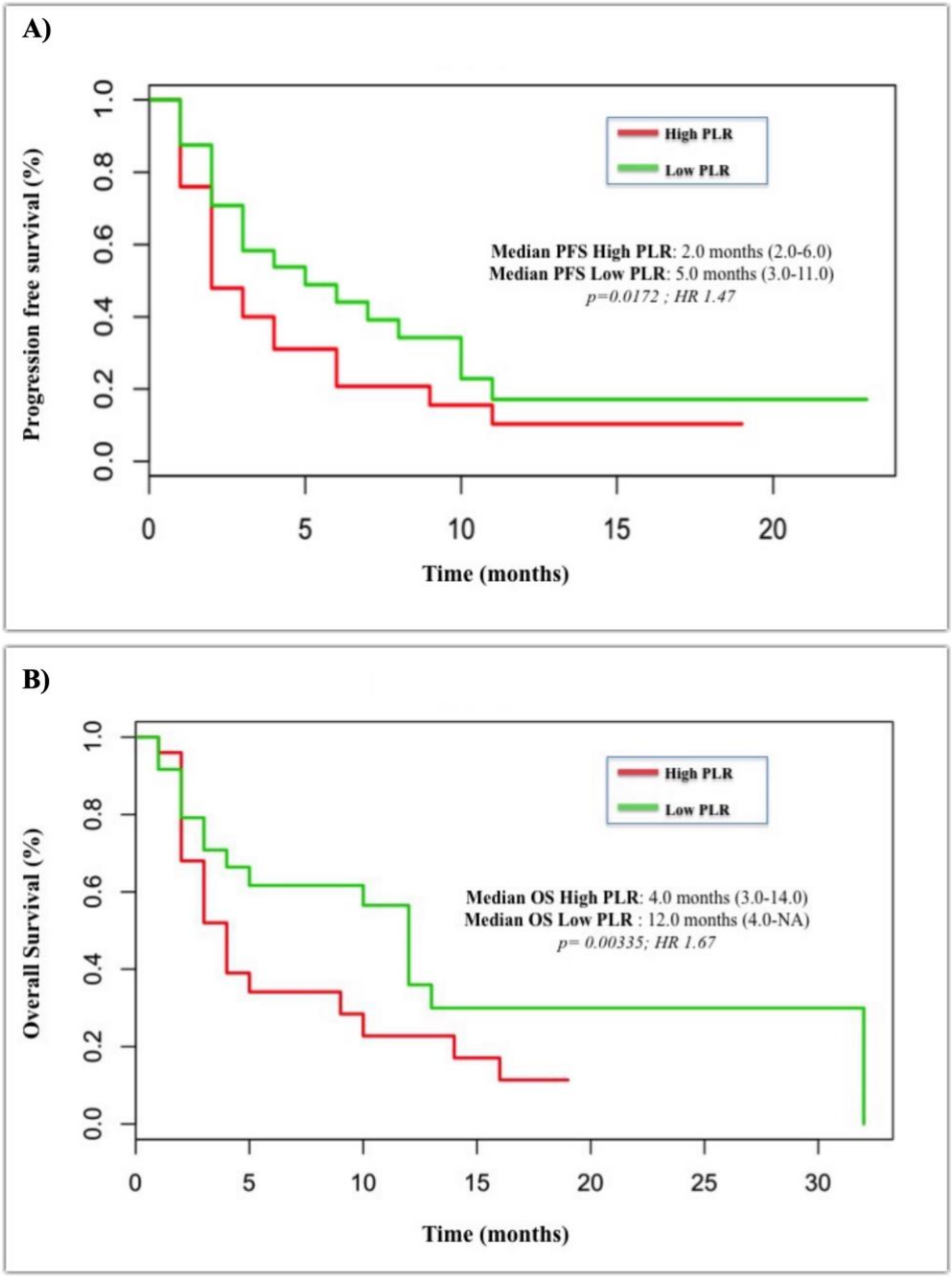

Figure 3 Kaplan-Meier curves for PFS (A) and OS (B) according to PLR levels in patients with NSCLC treated with chemotherapy or nivolumab as 2nd line (*From Russo A, et al. J Cell Physiol 2018*) [23]

In contrast, Diem et al. subdividing patients into three groups, according to PLR tertiles (PLR<193, PLR 193-328 and PLR>328) showed that patients with higher PLR values had

worse OS and ORR [27]. Recently, we reported in a large multicentre study that a pre-treatment PLR level ≥200 is associated with a statistically significant worse PFS (p=0.03) and OS (p=0.05), as well as a decreased response (p=0.04) and DCR (p=0.001) with nivolumab [**Fig. 4**].

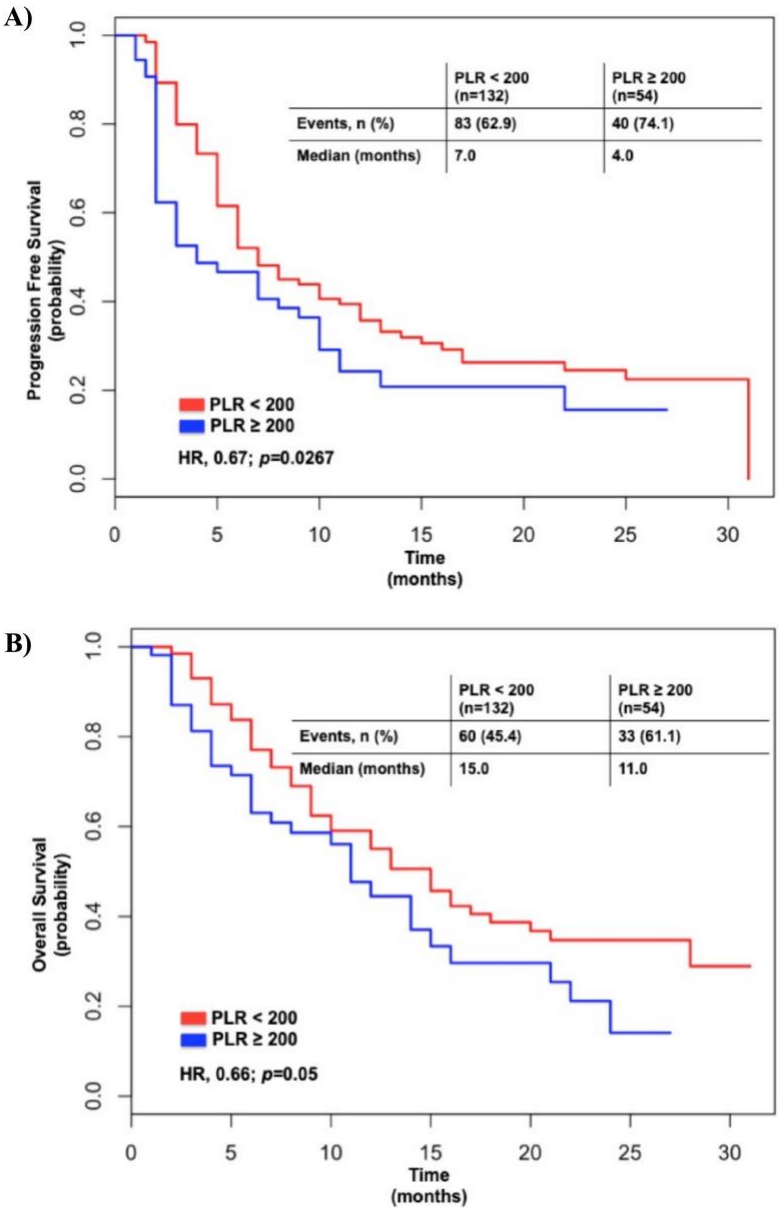

Figure 4 Kaplan-Meier curves for PFS (A) and OS (B) according to PLR levels (*From Russo A, et al. Targeted Oncology – under revision*)

In addition, we also evaluated in in a small retrospective study conducted at the Medical Oncology Unit of the A.O. Papardo (Messina, Italy) [25] the dynamic changes of PLR

levels during treatment with ICIs in NSCLC, reporting that PLR ≥200 at baseline and 12-wk was significantly associated with shorter OS (p=0.05 and p=0.004, respectively), but no in terms of PFS at all the three time points analyzed (baseline, 6 weeks and 12 weeks) [**Fig. 5**].

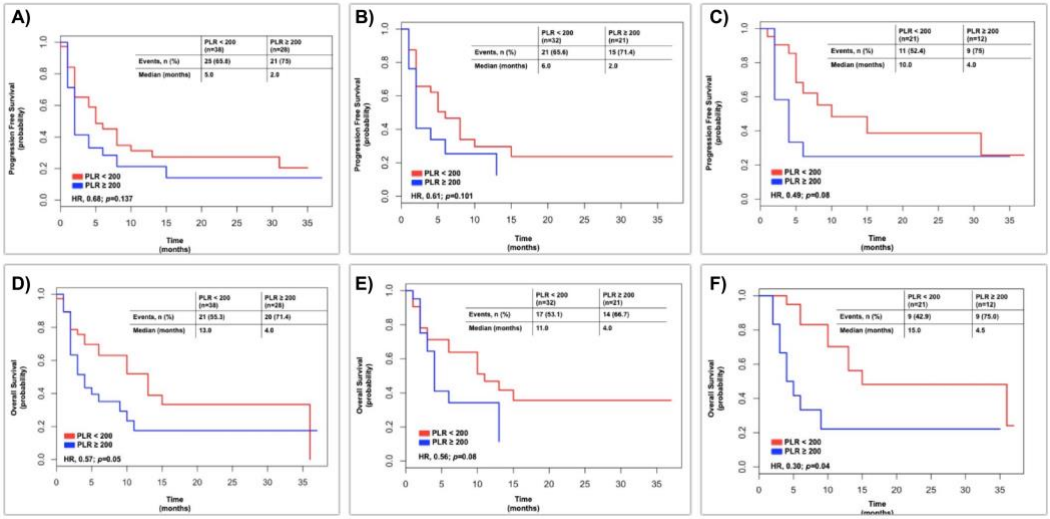

Figure 5. PFS and OS according to PLR levels changes over time (A-D baseline, B-E at 6 weeks, C-F at 12 weeks) (*From Russo A, et al. ASCO annual meeting 2019*) [25]

3.4 LDH levels and LIPI score in NSCLC patients treated with ICIs

Lactate dehydrogenase (LDH) is a marker of inflammation and tumour burden in patients with solid tumours. Recently, some Authors have reported inferior outcomes in NSCLC patients with high LDH levels treated with nivolumab [11, 24]. We recently evaluated the possible predictive role of LDH levels changes during treatment with ICIs in NSCLC at 6 weeks and 12 weeks. We reported that LDH ≥UNL (Upper Normal Limit) at baseline was associated with shorter PFS and OS (p=0.02 and p=0.03), as well as a reduction of LDH levels at 12-wk compared with baseline values (p=0.006 and p=0.004) [**Fig. 6**].

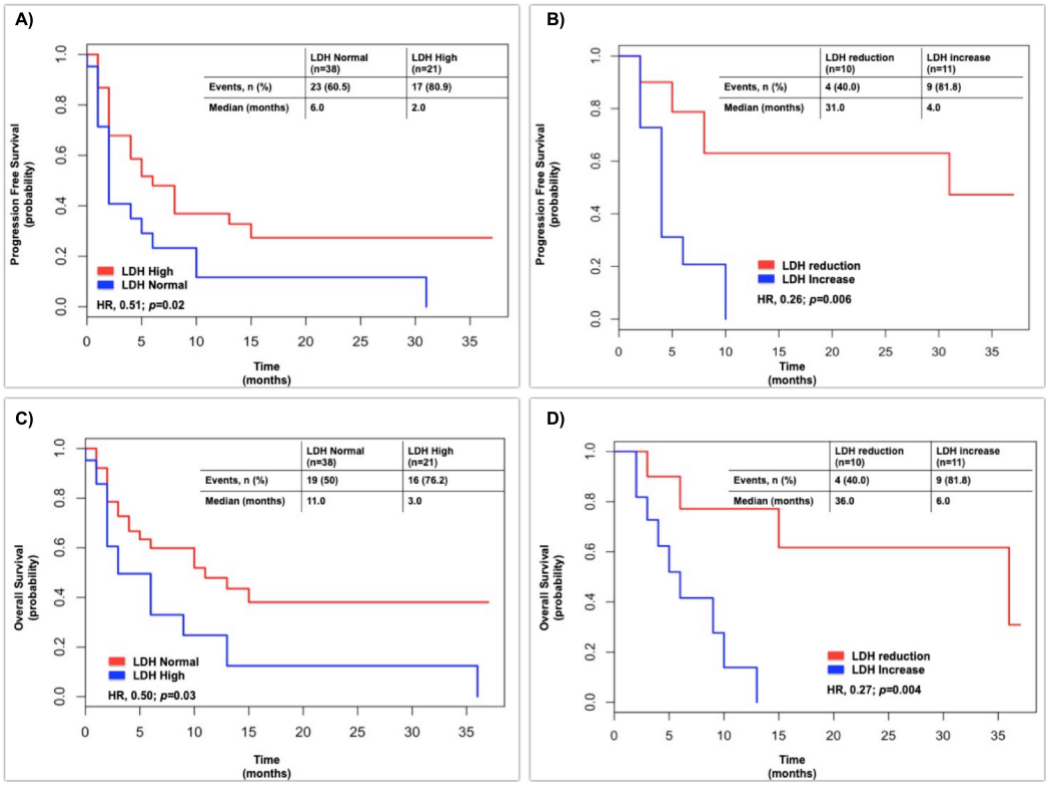

Figure 6. PFS and OS according to LDH levels at baseline (A-C) and changes at 12 weeks (*From Russo A, et al. ASCO annual meeting 2019*) [25]

In addition, recently some Authors have described a novel prognostic index, known as LIPI (lung immune prognostic index) based on dNLR greater than 3 and LDH greater than ULN, characterizing 3 groups (good, 0 factors; intermediate, 1 factor; poor, 2 factors) of patients. Pretreatment LIPI was correlated with worse outcomes for ICI, but not for chemotherapy in a larger retrospective study [24]. In contrast, another retrospective study evaluating the role of LIPI for prediction of atezolizumab outcome in a pooled analysis of 4 clinical trials [28], showed that LIPI is also a prognostic marker of survival and response for patients treated with chemotherapy and thus it is not specifically predictive for ICI treatment. Moreover, an exploratory pooled analysis of 11 randomized clinical trials in advanced NSCLC further confirmed that LIPI score is an important prognostic biomarker irrespective of treatment modality for patients with mNSCLC [29].

3.5 Conclusions

These routine available peripheral blood markers of inflammation, if validated in large prospective studies, may be an attractive biomarker that can be easily and quickly integrated in clinical practice, without additional costs, and may help clinical decision-making. In conclusions, patients with pre-treated NSCLC and high pre-treatment levels of NLR (≥5), PLR (≥200) and LDH levels ≥UNL may experience inferior outcomes when treated with ICIs. Therefore, in this poor prognosis subgroup of patients the use of alternative therapeutic strategies, such as the combination docetaxel/nintedanib or docetaxel/ramucirumab, may be a valuable option, especially in the case of negative PD-L1 expression and/or the presence of other additional poor prognostic factors (such as high tumour burden, liver and bone metastases, ≥2 previous lines of therapy, ECOG PS ≥1, never smoking status, and oncogene-addicted tumours) [30].

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CHAPTER 4

Liquid biopsy tracking of lung tumor evolutions over time

Extracted from:

"**Liquid biopsy tracking of lung tumor evolutions over time**"
Alessandro Russo, Diego de Miguel Perez, Muthukumar Gunasekaran, Katherine Scilla, Rena Lapidus, Brandon Cooper, Ranee Mehra, Vincenzo Adamo, Umberto Malapelle, and Christian Rolfo.

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"**Liquid biopsy in lung cancer screening: The contribution of metabolomics. Results of a pilot study.**"
Sandeep Singhal, Christian Rolfo, Andrew W. Maksymiuk, Paramjit S. Tappia, Daniel S. Sitar, **Alessandro Russo**, Parveen S. Akhtar, Nazrina Khatun, Rahnuma Parveen, Rashiduzzaman Ahmed, Rashid Ahmed Bux, Guoyu Huang, and Bram Ramjiawan.

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"Post-surgery Circulating tumor cells and AXL overexpression new poor prognostic biomarkers resected lung adenocarcinoma"

Diego de Miguel-Pérez, Clara Isabel Bayarri-Lara, Francisco Gabriel Ortega, **Alessandro Russo**, María José Moyano Rodriguez, Elizabeth Maza Serrano, José Antonio Lorente, Christian Rolfo, and María José Serrano.

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"Is there a room for personalized medicine in small cell lung cancer (SCLC)? Remarkable activity of pazopanib in a refractory FGFR1-amplified ED-SCLC patient"
Alessandro Russo, David Arias Ron, Marika Rasschaert, Hans Prenen, Ranee Mehra, Katherine Scilla, Patrick Pauwels, and Christian Rolfo.

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4.1 Introduction

The discovery of Epidermal Growth Factor Receptor (EGFR) mutations in 2004 [1, 2] and the subsequent demonstration that a targeted therapy is more efficacious than platinum doublet chemotherapy in molecularly selected patients [3, 4] represented a dramatic shift in the management of advanced non-small lung cancer (NSCLC), giving rise to the era of personalized medicine in lung cancer. These results profoundly influenced drug development in NSCLC, paving the way to a novel series of molecularly selected studies with specific tyrosine kinase inhibitors (TKIs) [5-7], expanding considerably the list of available oncogenic targets in NSCLC [8]. The consequence of the explosion of personalized medicine in NSCLC is the constant growing of the numbers of gene that should be tested for a correct therapeutic management of patients, but faces with the limited amount of tissue usually available in a disease that traditionally is diagnosed using small histological or even cytological samples. Unfortunately, in clinical practice up to 30% of the samples are inadequate for molecular testing [9] and, despite a broad agreement on the importance of biomarker testing in NSCLC patients, even the most common targetable drivers, such as EGFR and ALK, are not always assessed [10]. Liquid biopsy refers to a multitude of minimally invasive techniques that can allow a real-time bio-molecular characterization of the tumor through the analysis of human body fluids. Among the different components of the liquid biopsy [**Fig. 1**] cell-free DNA (cfDNA) is the most well studied and widely adopted source for tumor genotyping in lung cancer and has already entered clinical practice for detection of both EGFR sensitizing and resistance mutations [8, 11]. In contrast to tissue biopsy, liquid biopsy presents several undeniable advantages: minimally invasive, repeatable over time without risks for the patient, can better capture intra-tumor heterogeneity, and might offer a more timely picture of the actual status of the tumor compared with archival histological samples [12]. The current diagnostic algorithm considers cfDNA genotyping for EGFR mutational testing in case of insufficient tumor tissue at diagnosis of advanced/metastatic disease or at progression after 1st/2nd generation EGFR tyrosine kinase inhibitors (TKIs) for identification of secondary mutation T790M [8, 11]. However, the widespread use of plasma next generation sequencing (NGS), which allows a more comprehensive molecular characterization besides EGFR mutational testing only and the recent positive results of the NILE (Non-invasive versus Invasive Lung Evaluation) study [13] compared to standard of care tissue genotyping suggested a paradigm shift in the diagnostic algorithm of advanced NSCLC, moving from the old concept "*tissue first*" to a "*blood first*" approach.

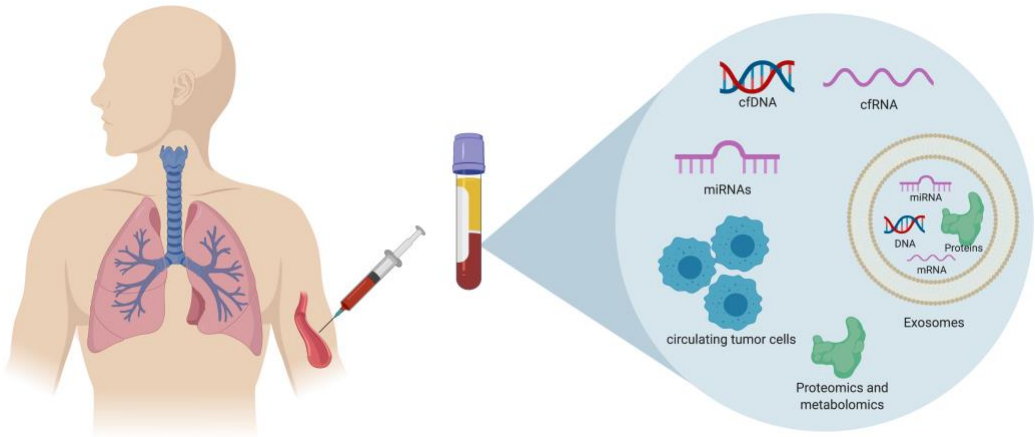

Figure 1 Liquid biopsy in lung cancer (*Credit: Created with Biorender*) [*From Russo A, et al. Expert Rev Mol Diagn 2019*].

Clinical indications of liquid biopsy are constantly growing and are revolutionizing the diagnostic algorithm of several solid tumors, including NSCLC. Furthermore, the impressive technological advances made in the last few years are expanding the potential applications moving from advanced/metastatic disease to early detection and minimal residual disease evaluation. In addition, considerable efforts are trying to extend its use also in small cell lung cancer (SCLC), an aggressive disease with dismal prognosis in which targeted therapies have traditionally failed to provide any significant benefit. Herein we provide a comprehensive overview of the role of liquid biopsy in lung cancer, focusing mainly only on the most-well studied members of the liquid biopsy family, cfDNA and circulating tumor cells (CTCs).

4.2 Liquid biopsy in early detection and evaluation of minimal residual disease (MRD) in NSCLC

Survival rates of NSCLC are largely dependent on tumor stage, with a 5-year overall survival rate that passes from 92% in stage IA1 to 0% in stage IVB [14]. Low dose computed tomography (LDCT) screening showed to reduce lung cancer mortality in large randomized clinical trials [15-17], improving the likelihood of detection of small non-calcified nodules, and thus of lung cancer at an earlier and potentially more curable [18, 19]. However, the risk of false positive results is not negligible and even using modern protocols, such as Lung-RADS 1.0, considering 1000 subjects screened in the National Lung Screening Trial (NSLT), only 41 lung cancer cases would have been diagnosed and

only 3 of them would have not died from lung cancer due to the screening [20]. There is an urgent need for the identification of biomarkers that can allow a better refinement of risk to improve the selection of subjects undergoing LDCT and characterization of indeterminate pulmonary nodules found during the screening [21]. Different studies have investigated the potential role of circulating tumor DNA (ctDNA) in early stage lung cancer [22-25]. ctDNA represents only a minor fraction of cfDNA and, in contrast with advanced/metastatic NSCLC, in which ctDNA variant allele fraction (VAF) can reach >10%, ctDNA-based screening for stage I-II NSCLC needs to detect mutations present at frequencies below the limit of detection of current technologies (VAF <0.5%) [26]. Further technological advancements are needed before clinical implementation of ctDNA profiling as screening tool for lung cancer and likely combinatorial approaches, for example exosomal RNA, might improve its sensitivity [26].

Cancer cells have a fundamentally different metabolism than non-cancerous cells and this difference is manifested in the endogenous metabolites they produce. Metabolomics aims to study global metabolic differences in biological systems by monitoring the levels of small molecular metabolites in biological fluids or tissues and has recently been applied to the discovery of tumor biomarkers for the diagnosis, treatment, and prevention of different solid tumors, including lung cancer [27]. The goal of metabolomics is to identify markers that can help distinguish between lung cancer and healthy patients, various lung cancer types and stages, and also aid in tumor detection [21, 27]. Previous studies have reported the utility of the enzyme spermidine/spermine N1-acetyltransferase-1 (SSAT-1) as a cancer detection tool. SSAT-1 is a key protein involved in the synthesis and homeostasis of the polyamines spermine and spermidine. These polyamines have specific roles in maintaining the membrane potential, controlling intracellular pH, and cell volume. SSAT-1 is upregulated in several solid tumors, including lung cancer. In a preliminary study, we evaluated a robust panel of 14 metabolites associated in the SSAT-1/polyamine pathway along with other endogenous metabolites that correctly discriminated between lung cancer patients from healthy controls [**Fig. 2**] [28].

These results demonstrate the utility of metabolomics for lung cancer detection and adding further evidence on the role of liquid biopsy in cancer interception.

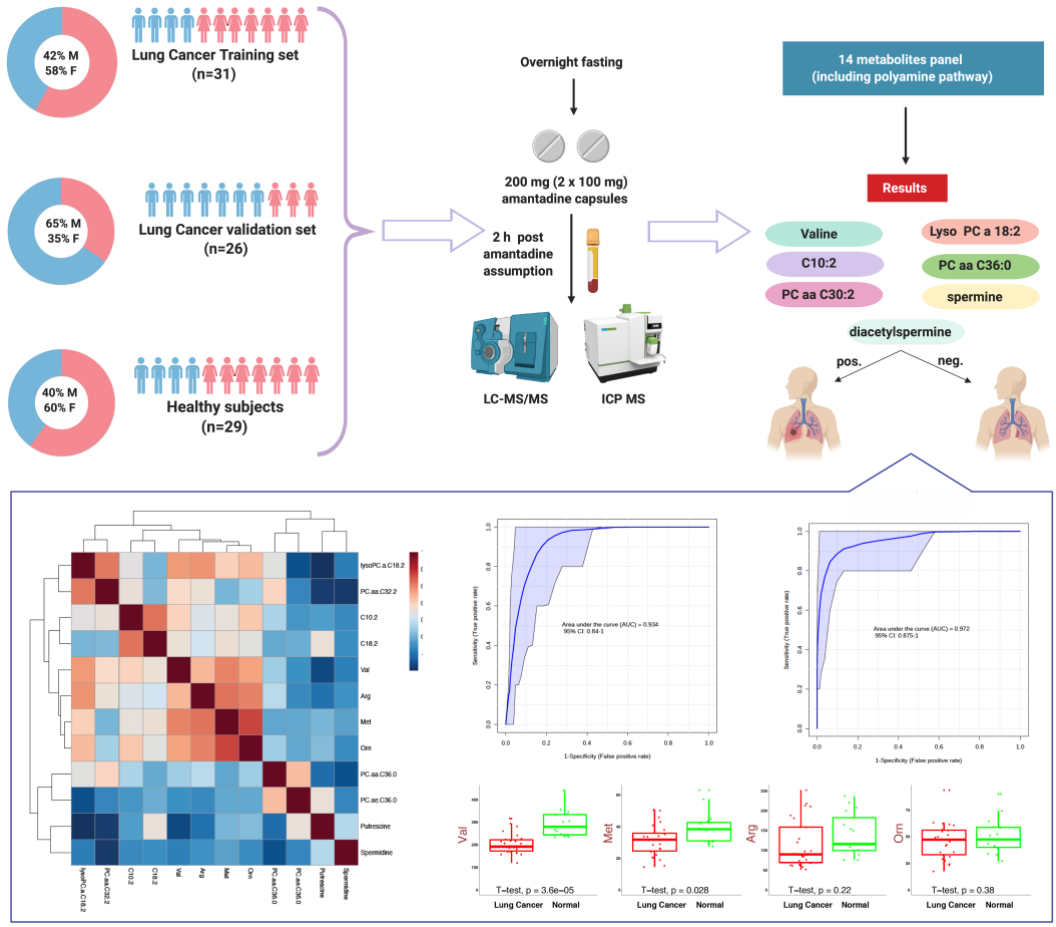

Figure 2 Metabolomics as early lung cancer detection tool. In the upper part of the figure is depicted the study design. In the bottom part are reported the main results (*Credit: Created with Biorender*) (*From Singhal S, et al. Cancers 2019*) [28].

Another extensively investigated field is the identification of minimal residual disease (MRD). With this term is described a clinical condition associated with increased risk of disease recurrence due to the presence of occult micrometastases after radical treatment for an early stage cancer in absence of any clinical and radiological sign of metastasis or residual disease. However, the role of liquid biopsy, especially ctDNA (mutations present at VAFs <0.1%) and CTCs, is challenged by the very low concentrations detectable in blood samples [26, 29]. For instance, in the TRACERx study was reported that detection of single nucleotide variations (SNVs) in ctDNA, using NGS-based assay panels synthesized for each patient, was associated with non-adenocarcinoma histology, necrosis, increased proliferative indices and lymphovascular invasion. In a cohort of 24 patients with longitudinal samples (pre- and post-surgery), detection of SNVs in ctDNA seemed correlated with disease recurrence (93% in patients with disease recurrence vs. 10% in patients without recurrence), preceded CT inconclusive for disease recurrence by several months in some cases, and reflected resistance to adjuvant chemotherapy [25]. Similarly,

in another study using a very sensitive approach (cancer personalized profiling by deep sequencing, CAPP-seq), ctDNA was detected in 94% of early stage NSCLC patients (Stage I-IIIA) experiencing disease recurrence and preceded radiographic progression in 72% of patients by a median of 5.2 months [24]. These data suggest the utility of ctDNA profiling for MRD identification and might impact adjuvant therapeutic strategy after curative intent surgery, changing clinical trial design. However, further technical improvements are needed before clinical implementation, especially considering the low ctDNA detection levels among stage I patients and the costs of these methodologies. Few studies have also evaluate the role of CTCs for MRD evaluation, suggesting that postoperative CTCs correlate with a shorter relapse free survival in early stage NSCLC both after surgery [30, 31] and stereotactic body radiotherapy [32].

We recently assessed the association between CTCs subpopulations and outcome of resected early stage lung adenocarcinoma (ADC) patients at three different time-points (CTC1-3) (before surgery, after one month, and after six months) in comparison to squamous cell carcinoma (SCC), as well as gene and miRNA tissue expression, immunoprofiling and epithelial-to-mesenchymal transition (EMT) markers [**Fig. 3**]. In the multivariate analysis, CTC2 was an independent prognostic factor for RFS and CTC3 and AXL were independent prognostic for OS in ADC. Neither the surgery nor the adjuvant treatment influenced the prognosis of these patients.

In addition, detectable CTC levels seems to precede radiologic evidence of disease recurrence in locally advanced NSCLC (stage II-III) treated with chemo-radiotherapy, as recently reported in a small retrospective study [33]. Future prospective studies with higher number of patients will define the place of CTCs detection in the diagnostic algorithm of early stage NSCLC.

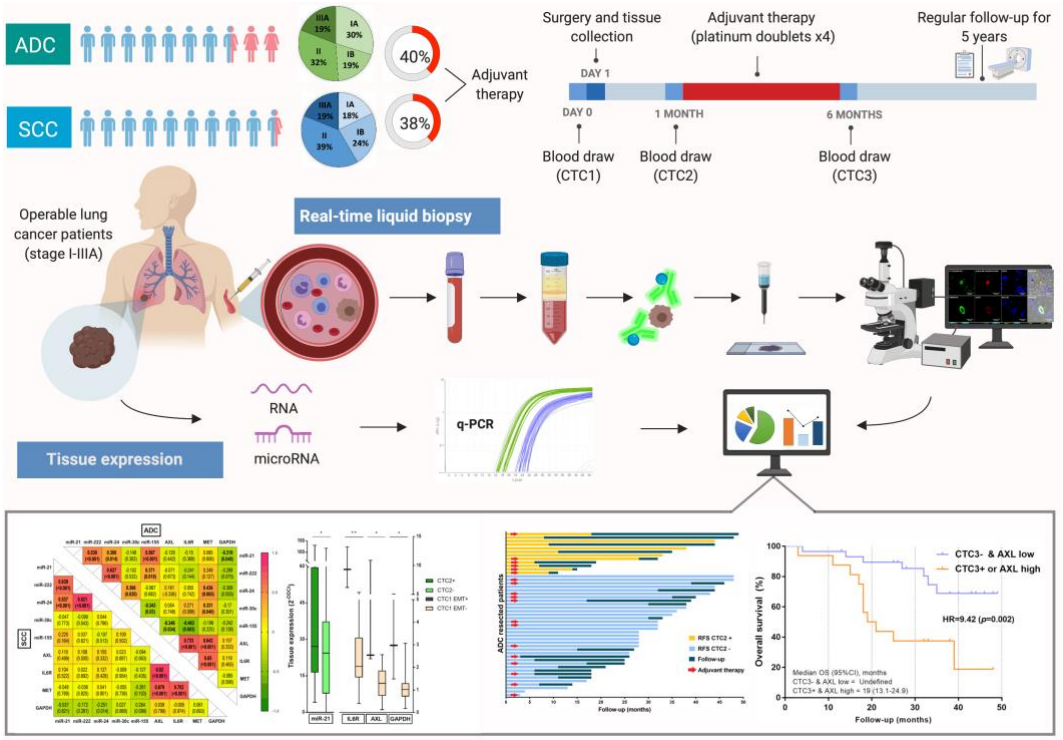

Figure 3 Post-surgery circulating tumor cells and AXL overexpression as new poor prognostic biomarkers in resected lung adenocarcinoma. In the upper part of the figure is depicted the study design. In the bottom part are reported the main results of the study (*Credit: Created with Biorender*) (*From de Miguel-Pérez D, et al. Cancers – under revision*).

4.3 Liquid biopsy in advanced/metastatic NSCLC

Over the last few years, isolation and analysis of cfDNA have emerged as an effective and promising tool for genomic profiling in advanced/metastatic NSCLC [34]. Current international guidelines support the use of cfDNA analysis for the identification of EGFR mutations in treatment-naïve advanced/metastatic NSCLC without sufficient tissue for tumor genotyping or after progression from first/second generation EGFR TKIs for the detection of secondary T790M mutation, reserving tissue biopsy in case of negative results [8, 11]). To date, the only FDA approved liquid biopsy test in lung cancer is the Cobas® EGFR mutation test v2 as a companion diagnostic test for detecting exon 19 deletions or exon 21 (L858R) substitutions in the EGFR gene [35]. A pooled analysis of three randomized studies evaluating erlotinib in first line (ENSURE, FASTACT-2, and ASPIRATION) showed a sensitivity of 72.1% (95% CI 67.8–76.1) and a specificity of 97.9% (95% CI 96.0–99.0) for Cobas® EGFR mutation test v2 compared with tissue [36]. This is RT-PCR based test that can identify 42 different EGFR gene mutations (exons 18-21), including exon 20 T790M. In a retrospective analysis of the AURA and AURA2 trials with osimertinib Cobas® EGFR mutation test v2 showed a positive percent agreement

(PPA) of 61% compared with tissue [37]. These results are inferior to that reported with sensitizing mutations, due to the heterogeneity of the mechanisms of acquired resistance to first/second generation EGFR TKIs, as well as the limit of detection of this assay (**Fig. 4**). Another RT-PCR based test, EGFR Therascreen®, showed good concordance (94.3%), specificity (99.8%), and sensitivity (65.7%) for the evaluation EGFR mutation in cfDNA compared with tissue in the phase IV IFUM study that evaluated gefitinib in first line EGFR-mutated Caucasian patients [38], demonstrating that cfDNA analysis can represent a valid option in case of insufficient tissue for EGFR mutational test. Based on these results, the European Medicine Agency (EMA) extended the use of gefitinib in patients with positive EGFR results on liquid biopsy only. To increase the sensitivity of PCR based methods and detecting selected individual point mutations, digital PCR techniques have been developed, such as BEAMing (beads, emulsion, amplification, and magnetics) or droplet digital PCR (ddPCR), which can identify and quantify alterations present at VAF of 0.01% or less in cfDNA [39]. A retrospective analysis of the phase I AURA trial showed that cfDNA analysis with BEAMing for T790M analysis was associated with similar outcomes on osimertinib (ORR 63%; median PFS 9.7 months) to that observed when treating with osimertinib on the basis of tumor genotyping results (ORR 62%; median PFS 9.7 months) [40]. These results first demonstrated plasma genotyping could be the initial step after acquired resistance to first/second generation EGFR TKIs and a tissue re-biopsy for tumor genotyping could be supplementary in case of negative T790M results. This concept was introduced in the IASLC statement on liquid biopsy in 2018 [11] and is considered a valid approach that could avoid a tumor biopsy for T790M genotyping in approximately 60-70% of the cases using a validated assay [40, 41].

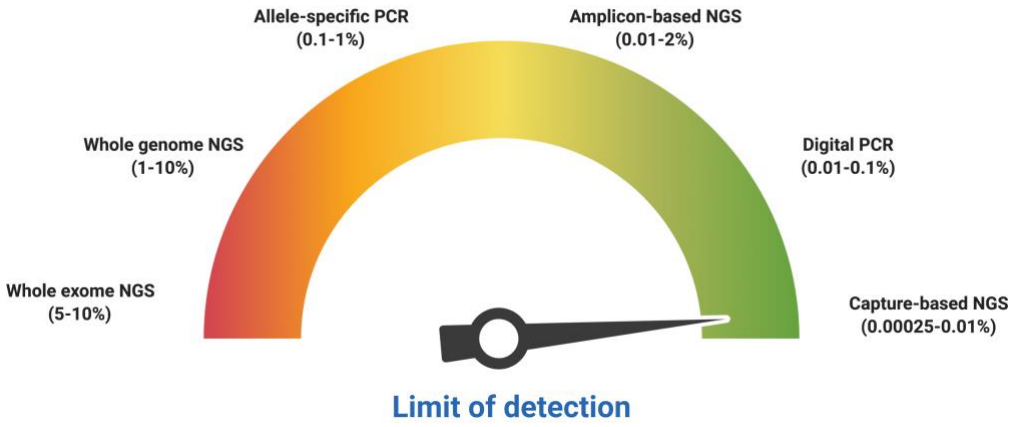

Figure 4 Limit of detection of different methods for cfDNA genotyping (*Credit: Created with Biorender*) [*From Russo A, et al. Expert Rev Mol Diagn 2019*].

The most significant limit of digital PCR is the detection of only selected individual point

mutations that do not allow a comprehensive genotype of treatment-naïve patients without sufficient tissue for molecular testing, missing potential therapeutic targets besides EGFR mutations, or a complete evaluation of the mechanisms of acquired resistance in oncogene-addicted NSCLC. Several commercially available plasma NGS have been developed and their use is growing fast, as they can allow the identification of a higher number of genetic aberrations compared with PCR-based methods, including in some cases also genetic rearrangements (hybrid capture based technologies) and with a high sensitivity (limit of detection of ~0.05% VAF). Recently, the NILE study reported that a 73-gene NGS panel (Guardant360) identifies guideline-recommended biomarkers (EGFR mutations, ALK fusions, ROS1 fusions, BRAF V600E mutation, RET fusions, ERBB2 mutations, MET exon 14 skipping mutations, MET amplifications, and KRAS mutations) at a higher rate at least as high as standard of care tissue genotyping (21.3% vs. 27.3%; $p<0.0001$ for non-inferiority), with high tissue concordance (>98.2% with 100% positive predictive value for FDA-approved targets, EGFR, ALK, ROS1, and BRAF), more rapidly (median turnaround time 9 vs. 15 days; $p<0.0001$), and completely (268 vs. 51 patients; $p<0.0001$) [13]. These results are reinforced by multiple studies showing the utility of plasma NGS for rescuing oncogene-addicted patients without sufficient tissue samples for molecular tests [42-45]. Furthermore, in oncogene-addicted NSCLCs, including EGFR mutated [46] and ALK/ROS1 rearranged patients [47, 48], the use of plasma NGS can allow a broader evaluation of the mechanisms of acquired resistance and might guide the therapeutic decision process. For instance, tumor genotyping for ALK mutations after failure of second generation ALK TKIs (alectinib, ceritinib) may identify patients who are more likely to derive clinical benefit from the third generation ALK inhibitor lorlatinib, as recently reported in an exploratory analysis of the phase II study [49]. The phase II NCI-NRG ALK MASTER Protocol (NCT03737994) will prospectively evaluate several combinations of different biomarker/ALK inhibitors in previously treated ALK-positive NSCLCs, based on the predicted sensitivity of each ALK secondary mutation. Furthermore, the changed therapeutic landscape of EGFR-mutated NSCLCs [50] with the introduction of osimertinib in first line will increase the utility of plasma NGS in patients with acquired resistance, due to the accumulation of novel mechanisms of escape [51] that can allow combinatorial approaches, as recently reported with savolitinib-osimertinib in the TATOON trial [52]. The ongoing phase II study SAVANNAH (NCT03778229) is evaluating the efficacy of osimertinib in combination with savolitinib in patients with EGFR mutations and MET-amplified who have progressed following treatment with osimertinib.

As minimally invasive method, serial liquid biopsies during systemic treatments can allow

a real-time monitoring of cancer patients. This strategy has been evaluated in different studies in oncogene-addicted NSCLCs during treatment with TKIs, demonstrating that liquid biopsy can identify the mechanisms of acquired resistance in advance compared with conventional radiographic imaging [53-55]. However, to date it is still unclear whether a change of treatment at molecular progression is associated with an advantage compared to the standard approach at radiographic progression. This issue will be addressed in the randomized phase II APPLE/EORTC 1613 trial [56]. In addition, some studies have reported that early EGFR mutations clearance in plasma using digital PCR is a prognostic factor for improved outcome with osimertinib in both TKI-naïve and TKI-pretreated EGFR-mutated NSCLC patients [57-59]. Similar findings were also reported in NSCLC patients treated with immunotherapy where changes of ctDNA levels during treatment were correlated with improved outcomes [60, 61]. However, to date the evidence supporting this strategy is relatively low and, to date, current guidelines do not recommend liquid biopsy as a monitoring tool [62].

4.4 Emerging challenges with cfDNA genotyping

One of the possible risks associated with cfDNA analysis is the identification of mutations that do not reflect tumor genotype, leading to false positive results.

Recently, clonal hematopoiesis (CH) has gained great attention as a potential source of false positives in liquid biopsy. This well-known phenomenon is characterized by an asymptomatic expansion of blood cells derived from a single hematopoietic stem cell harboring specific, disruptive, and recurrent somatic mutations, in individuals without clear diagnosis of hematological malignancies [63, 64]. More specifically, with the term clonal hematopoiesis of indeterminate potential (CHIP) is indicated the clinical condition in which somatic mutations in genes recurrently mutated in hematologic malignancies with a VAF $\geq$ 2% are detected in absence of a known hematologic malignancy or other clonal disorder and, similarly to the monoclonal gammopathy of undetermined significance (MGUS), is associated with an increased risk of disease progression to hematologic neoplasia of 0.5-1% per year [65]. Clonal hematopoiesis with somatic mutations has been described in 10% of subjects $\geq$ 65 years of age, but it is relatively uncommon in those younger than 50 years of age (~1%) and is usually associated with somatic mutations of genes implicated in hematologic malignancies, such as DNMT3A, TET2, ASXL1, TP53, JAK2, and SF3B1 [66-68]. However, less frequently CH can be associated with somatic mutations of driver genes in solid tumors, such as NOTCH2, FAT3, EXT2, ERBB4, KRAS and ARID2 [69, 70]. This has several important clinical implications not only for minimal

residual disease (MRD) profiling and early cancer detection where can incorrectly lead to the detection of non tumor-derived mutations [26], but also in patients with advanced cancer where CH can be a potential source of false positives and discordance between tissue and cfDNA genotyping [69, 70]. Even if no classically targetable mutations, such as EGFR or BRAF, have been described as of hematopoietic origin, the identification of KRAS mutations can be challenging in lung cancer, since they are usually mutually exclusive with other oncogenic drivers, leading to a mistakenly observation of the absence of a targetable mutation. Furthermore, the widespread use of highly sensitive commercially available plasma NGS platforms with limit of detection of ≤ 0.5% VAF, this phenomenon can be increasingly relevant, since most of the studies evaluating CH were based upon moderate-depth sequencing with a limit of detection ~2% VAF [70]. To minimize the potential impact of CH, recently a novel approach with ultra-deep NGS of plasma cfDNA with CH filtering has been described, demonstrating detection sensitivity comparable to that of established ddPCR methods [71].

CH might represent one of the potential sources of discrepancies between tissue and plasma genotyping, but other biological and technical causes can be singled out, including tumor heterogeneity and inherited differences between liquid biopsy platforms. For instance, a recent orthogonal comparison of 4 different plasma NGS platforms with matched tumor-normal tissue pairs from patients different solid tumors showed that most NGS assay discordance is a result of technical variations and, to a lesser extent, biologic factors such as CHIP and tumor heterogeneity. In particular, higher discordance results were observed at VAF below 1% [72]. Although the results of this study are limited by a small sample size, the high proportion of non-shedding tumors (58% of the samples, with 88% of the patients in stage I-II), and the inclusion of different solid tumors, it suggests the need for improvement in assay performance below this threshold and variants ≤1% VAF should be viewed with caution, particularly in the presence of previously unreported variants [72], bearing in mind also that conventional NGS approach cannot effectively (with both sensitivity and specificity of 95%) determine a cfDNA mutation with low VAF (<0.1%) to be of hematopoietic origin or tumor-derived when performing cfDNA genotyping [69]. Another potential source for discrepancy results is the different sensitivity of NGS assays in detecting oncogenic fusions in plasma cfDNA. A recent study, comparing the results of two commercially available NGS platforms (Guardant 360 and ctDx-Lung), hypothesized that differences in hybrid capture techniques and bioinformatic calling might be sources of variations in sensitivity [73]. However, use of plasma hybrid capture-based NGS has demonstrated to increase the identification of rare oncogenic drivers (including

rearrangements of ALK, ROS1 and RET) and can allow a complete genotype in a significant proportion of patients with insufficient tissue for molecular testing [13, 42, 45]. In addition, plasma NGS can provide useful information at progression for guiding treatment decisions [47, 48], especially for ALK rearranged NSCLCs [49]. Furthermore, recent studies suggested that tissue targeted RNA-based NGS might complement large panel DNA-based NGS testing in comprehensively uncovering targetable gene fusions, allowing the identification of actionable alterations, such as kinase fusions or MET exon14 skipping mutations, in 13% of cases apparently driver-negative by previous DNA sequencing testing [74]. Whether a similar approach can be useful in plasma NGS is unclear and further studies will clarify the potential role of cfRNA genotyping.

4.5 Emerging role of LB in advanced NSCLC: predicting immunotherapy efficacy

A novel potential application of LB is its use as predictive biomarker in lung cancer patients treated with immunotherapy. Over the last few years, immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 have emerged as the standard of care in different settings in advanced/metastatic NSCLC either as monotherapy or in combination with platinum-based chemotherapy [75]. Unfortunately, with the exception of PD-L1 immunohistochemical expression, no predictive biomarkers have been approved to date for these agents. Recently, the evaluation of tumor mutational burden (TMB) in tissue using whole exome sequencing (WES) and/or targeted NGS has been proposed as a predictive biomarker for ICIs targeting PD-1/PD-L1 either alone [76-78] or in combination with CTLA-4 inhibitors [79-81]. However, clinical implementation of this biomarker in clinical practice is hampered by several challenges, including sufficient tissue availability with only 34-59% of the samples were evaluable in recent clinical trials [78, 80-82] and spatial and temporal heterogeneity [83, 84]. TMB estimation on plasma (blood TMB, bTMB) can therefore overcome some of the limits of tissue TMB (tTMB) and has been recently proposed as a predictive biomarker of ICIs efficacy in NSCLC [**Tab. 1**].

Table 1 Studies evaluating bTMB as a predictive biomarker for immune checkpoint inhibitors in NSCLC [*From Russo A, et al. Expert Rev Mol Diagn – under revision*].

Study	bTMB evaluable (%)	Type of study	bTMB assay used	Number of genes analyzed	Type(s) of mutations	Cut-off used	bTMB high (%)
OAK **POPLAR**[1]	211 (73.5%) 583 (68.6%)	Retrospective	FoundationMedicine	394, 1.14 Mb	SNVs with VAF ≥0.5%	≥16 Mut/Mb	30% 27%
MYSTIC[2]	809 (72.4%)	Retrospective	GuardantOMNI	500, 2.1 Mb	SNVs and indels	≥20 Mut/Mb	26%
NCC-GP150[3]	50 (100%)	Retrospective	NCC-GP150	150	SNVs and indels	≥6 Mut/Mb	56%
B-F1RST[4]	119 (78.3%)	Prospective	FoundationMedicine	394, 1.14 Mb	SNVs with VAF ≥1%	≥16 Mut/Mb	23.5%

Abbreviations: *bTMB, blood TMB; SNV, single nucleotide variations; VAF, variant allele frequency; indels, insertions/deletions*

Using the 394-gene FoundationMedicine (FMI) bTMB assay, Gandara et al. retrospectively tested and validated the clinical utility of bTMB in pretreated NSCLCs in plasma samples from two randomized studies with atezolizumab monotherapy. Analyses performed in POPLAR samples and then confirmed in OAK samples demonstrated that bTMB is a predictive biomarker for PFS in patients receiving atezolizumab monotherapy in NSCLC and a value ≥ 16 as a clinically meaningful and technically robust cut-point [9]. As previously reported in tissue [78], bTMB was not associated with high PD-L1 expression [9]. An exploratory analysis of the randomized phase III MYSTIC trial retrospectively evaluated a second bTMB assay, using a 500-gene panel (Guardant OMNI) that incorporates somatic single nucleotide variants and insertions/deletions and accounts for low tumor shedding or low ctDNA input. In the intention-to-treat population 72.4% of the patients were evaluable for bTMB and ≥20 Mut/Mb was selected as cut-off for further exploration, based on the advantage observed in the durvalumab-tremelimumab subgroup compared with platinum-based chemotherapy in terms of both OS (21.9 vs. 10.0 months; HR 0.49) and PFS (4.2 vs. 4.4 months; HR 0.53). Furthermore, bTMB was independently of PD-L1 IHC expression [82]. In December 2018 FDA granted breakthrough device designation to GuardantOMNI. A third study evaluated a novel bTMB panel, NCC-GP150, designed and virtually validated using The Cancer Genome Atlas (TGCA) database and correlated bTMB estimated by NCC-GP150, including 150 genes, and tTMB measured by WES in 48 matched blood and tissue NSCLC samples, with a good correlation, especially when synonymous mutations were included. Furthermore, an independent cohort of 50 patients with advanced NSCLC was used to correlate bTMB estimates and outcome with PD-1/PD-L1 inhibitors, demonstrating that a bTMB ≥6 was associated with longer PFS (HR 0.39; 95% CI 0.18-0.84; p=0.01) and higher ORR (39.3% vs. 9.1%, p=0.02) compared

with bTMB <6 [85]. B-F1RST was the first prospective study to evaluate the impact of bTMB as a predictive biomarker for first line atezolizumab in PD-L1 all comers NSCLC patients [86]. Among 152 patients, patients with a VAF <1% were deemed not evaluable and bTMB was evaluated in 119 patients using the FMI bTMB assay. A bTMB cutoff score of ≥ 16 (23% of patients) showed a numerical improvement in clinical outcomes in patients with NSCLC treated with atezolizumab monotherapy, but no statistically differences between bTMB high vs. low: ORR 28.6% vs. 4.4% (p=0.0002), median PFS 4.6 vs. 3.7 months (HR, 0.66 [90% CI: 0.42, 1.02]; p=0.12), and median OS not estimable vs. 13.1 months (HR, 0.77 [90% CI, 0.41 1.43]; p=0.48), respectively for TMB ≥16 and <16. Interestingly, the non-biomarker evaluable population (n=33) presented a numerically higher ORR (34.5%) than TMB evaluable patients (10.1%), including those with a TMB ≥16 (28.6%) [86]. Several studies have reported lower cfDNA levels and low VAF in patients with low tumor burden [87, 88], a population that has been associated with improved efficacy with ICIs. Indeed, an exploratory analysis of patients with low cfDNA levels showed that VAF < 1% was associated with more favorable baseline prognostic factors than VAF ≥ 1%, likely accounting for better outcomes. However, after adjusting for baseline imbalances, ORR and median PFS did not differ significantly between subgroups [89]. Limits of the B-F1RST study are the absence of orthogonal comparison between bTMB and tTMB and local PD-L1 testing with any commercially available IHC test. The phase III trial B-FAST will further evaluate the predictive role of bTMB with FMI assay in first line, comparing atezolizumab with platinum-based chemotherapy in TMB high patients without oncogenic drivers.

Beside bTMB, cfDNA can offer other useful information in NSCLC treated with immunotherapy. For instance, a recent study suggested that ctDNA dynamics might predict the outcome of patients receiving ICIs. Using a Targeted Error Corrected sequencing (TEC-Seq), Anagnostou et al. evaluated the ctDNA dynamics in serial samples from 24 metastatic NSCLCs treated with ICIs and 14 resectable NSCLCs (Stage I-IIIA) undergoing nivolumab as neoadjuvant treatment [61]. To minimize the potential effect of clonal hematopoiesis, ctDNA analysis was focused only on genetic alterations identified through NGS in paired matched tissue samples. Three patterns of molecular response in ctDNA were observed: molecular response (dramatic reduction of ctDNA to undetectable levels), molecular resistance (associated with limited fluctuations or a rise of ctDNA levels), and molecular acquired resistance, (tumor-specific variants undetectable at the time of response followed by increase in VAF at the time of acquired resistance). In the metastatic cohort, molecular response was associated with longer PFS (p=0.001) and OS (p=0.008) and, in patients with

radiographic SD, correlated with clinical benefit, predicting more accurately the magnitude of therapeutic response than conventional radiographic imaging [61]. Similarly, other groups have reported a strong correlation between radiographic response to ICIs and a reduction in cfDNA level ≥50% compared to baseline and molecular response was observed significantly earlier than radiographic response and was associated with longer survival [60]. Furthermore, in the neoadjuvant cohort, molecular response was also associated with major or partial pathological response, with no pathological response observed in patients with molecular resistance [61].

This data suggests that cfDNA dynamics might complement standard imaging approaches in NSCLC patients treated with ICIs, allowing a better characterization of complex clinical scenarios, as pseudo-progressions and/or dissociated responses. In addition, ctDNA clearance could provide a valid tool for appropriate selection of patients that might benefit from elective immunotherapy discontinuation, an emerging therapeutic challenge in NSCLC [90].

Regarding the role of other components of the liquid biopsy in predicting the outcomes of NSCLC patients treated with immunotherapy data are relatively scant and limited mostly to the evaluation of PD-L1 expression on CTCs [46, 91, 92]. Some intriguing data have also been reported in melanoma patients with circulating exosomal PD-L1 [93] Further studies will clarify their role in lung cancer patients treated with immune checkpoint inhibitors.

4.6 Liquid biopsy in SCLC

In contrast with NSCLC, few therapeutic progresses have been made over the last three decades in SCLC and, only recently, after a countless series of failures a new therapeutic option finally joined the therapeutic armamentarium of extensive disease (ED) SCLC [94]. This lung cancer subtype is characterized by unique clinic-pathological characteristics, with a very aggressive behavior and usually a dismal prognosis. Large genomic studies have reveled that SCLC has one of the highest rates of somatic mutations among solid tumors, but unfortunately most of these aberrations, such as TP53 and RB1 mutations, are not targetable [95-97]. The absence of effective targeted agents and the peculiar features of this disease pushed the interest of liquid biopsy research in different directions compared with NSCLC. Few studies have evaluated to date the role of cfDNA in SCLC, showing the potential utility of liquid biopsy in this tumor as prognostic biomarker. Using a targeted deep sequencing of 430 cancer genes on pre-treatment tumor biopsies, as well as on plasma

samples collected prior to and during treatment from 22 SCLC patients, Nong et al. reported that the average VAF of clonal mutations was associated with PFS and OS rather than single gene mutations, suggesting that subclonal architecture of the tumor provides prognostic information in SCLC [98]. Another small study on 24 patients, using whole exome sequencing, reported that genomic alterations (SETBP1, PBMR1, ATRX, EP300, and ATM) on cfDNA of SCLC have prognostic impact [99]. However, plasma NGS can also provide useful information for treatment selection. Recently we reported a successful targeting of FGFR-1 amplified SCLC with the unselective FGFR inhibitor pazopanib [100], suggesting that in selected cases the use of liquid biopsy can allow the identification of oncogenic drivers also in this disease [**Fig. 5**].

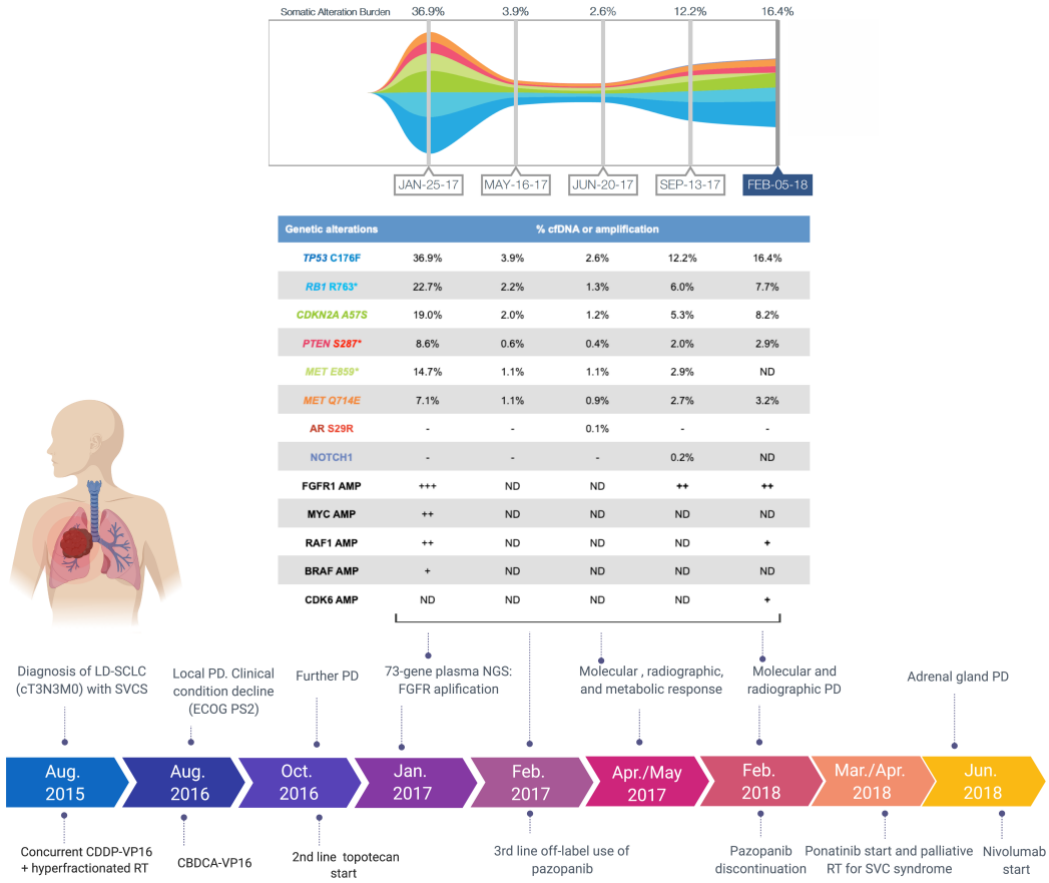

Figure 5 Timeline of treatment received and radiographic/molecular outcomes in a heavily pretreated ED-SCLC patient with FGFR1 amplification (*Credit: created with BioRender*) (*From Russo A, et al. JCO Precis Oncol 2019*) [100].

Furthermore, cfDNA analysis might be a useful source for bTMB evaluation, an emerging predictive biomarker for immune checkpoint blockage. An exploratory analysis of the randomized phase III trial IMpower133, which evaluated the addition of atezolizumab to carboplatin/etoposide in first line ED-SCLC, showed a consistent OS and PFS benefit with

the combination independently of bTMB, assessed with FMI assay, with similar magnitude above and below the pre-specified cutoffs of 10 and 16 mut/Mb. These data are in contrast with those reported with bTMB in NSCLC with the use of PD-1 inhibitors in monotherapy [9] or in combination with CTLA-4 blockage [82], as well as with tTMB in SCLC with nivolumab-ipilimumab in the Checkmate-032 study [101].

The high sensitivity of SCLC to carboplatin/etoposide and the myelosuppressive effects of chemotherapy have been hypothesized ad possible explanations for these apparent discrepancies.

Baseline CTCs are associated with worse prognosis in both limited [102, 103] and ED-SCLC [103]. Using a copy number aberrations (CNAs)-based classifier, Carter et al. also showed that CNAs in CTCs correctly identify chemosensitive and chemorefractory (relapse during treatment or within 3 months from completion) SCLC patients and is associated with PFS [104]. Future studies will elucidate the role of CTCs in SCLC, including patients treated with chemo-immunotherapy combinations.

4.7 Future directions and conclusions

Liquid biopsy has a firmly established role in advanced NSCLC through cfDNA genotyping. However, the indications of liquid biopsy are rapidly growing and accumulating evidence suggest a potential role as a drug monitoring tool in oncogene-driven NSCLCs, as predictive biomarker for immunotherapy, as well as a prognostic marker in early stage disease. The use of circulating tumor cells (CTCs), microRNAs (miRNAs), exosomes, and other components of the large family of liquid biopsy is under active evaluation, but their immediate application in lung cancer is still far from clinical practice.

4.8 References

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CHAPTER 5

Exosomal PD-L1 expression and dynamic changes of immune response associated cytokines as predictive biomarkers for immune checkpoint blockage with PD-1/PD-L1 inhibitors in advanced/metastatic non-small cell lung cancer

5.1 Background and rationale

Avoiding immune destruction is one of the hallmarks of cancer [1]. Therefore, it is not surprising that several strategies have been studied to restore the physiological mechanisms underlying the immune response against tumors. Unfortunately, most of these strategies traditionally failed to demonstrate a significant benefit in solid tumors, including non-small cell lung cancer (NSCLC) [2]. The development of immune checkpoint inhibitors (ICIs) during the last decade has dawn a new era in cancer immunotherapy, revolutionizing the treatment of several malignancies including advanced NSCLC. In contrast with other conventional medical treatments (i.e. chemotherapy and targeted therapies), immunotherapy is usually associated in unselected patients with lower response rates, but highly durable tumor responses [3], resulting in a long-term survival only in a minority of patients (~15-20% in unselected patients) [4-6]. Nivolumab, pembrolizumab and atezolizumab are monoclonal antibodies targeting the PD-1/PD-L1 axis which have been FDA-approved for use in advanced NSCLC based upon phase III trials which demonstrated improved survival outcomes with these agents as second line therapy compared to docetaxel chemotherapy [7-10]. Subsequent clinical trials examined ICI use in advanced NSCLC in the first line setting and the randomized phase III trial KEYNOTE-024 showed that pembrolizumab is superior to platinum-based chemotherapy in selected patients (PD-L1 tumor proportion score of ≥50%) [11]. More recently, different randomized phase III trials reported a survival advantage with the use of ICIs combined with platinum-based chemotherapy in unselected NSCLC patients without EGFR mutations or ALK translocations [12-14]. As a result of these studies, immunotherapy has become a mainstay of treatment for advanced NSCLC either as single agent or in combination with platinum-based chemotherapy in both treatment-naïve and pre-treated patients.

Despite these major breakthroughs, several open questions still remain opened and with the increasing use of these agents in clinical practice we are facing several new challenges. The costs of these new treatments are not negligible (the so called "*financial toxicity*") and in addition, albeit these therapies are usually associated with a more favorable safety profile than that observed with conventional anticancer agents, a significant proportion of patients may experience immune-related adverse events (irAEs), which in selected cases may be associated with life-threatening conditions [15]. Moreover, in some cases the use of these agents may be associated with unconventional patterns of response and, in some cases, with pseudo-progressions, leading to the proposal of novel radiological criteria for interpreting response in patients treated with immunotherapy [16]. In addition, a new pattern of progression has recently gained high attention, namely hyper-progression, which may

occasionally find in cancer patients treated with ICIs and it is associated with an acceleration of tumor growth during immunotherapy and a very dismal prognosis [17].

For these reasons, the identification of predictive biomarkers is essential in order to reduce the costs of these treatments and to avoid unnecessary toxicities to patients who cannot benefit from these drugs. Several biomarkers have been studied to help determine which patients will derive the most therapeutic benefit from PD(L)-1 inhibitors. However, predictive biomarkers for optimal patient selection are lacking, with PD-L1 immunohistochemical (IHC) expression being the main clinically applicable test at this time. A pooled analysis of the randomized phase III trials in NSCLC with ICIs in pre-treated NSCLC has shown that patients with PD-L1 positive tumors (PD-L1 tumor staining of ≥1%) have significantly higher overall response rates compared to PD-L1 negative tumors, suggesting that PD-L1 over-expression is a predictive biomarker [18]. However, PD-L1 immunohistochemical expression is not a perfect biomarker and despite a positive expression, a significant proportion of patients do not benefit from these agents, even when using more stringent cut-off values (ORR ~45% in patients with PD-L1 expression ≥50%) and, at the same time, patients with negative expression of PD-L1 may also experience a significant benefit from these compounds [7-9]. For instance, long-term survival analysis of nivolumab studies in pretreated NSCLCs recently showed 4-year overall survival with nivolumab was 14% (95% CI 11.17) for all patients (n=664), 19% (15.24) for those with at least 1% PD-L1 expression, and 11% (7.16) for those with less than 1% PD-L1 expression [6]. Furthermore, the use of PD-L1 expression as the only predictive biomarker for patient selection to ICIs is associated with several limitations, due to technical and biological issues [19-21]. Therefore, the identification of additional biomarkers is eagerly awaited.

The complex interactions between the immune system and cancer (the so called "*Cancer-Immunity cycle*") [22] makes difficult to identify a single biomarker that can discriminate responders from non-responder patients. Some authors have tried to bring back the aspects of cancer-immune interactions to seven parameters ("*Cancer immunogram*") from whom it is possible to identify several potential biomarkers [23]. One of these clinic-pathological parameters is the tumor foreignness, namely the presence of neoantigens present within the tumor that are recognized by the immune system as not-self. Indeed, mutations in the tumor genome can cause tumors to express mutant proteins that are tumor specific and not expressed on normal cells- referred to as neoantigens. These neoantigens are an attractive immune target because their selective expression on tumors, eliciting a higher anti-tumor response, minimizing immune tolerance as well as the risk of autoimmunity [24]. As a

consequence, tumors with a high mutational load, such as melanoma, lung cancer, and cancers with defects in the mismatch repair mechanisms, responds well to immunotherapy, due to an increased frequency of tumor neoantigens. Tumor mutational burden (TMB) is one of the emerging novel biomarker for ICIs and it is defined as the number of nonsynonymous mutations found in the genome of a single tumor; a high TMB is thought to result in greater tumor immunogenicity as more mutations are associated with a higher chance of neoantigens being presented to the immune system [25, 26]. Initial studies using whole exome sequencing (WES), showed that higher nonsynonymous mutation burden in NSCLCs treated with ICIs is associated with improved objective response, durable clinical benefit and improved progression free survival [27]. Subsequent studies have validated targeted next generation sequencing (NGS) panels as an alternative source for TMB estimation [28-30], including two FDA approved NGS tests (Foundation One and MSK-IMPACT). The concomitant use of PD-L1 IHC expression and TMB might increase the probability to identify the right patient candidate to immunotherapy [31, 32], albeit the wide clinical applicability in real world practice of this methodology is far from immediate, due to biological [33], technical [34] and financial issues. In addition, sufficient tissue availability may be an important obstacle, especially in lung cancer where the list of predictive biomarkers mandatory for the optimal patient selection is rapidly growing, but usually tumor samples are small biopsies or even cytological samples.

The new era of liquid biopsies is growing very fast, and less-known components, such as exosomes, may also be exploited as a source of biomarkers. Exosomes are cell-derived nanovescicles (30–100 nm of diameter) firstly described studying reticulocyte differentiation. Exosomes are formed by the inward budding of multivesicular bodies (MVB), component of the endocytic pathway and are released from different types of cells both under physiological and pathological conditions and, interestingly, have been well detected in several body fluids. They function as messenger particles, playing a role in cell-to-cell communication and to deliver proteins, lipids, mRNAs and miRNAs with biological activity in the target cells [35, 36].

We hypothesize that PD-L1 specific exosomes contains altered protein cargo, which can be used as non-invasive liquid biopsy marker for NSCLC. Proteomic profiling analysis will be performed using PD-L1 specific exosomes isolated at baseline and following treatment with ICIs. The differentially expressed proteins identified from PD-L1 specific exosomes will be used as non-invasive serum marker for immune response to these agents.

Several studies have demonstrated that PD-L1 expression is influenced by different signaling pathways, transcriptional factors, as well as epigenetic factors [37], in two

different ways: "*oncogene-driven*", a consequence of constitute activation due to activation of oncogenes or the loss of tumor suppressor genes, and "*immune-driven*", when there is a cytokine-induced stimulation as a mechanism of adaptive resistance of the tumor to the immune system action [20, 21]. Transforming growth factor-β (TGF-β), a pleiotropic cytokine with immunosuppressive effects on multiple cell types of the innate and adaptive immune system, has emerged as one of the potential key factors modulating response to ICIs [38]. Recently, increased TGF-β levels in the tumor microenvironment of colon cancer have been reported to represent a primary mechanism of immune evasion that promotes T-cell exclusion and blocks acquisition of the TH1-effector phenotype [39], suggesting a potential role for TGF-β as a target for immunotherapy as well as a marker of immune resistance. These data suggest that monitoring of cytokine levels in patients treated with ICIs may have a potential use as predictive biomarker to these agents.

The aim of the present study was to analyze the potential predictive role of some circulating biomarkers in NSCLC patients treated with PD(L)-1 inhibitors through the detection, quantification and kinetics of PD-L1 containing exosomes as well as pro- and anti-inflammatory cytokine profile analysis, using serial plasma samples collected at baseline and following ICIs treatment. In addition, we investigated the role of some systemic markers of inflammation, such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR), which recently were associated with poor prognosis in NSCLC patients treated with ICIs [40, 41].

5.2 Patients and methods

This was a retrospective multicenter study conducted at the Medical Oncology Unit of A.O. Papardo of Messina, Italy (Dir. Prof. Vincenzo Adamo) and at the Clínica del Country of Fundación Santa Fe de Bogotá, Colombia (Dr. Andrés Felipe Cardona) evaluating the potential predictive role of circulating biomarkers on the outcome of advanced/metastatic NSCLC patients treated with immune checkpoint inhibitors targeting the PD-1/PD-L1 axis. The global study design is summarized in **Fig. 1**.

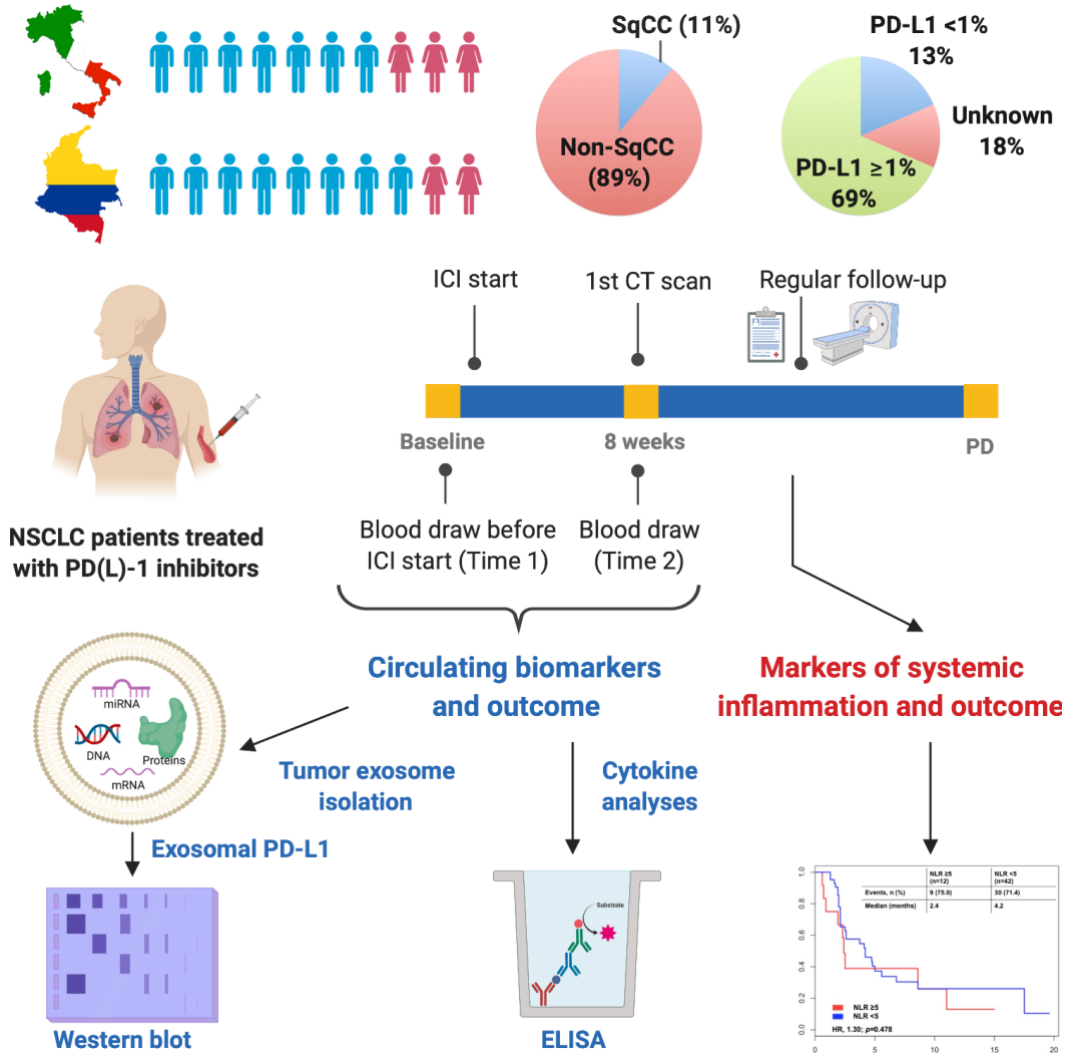


Figure 1 Study design and main characteristics of the two patient cohorts analyzed in the present study (*Credit: Created with Biorender*)

All patients consented to an Institutional Review Board–approved protocol. Inclusion criteria were as follows: age >18 years; cytological and/or pathological confirmed NSCLC; stage IIIB or IV (recurrent or metastatic) according to according to TNM [tumour, node, metastasis] American Joint Committee on Cancer [AJCC] version VIII; treatment with anti-PD(L)-1 inhibitors (nivolumab, pembrolizumab, atezolizumab, avelumab) either alone or in combination with chemotherapy or CTLA-4 inhibitors (ipilimumab); serial serum and plasma samples before treatment start and after 8 weeks (first radiographic examination). For each patients, the following data were collected: Demographics (age, sex, ethnicity); patient and disease characteristics (performance status, medical comorbidities, smoking history, histology, stage, bio-molecular status); baseline laboratory results (complete blood count, albumin, LDH, CEA, erythrocyte sedimentation rate, C-reactive protein, etc.);

treatment received for advanced disease and relative toxicities; outcomes (treatment response, progression free, overall survival; patterns of relapse).

NLR was calculated by division of absolute neutrophil and lymphocyte counts, while PLR was calculated by division of thrombocytes and lymphocytes. Patients were dichotomized according to pre-specified cut-off values of NLR ≥5 vs. NLR <5 and PLR ≥200 vs. <200, which have been previously validated [40, 41]. In patients with serial plasma samples before treatment start and after 8 weeks (first radiographic examination) were evaluated the dynamic changes of some immune response associated cytokines (TGF-β, INF-γ, and IL-6) that had been previously shown to influence PD-1 blockage in collaboration with Prof. Christian Rolfo (Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland, Baltimore, MD, USA). Multiplex cytokines analyses were performed at the Cytokine Core Laboratory, Bressler Research Building, University of Maryland (Baltimore, MD) and samples were prepared and aliquoted at the Translational Core Laboratory, Bressler Research Building, University of Maryland (Baltimore, MD). In summary, previously collected and snap-frozen patient plasma was thawed on ice and mixed thoroughly by vortexing. A 150 uL aliquot was transferred from each sample into new 1.5 mL centrifuge tubes, and spun at 3000 X G for 30 minutes at 4°C. The supernatant from each sample was transferred to fresh 1.5 ml centrifuge tubes, and subjected to cytokine analysis for TGF-β, IFN-γ, and IL-6. A 96 well plate (Greiner) was wet with 200ul of Assay Buffer and placed on a shaker for 10 minutes. The plate was then decanted and 25ul of Assay Buffer or appropriate buffer was added to each well and 25ul of standard/sample/control was added to the appropriate wells. Then 25ul of a mixture containing requested cytokines (1:50 dilution) that had been conjugated to beads was added. All plates contained at minimum high and low control in order to determine the validity of the plates. The plate was then placed on a shaker, at 4°C overnight. The plate was then placed on a magnetic washer, 200ul of Wash Buffer added to each well, the plate was set on a shaker at 500 rpm for 1 minute, and repeated an additional two times. After the last decanting step 25ul of detection antibody was added and the plate was placed on a shaker for one hour at room temperature. Then 25ul of Phycoerythrin (1:25 dilution) was added to each well and the plate was placed back on the shaker for 30 minutes. The plate was then washed three times and 150ul of Sheath Fluid was added to each well. The plate was then read using a Luminex MagPix reader and data was calculated using Luminex's exponent Software.

In addition, tumor exosomes isolation and exosomal PD-L1 expression were also evaluated in patients with matched serial samples at the Division of Cardiovascular Surgery,

University of Maryland School of Medicine, Baltimore, MD, USA. Exosomes were isolated from sera using the ultracentrifugation method. Briefly, 500 μl of serum was centrifuged at 3,000g for 20 minutes at room temperature to remove cells, and again at 10,000g for 20 minutes to remove cell debris. Serum was diluted with 1X PBS and centrifuged at 100,000g for 70 minutes at 4°C. The exosome pellet was washed twice with 1X PBS and centrifuged at 100,000xg for 70 minutes. The purity of the exosomes was determined using sucrose cushion method. Finally, the isolated exosomes were lysed with RIPA buffer; protein concentration was measured with the bicinchoninic acid (BCA) method for immunoblot. Exosomes isolated from serum of were analyzed to determine the presence of PDL1 molecules by immunoblot. Briefly, 20µg of total exosome proteins were resolved in 12% Bis-Tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA) and were transferred into a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk prepared in 1X PBS. Abs specific to co-stimulatory molecules PDL1 (Abcam ab213524) and CD9 (Bio Legend 312102) were used to detect the specific protein in the exosomes. Goat-anti-rabbit Ab conjugated with HRP was used as a secondary Ab. The blots were developed using chemiluminescent HRP substrate (Millipore WBKLS0500) and exposed using the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE). The PDL1 band intensity was quantified using ImageJ software and normalized with CD9.

Categorical variables were compared using chi-square or Fisher's exact test. Overall survival (OS) was defined as time from nivolumab start to death and Progression Free Survival (PFS) as time from treatment start to Progression Disease (PD) or death for any cause. OS and PFS survival were estimated using the Kaplan–Meier method. Survival curves were compared using the log-rank test. To estimate the hazard ratio (HR), Cox regression analysis was used. Analyses were carried out using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 8.1.2. Statistical significance was assumed if $p<0.05$.

5.3 Results

A total of 54 NSCLC patients treated with immune checkpoint inhibitors for advanced/metastatic disease were included in the present analysis. No significant differences were reported between the two patient cohorts, except for a slightly higher proportion of current/former smokers and non-squamous NSCLCs in the Colombian cohort (**Tab. 1**).

Characteristics	Colombian cohort (n = 21)	Italian cohort (n = 33)	p value
Age – yr			
Median	63	70	
Range	42-76	38-86	
Sex – no. (%)			
Male	17 (80.9%)	23 (69.7%)	p=0.36
Female	4 (19.1%)	10 (30.3%)	
Smoking status – no. (%)			
Current/Former smokers	21 (100%)	27 (81.8%)	p=0.04
Never smokers	0 (0.0%)	6 (18.2%)	
Histology – no. (%)			
Squamous	0 (0.0%)	6 (18.2%)	p=0.04
Non-Squamous	21 (100 %)	27 (81.8%)	
EGFR mutational status – no. (%)			
Unknown	4 (19.0%)	2 (6.1%)	
EGFR mutated	0 (0.0%)	1 (3.0%)	
EGFR wild type	17 (81.0%)	30 (90.9%)	p=0.29
ALK rearrangements – no. (%)			
Unknown	4 (19.0%)	10 (30.3%)	
ALK rearranged	0 (0.0%)	0 (0.0%)	
ALK not rearranged	17 (81.0%)	23 (69.7%)	p=0.36
PD-L1 IHC status – no. (%)			
Unknown	4 (19.0%)	6 (18.2%)	
PD-L1 <1%	3 (14.3%)	4 (12.1%)	
PD-L1 ≥1%	14 (66.7%)	23 (69.7%)	p=0.36
ECOG PS – no. (%)			
0	8 (38.1%)	12 (36.4%)	p=0.90
1	13 (61.9%)	19 (57.5%)	p=0.75
2	0 (0.0 %)	2 (6.1 %)	p=0.25
Line(s) of previous treatment			
Median	1	1	
Range	0-3	0-3	

Table 1 Clinic-pathological characteristics of the two patient cohorts. Abbreviations: *yr, year; no, number; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; PD-L1, programed death ligand-1; IHC, immunohistochemistry; ECOG PS, Eastern Cooperative Oncology Group Performance Status.*

Most of the patients received PD(L)-1 inhibitors as monotherapy (80.9% of the Colombian cohort and 100% of the Italian cohort), with only a minority of patients receiving immunotherapy in combination with chemotherapy (1 patient) or dual PD-1/CTLA-4 blockage (3 patients). The outcome of both patient cohorts is summarized in **Tab. 2**. No statistically significant differences were reported between the two groups.

Summary of ICIs activity	Colombian cohort (n=21)	Italian cohort n=33)	p value
Type of response – no. (%) Complete response (CR) Partial response (PR) Stable disease (SD) Progressive disease (PD)	evaluable 21/21 0 (0.0%) 6 (31.6%) 4 (21.0%) 9 (47.4%)	evaluable 31/33 0 (0.0%) 5 (16.2%) 13 (41.9%) 13 (41.9%)	
Objective Response Rate (ORR) - %	31.6%	16.2%	p=0.28
Disease Control Rate (DCR) - %	52.6%	58.1%	p=0.62
Progression Free Survival (PFS) – mos Median CI 95%	 4.2 2.1-17.5	 2.6 2.3-NA	 p=0.441
Overall Survival (OS) – mos Median CI 95%	 19.9 3.93-NA	 10.0 3.1-NA	 p=0.663

Table 2 Summary of treatment outcomes in both cohorts. Abbreviations: *ICIs, immune checkpoint inhibitors; mos, months*

Median progression-free survival (PFS) and overall survival (OS) in the overall study population were 4.1 months (CI 95%, 2.5-8.6) and 10.0 months (CI 95%, 4.7-NA), respectively (**Fig. 2**).

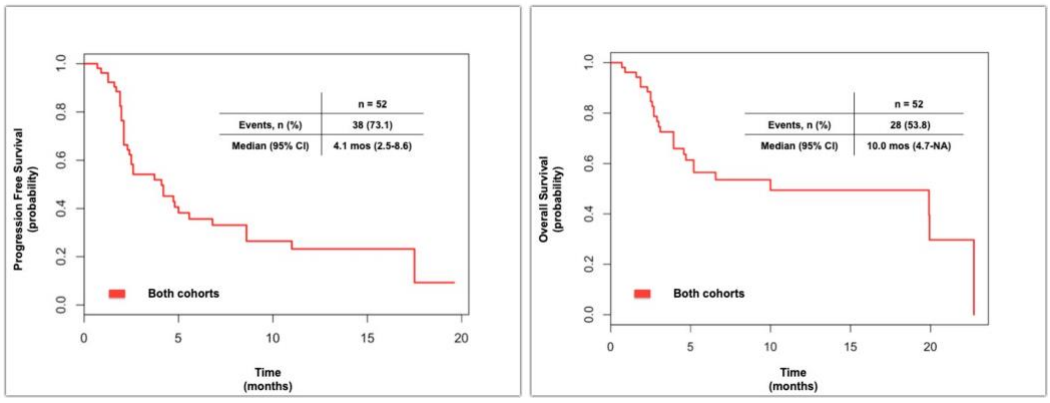
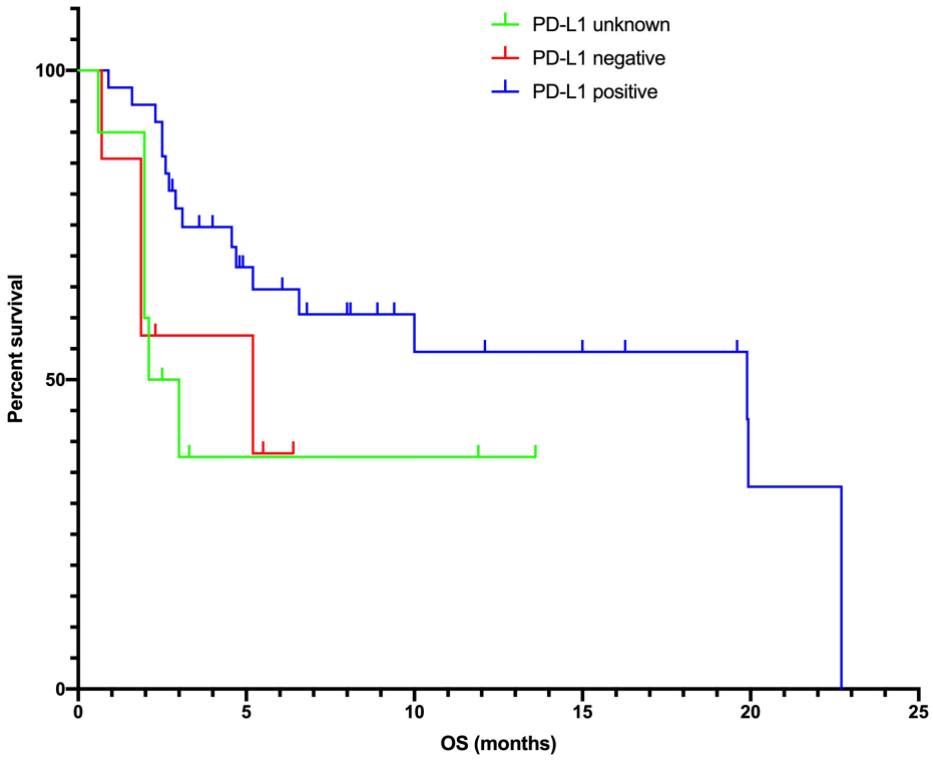


Figure 2 Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) in the study population

Based on PD-L1 IHC status, positive expression (TPS ≥1% assessed with SP263 or 22C3 Clone) was associated with longer median OS (19.9 months) compared with PD-L1 negative (<1%) (5.2 months) and unknown (2.55 months) status (log-rank test for trend *p*=

0.04) (**Fig. 3**).



We first assessed the prognostic role of baseline neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLT) in the overall study population. High baseline NLR (≥5) was associated with statistically significant shorter OS and a numerically but not statistically shorter PFS (**Fig. 4A** and **Fig. 4B**). Similarly, high baseline PLR (≥200) was associated with poorer outcomes, albeit these differences did not translate in a statistically significant difference, likely due to the small sample size and the relatively immaturity of the OS data in the PLR low subgroup (43.7% of events compared with 63.6% in the PLR high group) (**Fig. 4C** and **Fig. 4D**).

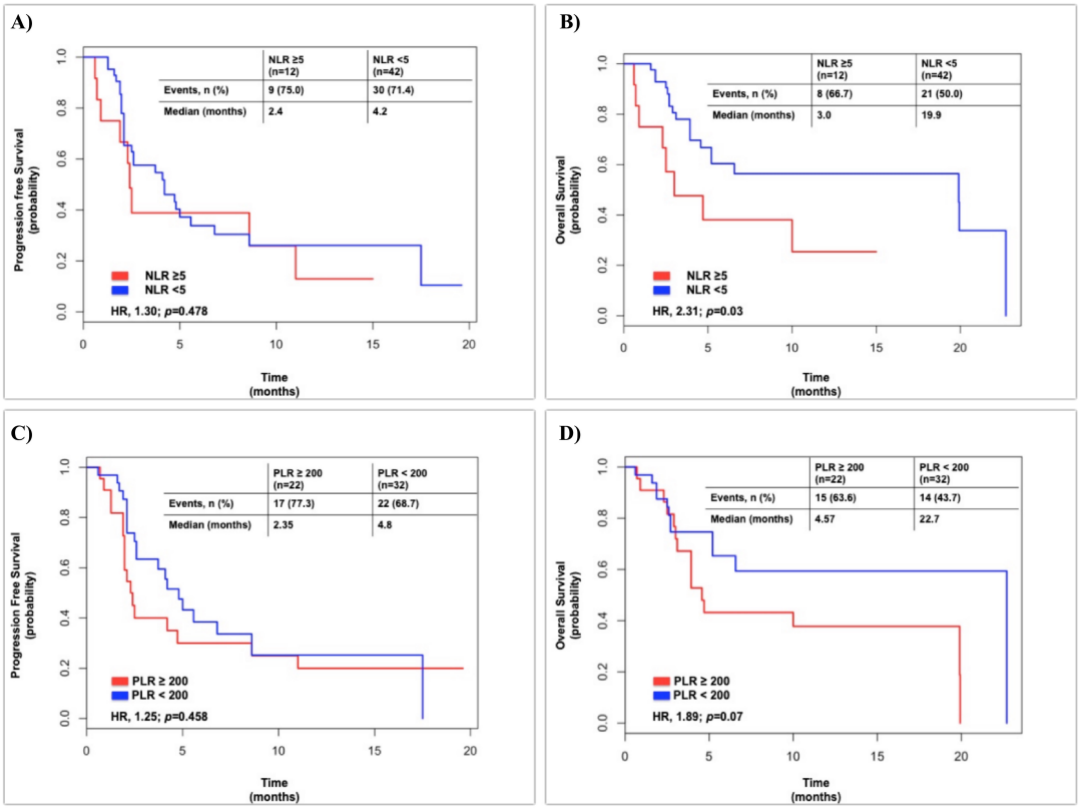


Figure 4 Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) according to baseline neutrophil-to-lymphocyte ratio (NLR) (A-B) and platelet-to-lymphocyte ratio (PLR) (C-D).

Furthermore, we investigated potential differences in the response to ICB by sex. We did not find any significant difference in both PFS and OS between male and female patients (**Fig. 5**).

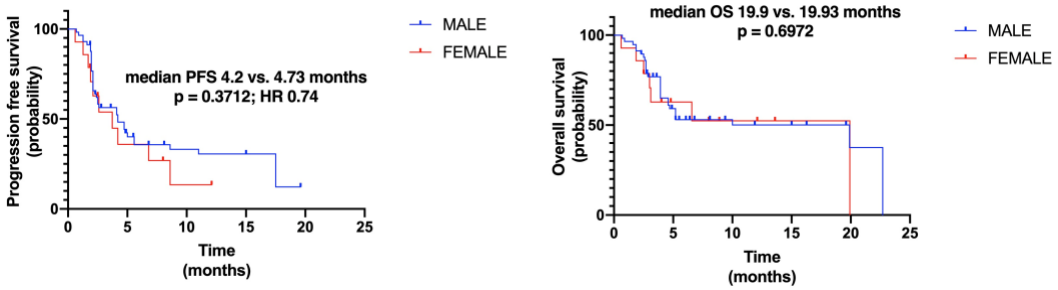


Figure 5 Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) according to sex.

A subset of patients (n=32, 59.2%) had matched paired plasma samples at two time points (before immunotherapy start and after 8 weeks) for cytokines analyses. All these patients received single agent anti-PD-1 (nivolumab or pembrolizumab). Differences between the

groups were estimated with unpaired t test. No statistically significant differences were reported baseline samples and after 8 weeks of PD-1 blockage (p=0.48) in the plasma levels of TGF-β (**Fig. 6**).

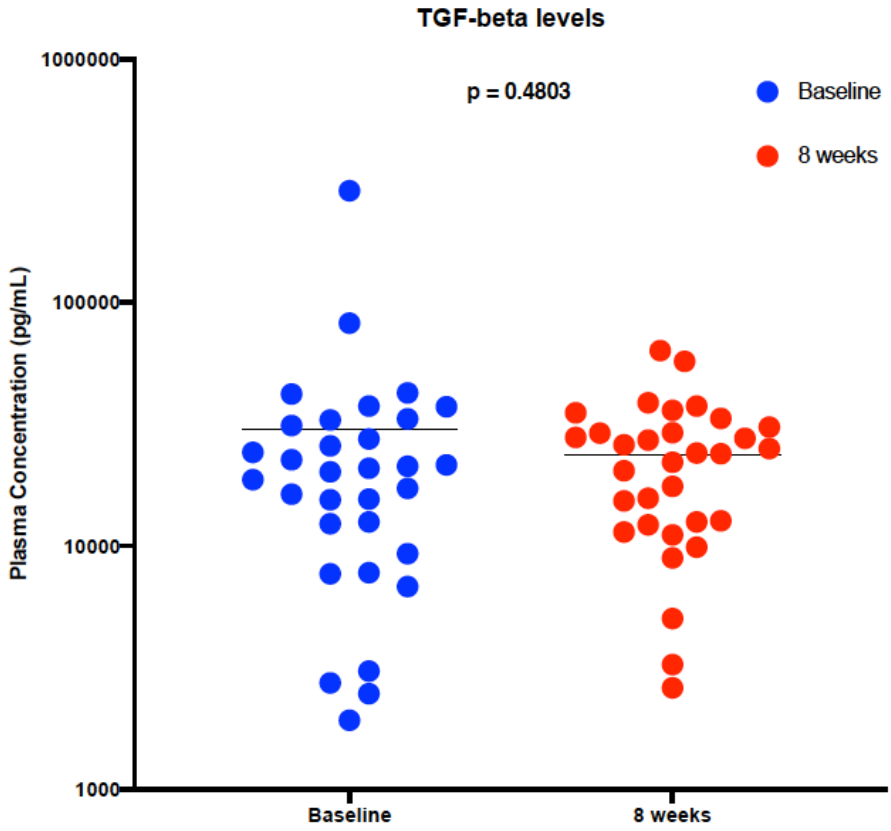


Figure 6 Plasma levels of TGF- β in NSCLC patients treated with ICIs targeting the PD-1/PD-L1 axis at baseline (blue) and after 8 weeks of treatment (red).

Similarly, no statistically significant differences were reported in TGF-β levels between baseline samples and after 8 weeks of ICB according to the response to PD-1 inhibitors (**Fig. 7**).

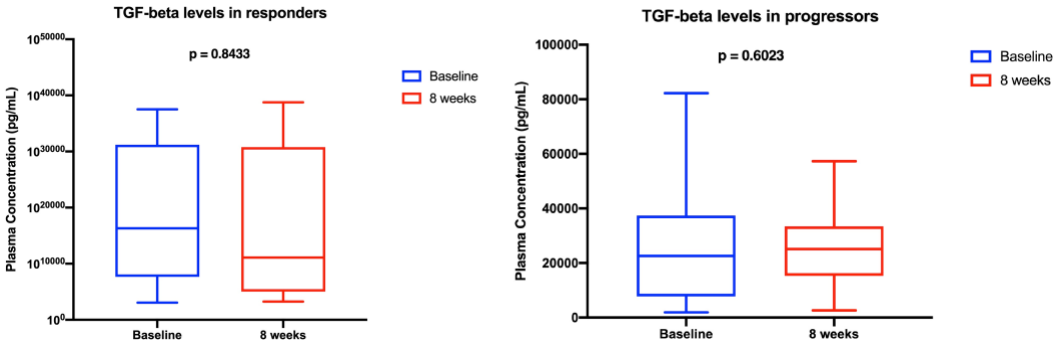


Figure 7 Differences of plasma levels of TGF-β between baseline (blue) and after 8 weeks according to radiographic response to ICB

We also correlated the levels of TGF-β upper normal limit (903 to 63000 pg/ml) with PFS and OS, reporting a worse OS in patients with high TGF-β levels, albeit the difference was not statistically significant due to the small sample size (**Fig. 8**).

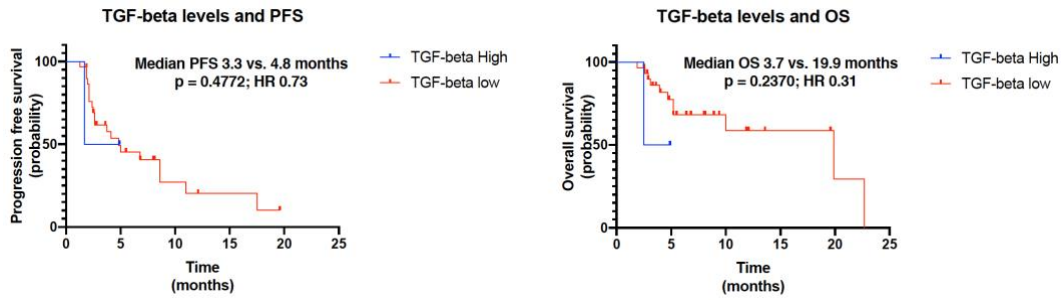


Figure 8 Kaplan-Meier curves of PFS (right) and OS (left) according to TGF-β levels over (blue) or within the normal limit range (903 to 63000 pg/ml) (red)

Interestingly, TFG-β ratio between levels at 8 weeks and baseline significantly correlated with the outcome, in terms of both PFS and OS. Patients with higher TFG-β ratio were associated with longer PFS (p=0.015) and OS (p=0.0034) (**Fig. 9**).

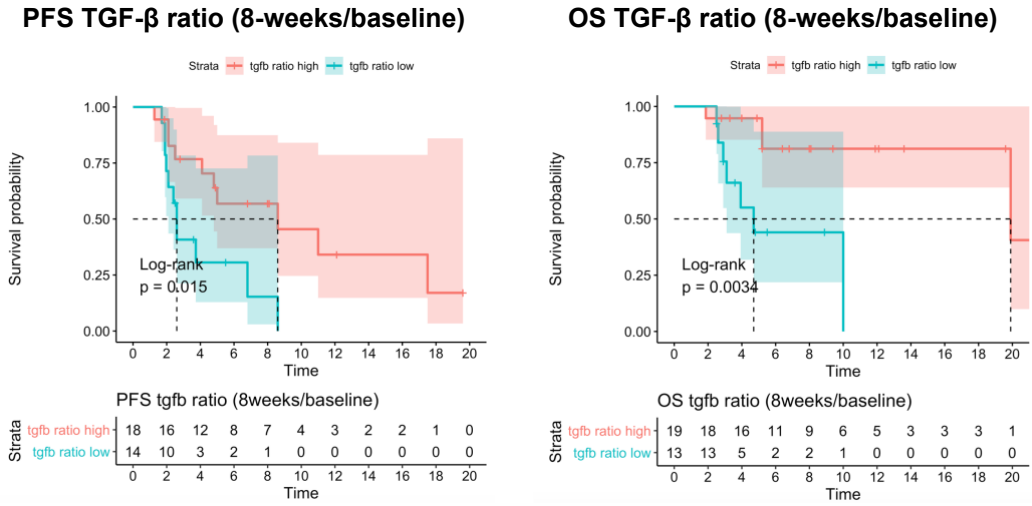


Figure 9. PFS and OS curves for TGF-β ratio (8 weeks/baseline)

We then analyzed plasma levels of IL-6 evaluating potential differences during treatment with PD-1 blockage. No significant differences were reported between pre-treatment levels of IL-6 and after 8 weeks of treatment (p=0.6756) (**Fig. 10**), independently of treatment response (**Fig. 11**).

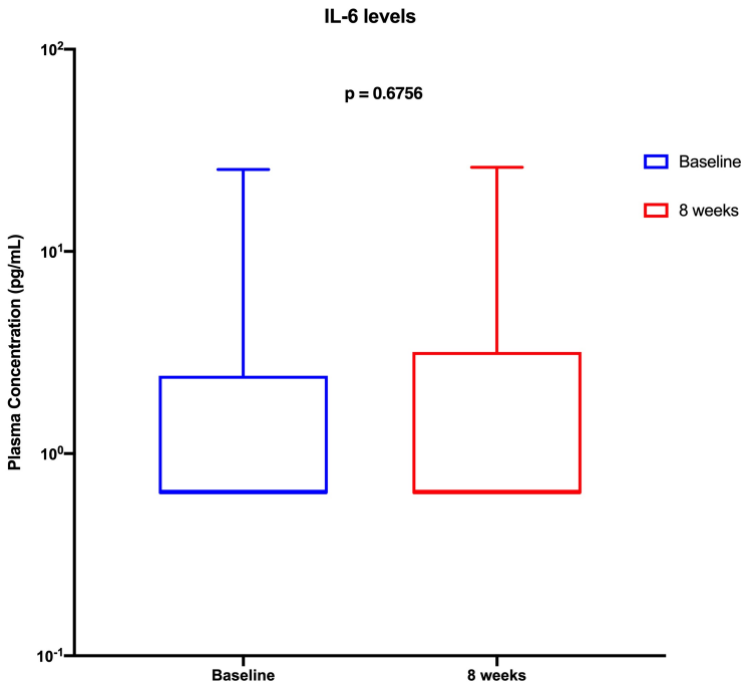


Figure 10 Plasma levels of IL-6 at baseline (blue) and after 8 weeks of treatment (red) with PD-1 inhibitors.

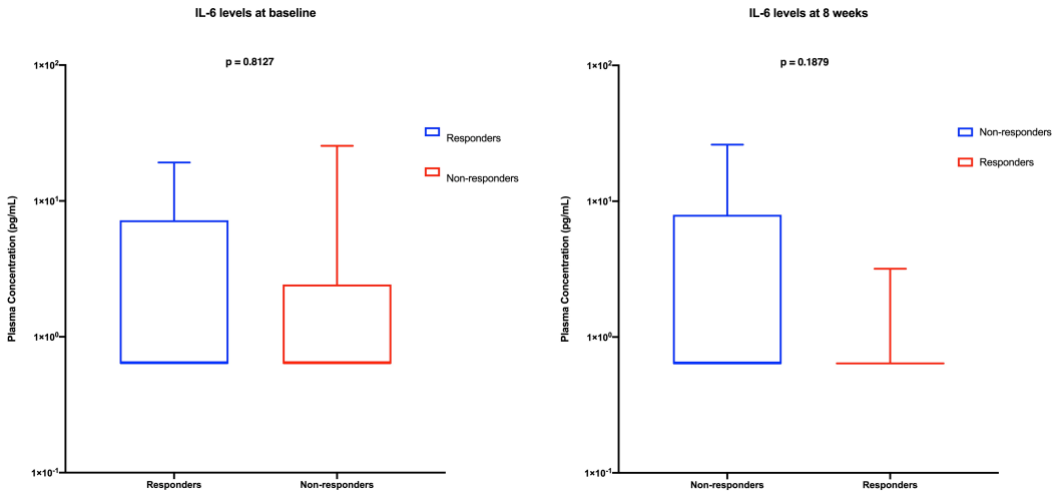


Figure 11 Comparison of IL-6 plasma levels between responders and non-responders to ICB at baseline and after 8 weeks of treatment

Interestingly, high IL-6 levels were associated with shorter PFS and OS compared with normal IL-6 levels (non-detectable to 12.5 pg/ml) and these differences were statistically

significant (p=0.04 for both) (**Fig. 12**).

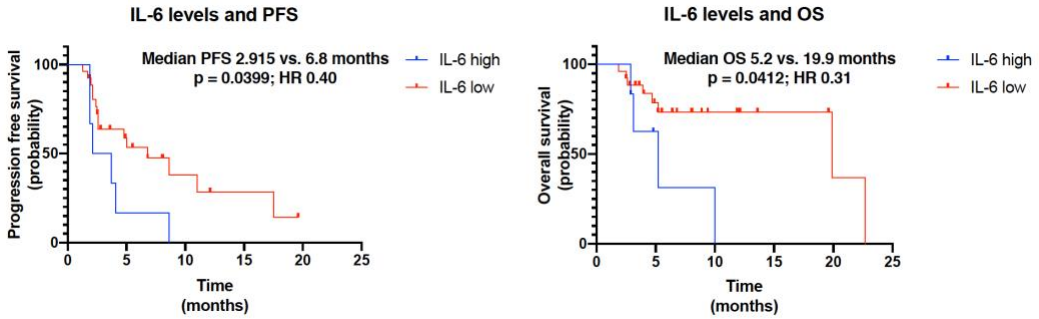


Figure 12 Kaplan-Meier curves of PFS (right) and OS (left) according to IL-6 levels over (blue) or within the normal limit range (non detectable to 12.5 pg/ml) (red)

Finally, we evaluated potential differences of IFN-γ plasma levels before and after ICB, reporting not statistically significant differences (p=0.1624) (**Fig. 13**).

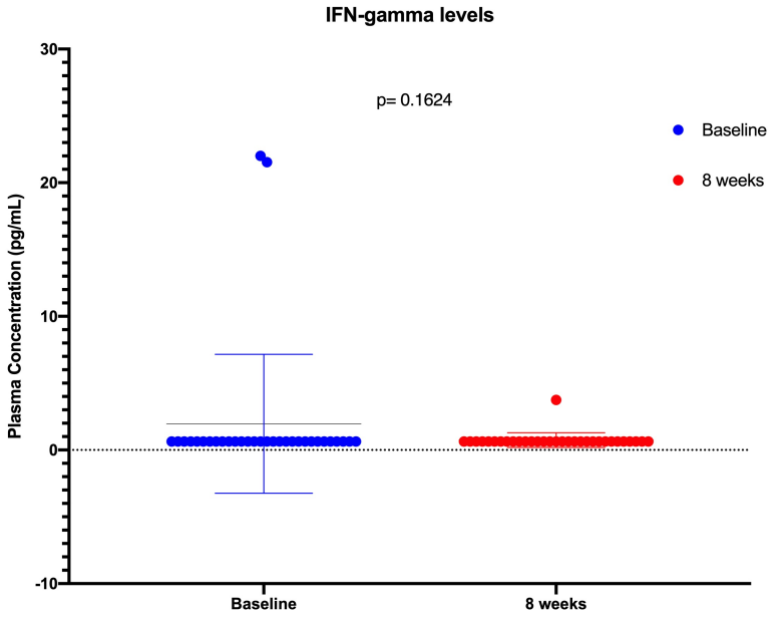


Figure 13 Plasma levels of IFN-γ at baseline (blue) and after 8 weeks of treatment (red) with PD-1 inhibitors

All responder patients had undetectable plasma levels either at baseline or after 8 weeks of treatment. No statistically significant differences (p=0.16) were also reported in non-responding patients either at baseline or after treatment with ICIs (**Fig. 14**), with most of the cases showing non-detectable levels (<0.64 pg/ml).

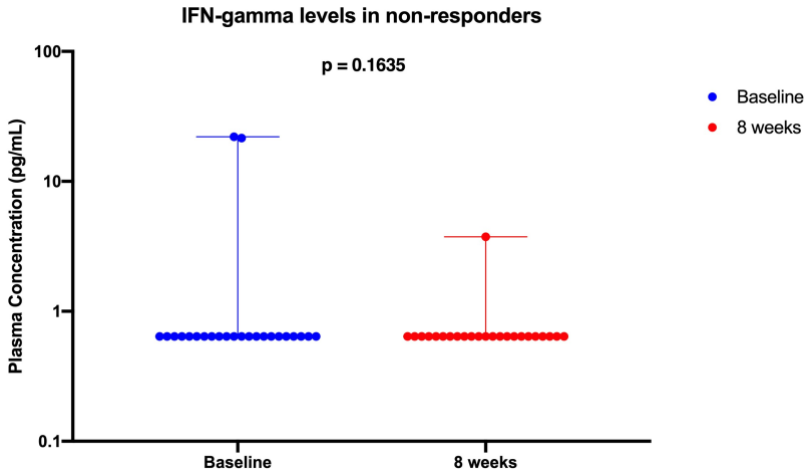


Figure 14 IFN-γ plasma levels in healthy controls (green) and non-responding NSCLC patients at baseline (blue) and after 8 weeks (red)

We finally correlated the levels of IFN-γ upper normal limit (not detectable) with PFS and OS, reporting a non-statistically significant difference in terms of PFS (p=0.22) (**Fig. 15**). Data on OS were not mature at the time of the present analysis.

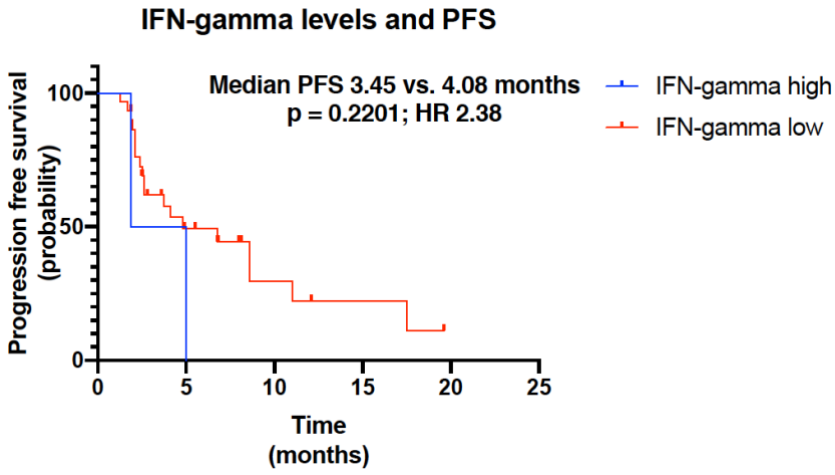


Figure 15 Kaplan-Meier curve of PFS according to IFN-γ over (blue) or within the normal limit range (non-detectable) (red)

We then analyzed the correlation between cytokines levels at baseline and after 8 weeks with PFS and OS. Cox regression analysis showed no correlation between IFN-γ, TGF-β and IL-6 with both PFS and OS (**Tab. 3**).

Variables	p values for PFS	p values for OS
IL-6 at baseline	0.8232	0.9880
IL-6 at 8 weeks	0.1610	0.1390
IFN- γ at baseline	0.4324	0.5296
IFN- γ at 8 weeks	0.6110	0.5278
TGF-β at baseline	0.7654	0.6801
TGF-β at 8 weeks	0.8341	0.9234

Table 3. Correlation between cytokine levels and survival

Finally, we evaluated exosomal PD-L1 fold change on 25 patients from the Italian cohort with matched paired samples at baseline and after 8 weeks of PD-1 blockade. Western blot results for exosomal PD-L1 are depicted in **Fig. 16**.

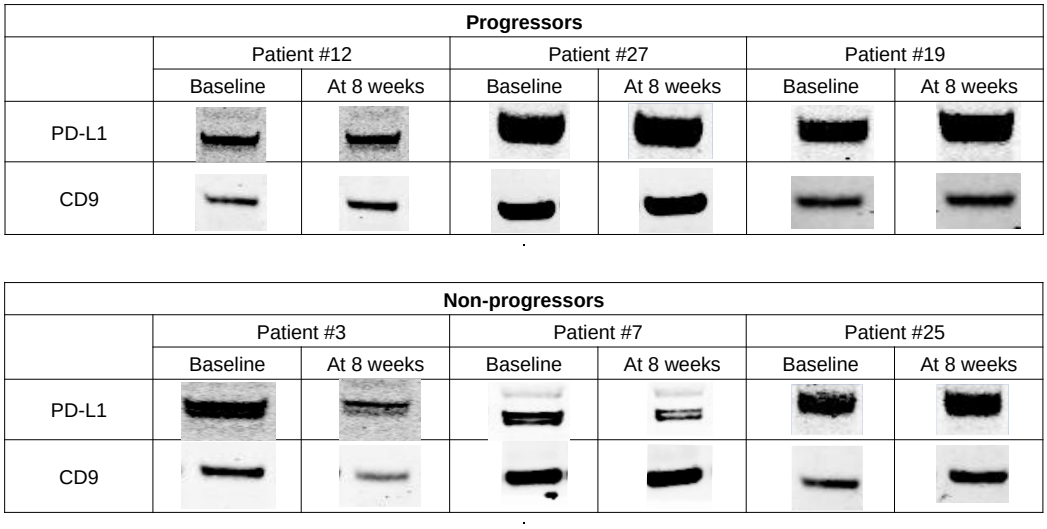


Figure 16. Western blot results for exosomal PD-L1. CD9 as loading control

No statistically significant differences were reported for PD-L1 changes between pretreatment samples and at 8 weeks in both progressive patients (p=0.3783) and non-progressing patients (p=0.8066) (**Fig. 17**).

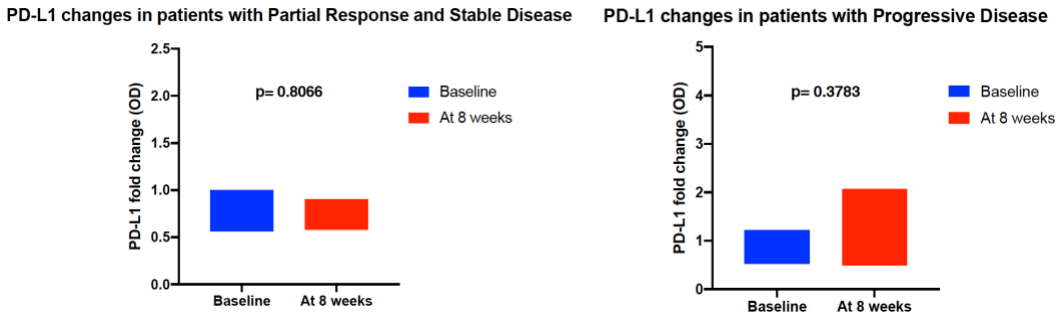


Figure 17 PD-L1 changes between baseline (blue) and after 8 weeks of ICB in patients with PR/SD disease (left) or PD (right)

Interestingly, when considering PD-L1 changes at 8 weeks between patients with progressive disease and partial response/stable disease (non-progressors), a trend towards PD-L1 reduction was observed in the latter group, albeit this difference was not statistically significant (p=0.1941) (**Fig. 18**).

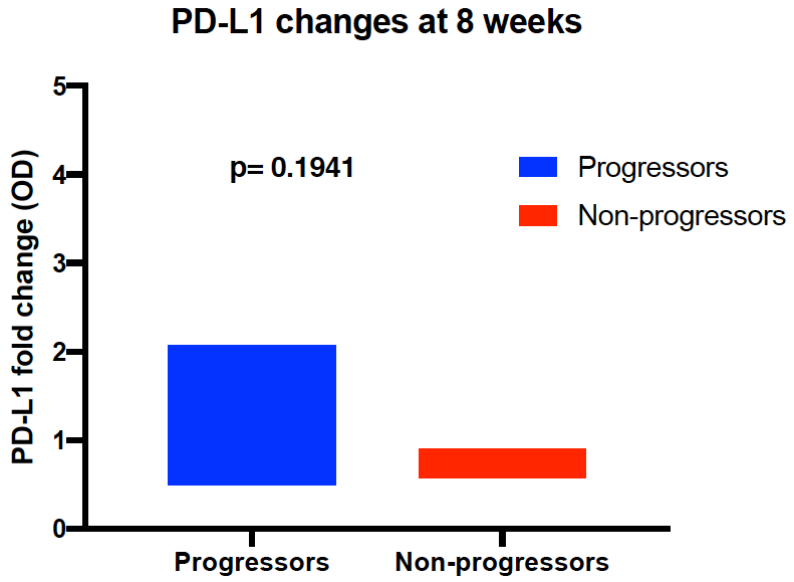


Figure 18 PD-L1 changes after 8 weeks of ICB in patients with PR/SD disease (red) or PD (blue)

Interestingly, dynamic changes during treatment of exosomal PD-L1 expression, assessed as exosomal PD-L1 ratio (**Fig. 19**) between pretreated samples and after 8 weeks of treatment, predicted the outcome of ICI treated patients in terms of both PFS and OS.

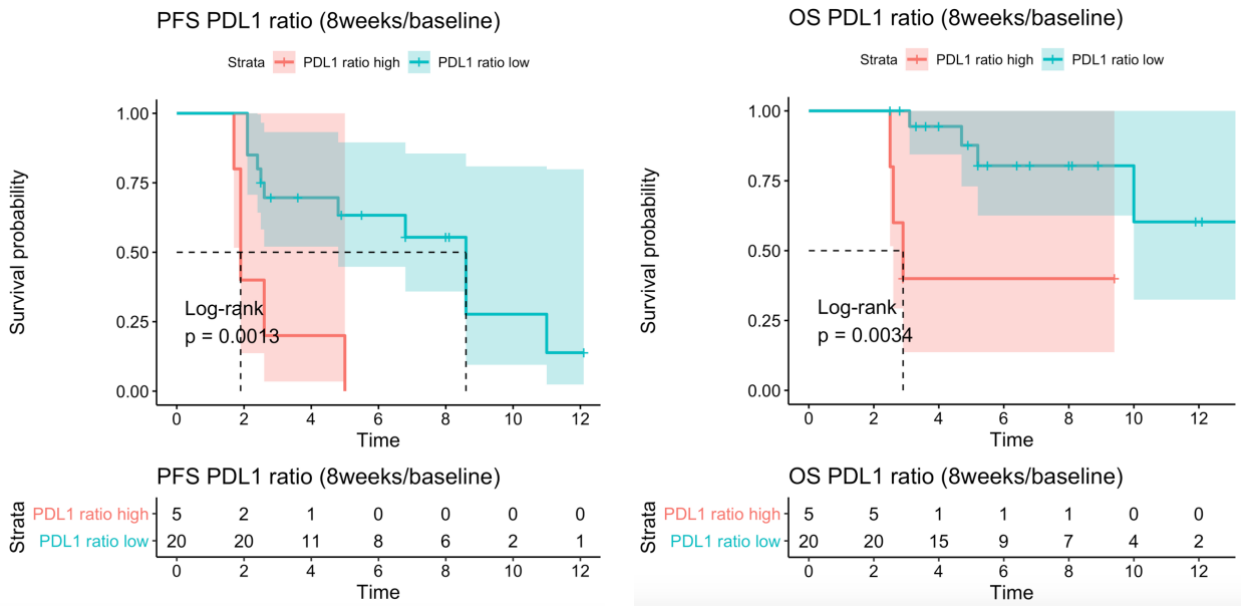


Figure 19. PFS and OS curves for exosomal PD-L1 ratio (8 weeks/baseline)

5.4 Discussion

During the last few years, immune checkpoint inhibitors targeting PD-1/PD-L1 have revolutionized the therapeutic landscape of advanced NSCLC with unprecedented results in multiple clinical settings. However, with the exception of PD-L1 IHC expression, there is a lack of reliable predictive biomarkers that can allow an optimal selection of patient candidate to these agents. The recent failure of tumor mutational burden (TMB) in predicting the activity of ICIs in addition to chemotherapy in the KEYNOTE-189 [42] and KEYNOTE-021 [43] trials raised several concerns on the validity of this beforehand promising biomarker, supporting the evaluation of alternative markers of efficacy. In this preliminary study we evaluated different clinical and circulating biomarkers that can predict the outcomes of NSCLC patients treated with ICIs.

NLR and PLR and ICIs outcome. First, we assessed the prognostic role of some markers of systemic inflammation that we and others [40, 41, 44-46] previously had associated with poor outcome after ICB in advanced NSCLC. Tumor-associated inflammation is a well-established hallmark of cancer [1] and accumulating evidence suggest that neutrophils are key modulators of immune system in cancer, generally associated with poor outcomes [47, 48]. Our results confirm the poor prognostic role of high pretreatment neutrophil-to-lymphocyte ratio (NLR ≥5) and platelet-to-lymphocyte ratio (PLR ≥200) with a very

dismal OS compared with low NLR and/or PLR. A prospective validation of these easy to measure parameters is warranted and might be of patient stratification for clinical trials beside other well-known prognostic factors. In addition, the recent evidence of dynamic changes of these parameters [40, 41] during ICB suggests also a potential predictive role for ICIs and should be confirmed in prospective studies.

Cytokines dynamics and outcome. We then evaluated the potential predictive role of different circulating biomarkers in serial plasma samples of 32 NSCLC patients treated with PD-1 inhibitors as single agent.

Several studies have demonstrated that PD-L1 expression is influenced by different signaling pathways, transcriptional factors, as well as epigenetic factors [22], in two different ways: "*oncogene-driven*", a consequence of constitute activation due to activation of oncogenes or the loss of tumor suppressor genes, and "*immune-driven*", when there is a cytokine-induced stimulation as a mechanism of adaptive resistance of the tumor to the immune system action [20, 21]. Therefore, the use of tissue biopsy cannot recapitulate the exact status of the tumor microenvironment at the time of treatment with ICIs that in some cases can be several months or even years after tumor collection. The use of liquid biopsy can overcome some of the limits of tissue biopsy allowing a dynamic monitoring of the complex interaction between immune system and tumor rather than a static picture of the tumor microenvironment. Therefore we evaluated in the present study different circulating biomarkers to predict the outcome of NSCLC patients treated with ICIs selecting two different time points, baseline and after 8 weeks of treatment, based on the evidence that median time to response to ICIs was ~2 months in all randomized clinical trials in advanced NSCLC with PD-1/PD-L1 inhibitors as single agent [7-11]. A third time point for biomarkers analysis was planned at disease progression in order to evaluate the potential mechanisms of acquired resistance to these agents and will be object of future investigations in a larger cohort of patients. In the present preliminary analysis, we investigated a small panel of cytokines, which were previously correlated with activity on PD-1/PD-L1 axis as well as with resistance to ICB in solid tumors, including TGF-β, IFN-γ and IL-6. Transforming growth factor-β (TGF-β), a pleiotropic cytokine with immunosuppressive effects on multiple cell types of the innate and adaptive immune system, has emerged as one of the potential key factors modulating response to ICIs [38]. Recently, increased TGF-β levels in the tumor microenvironment of colon cancer have been reported to represent a primary mechanism of immune evasion that promotes T-cell exclusion and blocks acquisition of the TH1-effector phenotype [39] and lack of response

to atezolizumab in urothelial carcinoma was associated with a signature TGF-β signaling in fibroblasts [49]. This data suggests a potential role for TGF-β as a target for immunotherapy as well as a marker of immune resistance.

IFN-γ is produced predominantly by T cells and NK cells in response to a variety of inflammatory or immune stimuli and also contributes to cancer immune-evasion by promoting the expression of tolerant molecules in tumor cells, such as PD-L1, CTLA-4 and IDO1 [50]. As PD-1 can attenuate T-cell-mediated antitumor responses, preclinical studies reported that blockade with an anti-PD-1 antibody increases IFN-γ at the tumor site [51]. Therefore, some studies evaluated the expression of IFN-γ as predictive biomarker for immunotherapy. For instance in the IMpower150, patients who had high expression of an effector T-cell (Teff) gene signature in the tumor (Teff-high WT population), defined as the expression of PD-L1, CXCL9, and IFN-γ messenger RNA, as determined with the use of RNA sequencing, was associated with improved outcome with addition of atezolizumab to carboplatin-paclitaxel-bevacizumab (ABCP) compared to carboplatin-paclitaxel-bevacizumab (BCP) alone (median PFS 11.3 months vs. 6.8 months; HR 0.51; P<0.001). However, the PFS benefit was also observed in the ABCP group than in the BCP group in the entire intention-to-treat population (including those with EGFR or ALK genetic alterations) and among patients with low or negative PD-L1 expression, those with low Teff gene-signature expression (GSE), and those with liver metastases [52]. For these reasons, ABCP combination gained FDA approval in upfront setting of advanced non-squamous NSCLC regardless of PD-L1 or Teff GSE.

High levels of IL-6 have been associated with poor outcomes in several solid tumors, including NSCLC and recently have been correlated with high levels of PD-1 and deregulated STAT-1 signaling [53]. Preclinical studies have associated IL-6 with resistance to ICIs and increased PD-L1 expression. Furthermore, blocking IL-6 and TGF-β enhances the activity of immunotherapy [54, 55]. These data suggest that monitoring of cytokine levels in patients treated with ICIs may have a potential use as predictive biomarker to these agents. However, to date only few studies evaluated the potential role of these cytokines and their potential dynamic changes during ICB in NSCLC. A small retrospective study evaluating a panel of 12 cytokines and showed that increased cytokine levels (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6 and IL-8) at the time of diagnosis and at 3 months after initiation of ICIs (nivolumab or pembrolizumab) were significantly correlated with improved response to immunotherapy and prolonged OS, but not with PFS [56]. However, here we did not find any significant correlation between cytokines levels and outcomes during ICB, despite similar sample size and treatment. Potential reasons of this discrepancy might be

the different time points for cytokine analysis (after 3 months vs. 8 weeks), as well the inclusion of two different ethnical populations (Caucasian and Hispanics) that are known to be associated with some distinct clinic-pathological characteristics in advanced NSCLC, as for instance EGFR mutations frequency [57]. Interestingly, a small Japanese study reported that serum levels of IL-6/TNF-α in 10 NSCLC patients treated with PD-1 inhibitors were not significantly different between pre- and post-initial PD1-1 blockage [IL-6 20.3 (2.6-49.9) and 22.9 (3.6-96.1) pg/mL, $p = 0.453$; TNF-α 1.6 (0.7-6.3) and 3.3 (0.7-9.6) pg/mL, $p = 0.329$]; however, high IL-6 levels were associated with higher responses (ORR 57%) [58]. These results are in line with our analysis, showing poorer outcomes among patients with IL-6 levels in terms of both PFS and OS. Unfortunately, the low number of patients with high IFN-γ and TGF-β in our study does not allow drawing definitive conclusions on their role in NSCLC patients treated with ICIs, albeit a trend toward a shorter survival was observed.

Exosomal PD-L1 and PD-1 blockage. The new era of liquid biopsy is growing very fast and has already entered clinical practice in advanced NSCLC through circulating tumor DNA genotyping [59]. However, this large family of circulating biomarkers includes a great variety of components that may also be exploited as a source of tumor biomarkers. Exosomes are cell-derived nanovescicles (30–100 nm of diameter) firstly described studying reticulocyte differentiation. Exosomes are formed by the inward budding of multivesicular bodies (MVB), component of the endocytic pathway and are released from different types of cells both under physiological and pathological conditions and, interestingly, have been well detected in several body fluids. They function as messenger particles, playing a role in cell-to-cell communication and to deliver proteins, lipids, mRNAs and miRNAs with biological activity in the target cells [60, 61]. We hypothesize that PD-L1 specific exosomes contains altered protein cargo, which can be used as non-invasive liquid biopsy marker for NSCLC. Here we evaluated exosomal PD-L1 at baseline and following treatment with ICIs in 25 NSCLC patients. Albeit the difference of expressed proteins identified from PD-L1 specific exosomes was not statistically significant between progressors and non-progressors likely due to the small sample size, a trend towards reduced expression at 8 weeks was observed in the latter subgroup, suggesting that exosomal PD-L1 might be used as non-invasive serum marker for immune response to these agents. Further studies in larger populations are needed to confirm these findings. To the best of our knowledge no previous studies have explored the potential utility of exosomes as predictive biomarkers for immunotherapy in NSCLC. Recently, a small

retrospective study in melanoma patients suggested that circulating exosomal PD-L1 might represent a potential rationale-based and clinically accessible predictor for clinical outcomes of anti-PD-1 therapy. In patients with metastatic melanoma, the level of circulating exosomal PD-L1 positively correlated with that of IFN-γ and changes during the course of anti-PD-1 therapy. Indeed, the magnitudes of the early on-treatment increase in circulating exosomal PD-L1 might represent as an indicator of the adaptive response of the tumor cells to T cell re-invigoration, stratifying clinical responders from non-responders. High levels of exosomal PD-L1 might reflect the "exhaustion" of patient T cells to a breaking point, by which the T cells can no longer be re-invigorated by the anti-PD-1 treatment [62]. This data is hypothesis generating and should be confirmed in further studies.

Study limitations and future perspectives. One of the limitations of the present study is the small sample size and further studies in larger patient populations are needed to further confirm these findings. In addition, we included in this preliminary analysis only a small panel of plasma cytokines, but the evaluation of a broader panel that includes all the key regulators of PD-1/PD-L1 axis (IL-2Ra, CD27, B7.2, CTLA-4, PD-L1, PD-L2, PD-1, Tim-3, LAG-3, Galectin-9, etc.) and CD8+ T cells activity (Il-2, IL-4, IL-10, IL-6, IL-17A, TNF-α, etc.) is planned. Another crucial aspect that we will investigate is the existence of potential racial and sexual differences in the response to ICIs in advanced NSCLC. Limited studies have evaluated racial disparities in the molecular landscape of lung cancer, with variable results. Literature data supported a gender difference, in addition a faster immunoresponse in African Americans (AAs) to vaccines and infections [63-65], but not studied in Hispanics. Currently, there is no data evaluating the possible differential response to immunotherapy in these subpopulations. In addition, there are very limited reports regarding the role of immune related biomarkers among different racial populations. Unfortunately, clinical trials that have evaluated ICIs in NSCLC included a disproportionately low number of minority patients including AAs. In each of the phase III clinical trials which led to the FDA approval of nivolumab, pembrolizumab and atezolizumab as second line therapy for advanced NSCLC [7-10], AA patients represented 3% or less of the total enrolled participants. The KEYNOTE-024 and KEYNOTE-189 trials do not specify the race of the patients enrolled, but rather report the geographic "*region of enrollment*" worldwide [11, 14]. With minority underrepresentation in these clinical trials, questions regarding differential treatment response or side effect profile of immunotherapy agents in different racial populations have gone unanswered. Therefore, there is a strong

need to evaluate for differences in immunologic response among advanced NSCLC patients of different races before and during oncologic treatment. In addition, recent reports suggest that sex may play an important role in immunotherapy efficacy. Several lines of evidence suggest that the immune system of males and females reacts substantially differently [66] and recently strong patterns of gene-expression profile (GEP) of immune genes in tumors from female patients have been reported, suggesting gender-specific patterns of response to ICIs [67]. However, available clinical data are conflicting. A recent meta-analysis of randomized clinical trials evaluating the addition of PD(L)-1 blockage to platinum-based chemotherapy in 1st line NSCLC showed that women derived a statistically significantly larger benefit from the addition of chemotherapy to anti–PD-1/PD-L1 as compared with men (HR 0.48 and 0.76, respectively) [68]. However, a large US retrospective real-world study presented at the 2019 World Conference on Lung Cancer (WCLC) showed that sex alone does not impact the benefit of immunotherapy treatment in stage IV NSCLC, suggesting that other underlying biological differences which may impact immunotherapy benefit [69]. Similarly, in the present study, albeit limited by a small sample size, we did not find any significant difference in the outcome of female and male patients treated with PD-1 inhibitors. In addition, a meta-analysis of randomized clinical trials in pretreated NSCLC patients reported that PD-1 inhibitors significantly improved the PFS in male patients when compared with chemotherapy (HR=0.76; 95% CI 0.68 to 0.86; p<0.00001), but not in women (HR=1.03; 95% CI 0.89 to 1.20; p=0.69) [70].

Racial and sexual differences in the response to immunotherapy and the underlying biological mechanisms will be investigated in a larger patient's cohort that will include in addition to the two cohorts of patients analyzed in the present preliminary study 78 patients enrolled in the phase II study PROLUNG that was recently presented at the WCLC 2019 [71]. We planned to include a broader panel of cytokines that regulate the PD-1/PD-L1 axis as well as CD8+ T cells activity, miRNAs discovery and proteomics analyses on tumor exosomes and correlation of exosomal PD-L1 with tissue expression and patient outcomes. Various studies have demonstrated the role of miRNA and mRNA in signaling pathways in NSCLC. Therefore, gene chip analysis for mRNA and microarray for miRNA will be performed using PD-L1 exosomes to determine a diverse range of transcripts (mRNA) and miRNA that regulates signaling molecules involved in inflammatory, apoptotic pathways in NSCLC patients. Analysis will be performed using PD-L1 exosomes isolated at baseline and after treatment to study the differentially regulated mRNA and miRNA that will define the role for PD-L1 exosomes in NSCLC patients. Ideal candidate molecule will be used as non-invasive marker for NSCLC patients. The qualitative analysis of tumor exosome

content, including miRNAs, mRNAs and proteins, may provide useful information on the complex interactions of cancer-immune system in these patients and may generate future studies investigating their potential exploitations as biomarkers of activity to these compounds as well as a pharmacological target.

5.5 Conclusions

In summary, we reported in this preliminary study that increased levels of inflammatory cytokines, such as IL-6, IFN-γ and TGF-β are associated with poorer outcomes in advanced NSCLC patients treated with ICIs targeting PD-1/PD-L1. Furthermore, changes of exosomal PD-L1 are observed during immune checkpoint blockage and might represent a potential biomarker of activity to these agents. The project is still ongoing and a more comprehensive characterization of the immune profile of NSCLC patients treated with ICIs will be provided with a larger patient cohort. The use of a broader panel of cytokines and a more extensive characterization of tumor exosome content through miRNA and proteomic analysis in addition to the data presented here could allow us to better characterize the biological bases of response to PD-1/PD-L1 blockage in NSCLC. In addition, the evaluation of plasma samples at disease progression might further define the underlying mechanisms of acquired resistance to ICIs that are to date largely unknown.

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