SECOND LINE TREATMENT OF NON-SMALL CELL LUNG CANCER: CLINICAL, PATHOLOGICAL AND MOLECULAR ASPECTS OF NOVEL PROMISING DRUGS

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SECOND LINE TREATMENT OF NON-SMALL CELL LUNG CANCER: CLINICAL, PATHOLOGICAL AND MOLECULAR ASPECTS OF NOVEL PROMISING DRUGS

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Lung cancer still remains a challenging disease with a higher mortality rate in comparison to other cancers. The discovery of oncogene addicted tumours and targeted therapies responsive to these targets lead to a meaningful change in the prognosis of these diseases. Unfortunately, these newer therapeutic options are reserved to a minor part of lung cancer patients harbouring specific mutations. In the so called wild type population, the first line options bring the median overall survival to go beyond 1 year, and in the population receiving the maintenance therapy over 16 months. Given these results, more than 60% of patients may receive a second line therapy with further opportunities to improve the length and quality of life.

For patients not harbouring targetable DNA mutations newer options will be available for second line therapeutic schemes and two major assets seem to be promising: immune modulation and anti-angiogenetic agents. In particular, anti PD1/PDL1 antibodies, VEGFR antibodies and TKIs, these latter combined with standard chemotherapy docetaxel advance the median overall survival of 12 months. These drugs have a different mechanism of action, various adverse events and their activity is different depending on the types of population. However, the biomarkers' activity and efficacy prediction are not fully or totally understood. In addition, also for patients with DNA targetable mutations new drugs seems to be promising for the use in the second line therapeutic protocols. In particular, drugs selectively directed against ALK translocation and mutational events and EGFR T790M secondary mutations seems to be very promising.

In this Research Topic we critically discuss the older therapies and the historical development of second line, putting in to perspective the new agents available in clinical practice. We discuss their importance from a clinical point of view, but also consider and exploit the complex molecular mechanisms responsible of their efficacy or of the subsequently observed resistance

phenomena. In this perspective, the undercovering and characterization of novel predictive biomarkers by NGS technology, the characterization of novel actors in the signal transduction pathway modulating the response of the cells, the optimization of new diagnostic tool as the evaluation of liquid biopsy and the implementation of more suitable pre-clinical models are crucial aspects dissected too.

Nivolumab, nintedanib and ramucirumab probably will give the opportunity to improve the efficacy outcomes for the treatment of wild type tumours in second line therapeutic schemes, but many aspects should be debated in order that these agents are made available to patients, planning ahead a therapeutic strategy, beginning from the first line therapy, to the subsequent ones in a logical and affordable manner. As well, for treatment of mutated tumours, mutated EGFR irreversible inhibitors such as rociletinib and AZD9291, and ALK targeting drugs ceritinib and alectinib will also play an important role in the immediate future. Probably the right way is to give all the available opportunities to patients, but challenges and pitfalls should be carefully debated, and by launching this Research Topic we tried to give some practical insights in this changing landscape.

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EDITORIAL
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Editorial: Second Line Treatment of Non-Small Cell Lung Cancer: Clinical, Pathological and Molecular Aspects of Novel Promising Drugs

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Keywords: NSCLC, liquid biopsy diagnostics, precision medicine, molecular markers, immunotherapy, antiangiogenic therapy

Editorial on the Research Topic

Second Line Treatment of Non-Small Cell Lung Cancer: Clinical, Pathological and Molecular Aspects of Novel Promising Drugs

The advent of precision medicine and predictive molecular pathology led to a revolution in clinical management of patients with non-small cell lung cancer. The discovery of oncogene addiction allowed the development of targeted therapies that represent newer therapeutic options reserved to those patients harboring specific gene alterations, such as EGFR mutations, ALK, and ROS1 translocations (Lazzari et al.; Sullivan and Planchard; Tran and Klempner; Köhler). In addition, the introduction of immunotherapy, with anti PD-1 Pembrolizumab, in first-line treatment of NSCLC represents the best choice for EGFR, ALK, and ROS1 wild-type patients expressing PD-L1 on ≥50% of neoplastic cells (Cortinovis et al.). Despite the survival improvement achieved with these new therapeutic options in first-line treatment, about 30% of patients do not obtain a tumor response (Lazzari et al.). Moreover, those patients, initially sensitive to these treatments, acquire resistance and develop tumor progression. Approximately 60% of the patients progressing from first-line therapy receiving further systemic treatment in the second-line setting (Lazzari et al.; Cortinovis et al.) Also in second line, the armamentarium for the treatment of patients with NSCLC, includes a pletora of new drugs, such as immune checkpoint inhibitors (Pembrolizumab, Nivolumab) (Cortinovis et al.), third generation tyrosine kinase inhibitors (Osimertinib) (Molina-Vila et al.; Zugazagoitia et al.), and anti-angiogenic agents (Nintedanib and Ramucirumab) (Corrales et al.; Manzo et al.).

This exciting therapeutic scenario for NSCLC patients still has unsolved questions and challenging issues, in particular regarding the optimal selection of the patient population through the individualization of the correct methodology and biological source of material (tissues vs liquid biopsy) for clinical relevant biomarkers assessment (Molina-Vila et al.). Probably the right way is to give all the available opportunities to patients, but challenges and pitfalls should be carefully debated.

Taken together, the papers published in Research Topic "Second Line Treatment of Non-Small Cell Lung Cancer: Clinical, Pathological and Molecular Aspects of Novel Promising Drugs" represent a critical discussion focused on the older therapies and the historical development of second line, putting into perspective the new agents available in clinical practice, defining their importance from a clinical point of view, but also to consider and exploit the complex molecular mechanisms responsible of their efficacy or of the subsequently observed resistance phenomena, to support the oncologist to design the best therapeutic strategies for NSCLC patients.

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REVIEW
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Historical Evolution of Second-Line Therapy in Non-Small Cell Lung Cancer

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Innovative therapeutic agents have significantly improved outcome with an acceptable safety profile in a substantial proportion of non-small cell lung cancer (NSCLC) patients, who depend on oncogenic molecular alterations for their malignant phenotype. Despite the survival improvement achieved with first-line chemotherapy, about 30% of patients do not obtain a tumor response. Moreover, those patients, initially sensitive to treatment, acquire resistance and develop tumor progression after a median of about 5 months. Approximately 60% of the patients progressing from first-line chemotherapy receive further systemic treatment in the second-line setting. Moreover, new options have emerged in the second-line armamentarium for the treatment of patients with NSCLC, including immune checkpoint inhibitors and antiangiogenic agents. The current review provides an overview on the clinical studies that gained the approval of chemotherapy agents (*docetaxel and pemetrexed*) and epidermal growth factor receptor gene–tyrosine kinase inhibitors as second-line treatment options for NSCLC patients, not carrying molecular alterations.

Keywords: non-small cell lung cancer, second line, docetaxel, pemetrexed, erlotinib, angiogenesis, immunotherapy

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INTRODUCTION

Lung cancer is the leading cause of cancer death in the world (1) and non-small cell lung cancer (NSCLC) accounts for approximately 85% of cases. The majority of patients are diagnosed with advanced or metastatic disease. Despite the progresses in the treatment of NSCLC, the prognosis remains poor, with an estimated 5 years overall survival (OS) of only 16%.

For a long time, platinum doublet chemotherapy has been the standard first-line treatment option for NSCLC patients (2, 3). Until 2005, treatment choice was mainly based on the distinction between NSCLC and small cell lung cancer. The approval of bevacizumab in 2006 (4, 5) and pemetrexed in 2008 (6) raised the issue that discriminating between squamous and non-squamous histology was a crucial element for therapeutic selection, since bevacizumab and pemetrexed can be administered to patients with non-squamous tumors only, for safety and efficacy reasons.

During the past 10 years, thanks to the technological advances, our knowledge on NSCLC tumor biology has improved (7). Different driver molecular alterations, responsible for the development of oncogene-addicted NSCLC tumors, have been identified, especially in the subgroup of patients with adenocarcinoma (8–12). Currently, NSCLC is not considered a single homogenous entity, but as a heterogeneous disease, including rare molecularly classified lung tumors, that are susceptible to targeted inhibition (13–17). Patients who carry activating mutations in the epidermal growth factor

receptor gene (EGFR) or translocations in the anaplastic lymphoma kinase gene are treated with their specific tyrosine kinase inhibitors (TKIs), while platinum-based doublet chemotherapy with or without bevacizumab remains the first-line standard of care for patients in whom no molecular alteration is identified.

Despite the survival improvement achieved with first-line chemotherapy (18), about 30% of patients do not obtain a tumor response. Moreover, those patients, initially sensitive to treatment, acquire resistance and develop tumor progression after a median of about 5 months (19). Approximately 60% of the patients progressing from first-line chemotherapy receive further systemic treatment in the second-line setting. Currently, second-line therapy is based on docetaxel, pemetrexed, erlotinib, nivolumab, or the combination of docetaxel with nintedanib or ramucirumab. The current review provides an overview on the clinical studies that gained the approval of chemotherapy agents and EGFR–TKIs as second-line treatment options for NSCLC patients, not carrying molecular alterations.

DOCETAXEL AND PEMETREXED

The TAX317 study (20) was the first phase III trial showing a survival advantage of second-line chemotherapy in NSCLC patients, previously treated with platinum-based regimen (**Table 1**). One hundred three patients, stratified according to Eastern Cooperative Oncology Group performance status (ECOG PS) and best response to first-line chemotherapy, were randomized between two different doses of docetaxel (100 and 75 mg/m^2) and best supportive care. Docetaxel was associated with significantly longer OS and time to progression (TTP), compared with best supportive care. The advantage was significantly greater in the group receiving docetaxel at the dose of 75 mg/m^2, probably due to the higher frequency of febrile neutropenia and deaths observed in patients under treatment with 100 mg/m^2. These results were confirmed by the phase III TAX320 study (**Table 1**), which compared docetaxel at the dose of 100 or 75 mg/m^2, with vinorelbine or ifosfamide in 373 NSCLC patients, who had previously failed platinum-containing chemotherapy (21). Docetaxel was associated with longer TTP and progression-free survival (PFS). Even though OS did not differ between the three regimens, a significant greater percentage of patients receiving docetaxel at the dose of 75 mg/m^2 was alive during the first year, compared with those randomized in the vinorelbine or ifosfamide arms (**Table 1**). Based on these data, docetaxel at the dose of 75 mg/m^2 has become the reference control arm for second-line chemotherapy for patients with advanced NSCLC.

With the aim to reduce the frequency of grade 3–4 hematologic adverse events, observed in a high proportion of the patients enrolled in the TAX317 and TAX 320 trials (54 and 67%, respectively) (20, 21), two docetaxel schedules (75 mg/m^2 administered every 3 weeks and 33.3 mg/m^2 administered weekly) were investigated in the phase III DISTAL-1 study (**Table 1**) (22). No significant difference was observed in terms of OS or global quality of Life (QoL), even though the weekly docetaxel resulted in significantly lower incidence of leukopenia, neutropenia, and hair loss, but higher occurrence of non-neutropenic infections. Moreover, an improvement in some of the QoL items, such as pain and cough, were reported with the weekly regimen (**Table 1**). In order to better compare the efficacy and the safety profile of the weekly and three-weekly docetaxel regimens, an individual patient data meta-analysis, including three phase III and two phase II randomized trials, enrolling 865 patients, was performed (24). No difference in terms of OS or objective response rate (ORR) was found, but a significant advantage in terms of severe and febrile neutropenia was confirmed in favor of the weekly schedule, thus suggesting that weekly docetaxel represents a valid alternative to the three-weekly administration (**Table 1**).

Another therapeutic opportunity in the second-line setting is represented by the antifolate pemetrexed (25). Based on the results of a phase III trial, showing the non-inferiority of pemetrexed in terms of PFS, OS, and ORR and a more favorable toxicity profile over docetaxel, with fewer grade 3–4 neutropenia and febrile neutropenia (23), in 2004, pemetrexed was approved in the USA and Europe for the second-line treatment of patients with advanced NSCLC (**Table 1**). A previous retrospective analysis, focusing on the toxicities observed in 246 patients treated between 1995 and 1999 with pemetrexed, indicated that high pretreatment plasma homocysteine levels were associated with severe toxicity. This finding suggested that decreasing homocysteine levels, through the use of folate and vitamin B12 supplementation, would have improved pemetrexed safety profile without decreasing its efficacy (26). The favorable toxicity profile of pemetrexed was confirmed in the subset analysis performed in 86 out of 571 patients with ≥70 years, enrolled in the phase III registration trial (27).

TABLE 1 | Clinical trials exploring second-line chemotherapy.

Study	Treatment	*N* of pts	Major toxicities	Progression-free survival HR (95%CI)	Overall survival HR (95% CI)
TAX317 (20)	Docetaxel (100 or 75 mg/m^2) vs best supportive care	103	Leukopenia, neutropenia, hair loss	–	$p = 0.01$
TAX320 (21)	Docetaxel (100 or 75 mg/m^2) vs vinorelbine or ifosfamide	373	Leukopenia, neutropenia, hair loss for docetaxel	$p = 0.005$	5.5 vs 5.7 m (NS)
DISTAL-01 (22)	Docetaxel (75 mg/m^2 q21) vs docetaxel (33 mg/m^2 weekly)		Weekly: non-neutropenic infection 3-weekly: leukopenia, neutropenia, hair loss	–	HR 1.04 (0.77–1.39) $p = 0.80$
JMEI (23)	Pemetrexed (500 mg/m^2) vs docetaxel (75 mg/m^2)		Leukopenia, neutropenia, hair loss for docetaxel	HR 0.97 (0.82–1.16)	HR 0.99 (0.8–1.20)

NS, not significant; HR, hazard ratio.

A following phase III study, exploring cisplatin–pemetrexed as a first-line option, showed that pemetrexed is more effective in patients with non-squamous histology, due to the low expression of thymidylate synthase, a gene involved in the synthesis of folate and responsible for pemetrexed resistance in patients with lung squamous tumors (6, 28). Accordingly, the second-line indication for pemetrexed was revised to include patients with advanced non-squamous histology only.

In order to improve the therapeutic options, several trials have explored the efficacy and safety of doublet chemotherapy. An individual patient data analysis, including 847 patients, enrolled in six randomized trials (four phase II and two phase III), comparing mono-chemotherapy with doublet chemotherapy, was performed (29). Even though there was a statistically significant PFS improvement (of about 2 weeks) and a double RR with combination regimen, no survival prolongation was observed. These findings do not appear clinically relevant, and mono-chemotherapy has remained the standard of care for second-line treatment.

CHEMOTHERAPY OR EGFR–TKIs IN SECOND-LINE SETTING

In 2005, the phase III BR.21 trial (**Table 2**) compared the efficacy of the EGFR–TKI erlotinib with best supportive care in previously treated 731 advanced NSCLC patients, with ECOG PS 0-3. Significant improvement in terms of OS, PFS, and QoL was observed in the erlotinib arm (30). For a long time, the identification of molecular and clinical features, able to predict which patients could benefit more from EGFR–TKIs, has been the focus of much research. Based on the clinical data from patients, enrolled in the BR.21 trial, who early progressed (<8 weeks), or died (within 3 months from randomization) under erlotinib, a prognostic score, including 10 factors (smoking history, ECOG PS, weight loss, anemia, lactic dehydrogenase, response to prior chemotherapy, time from diagnosis, number of prior regimens, EGFR copy, and ethnicity), was built (31). Only 10% of the patients were classified in the low risk group and had high significant survival advantage with erlotinib over placebo. Moreover, the retrospective analysis of Kirsten rat sarcoma viral oncogene homolog, EGFR mutations, and EGFR gene copy number by fluorescence *in situ* hybridization (FISH) showed that EGFR mutations and high copy number were predictive of response to erlotinib, but only EGFR FISH resulted as a significant predictive marker of differential survival benefit (32). Despite EGFR activating mutations being identified in 2004 (8, 33), their role, as predictive biomarkers of sensitivity to EGFR–TKIs, was recognized only in 2009, following the results from the phase III IPASS study. The study reported a significant PFS and ORR advantage of gefitinib over platinum-based first-line chemotherapy in EGFR mutant patients and a detrimental effect in the EGFR wild-type subgroup (34). These findings shifted the development of EGFR–TKIs toward the first-line treatment of EGFR oncogene-addicted tumors and raised the question if erlotinib was an appropriate therapeutic option for EGFR wild-type patients or patients with unknown molecular status in the second-line setting. Other considerations include understanding the differences between QoL and toxicity profile for EGFR–TKIs in comparison to standard of care in second-line and beyond.

Erlotinib has advantages in terms of toxicity, route of administration, and QoL. Its efficacy was compared with docetaxel and pemetrexed in different Phase III trials. Even though these studies had a different statistical design, the results were similar.

The TITAN trial (**Table 2**) was designed to demonstrate a 25% improvement in median OS of erlotinib vs chemotherapy (docetaxel and pemetrexed) in 648 unselected NSCLC patients, who had progressed during first-line platinum doublet chemotherapy (35). Due to the slow accrual, the trial was prematurely closed, enrolling 424 patients only. No significant difference in terms of OS or PFS was seen between erlotinib and chemotherapy. Tumor samples were mandatory to enter the trial, and EGFR mutation status was available in 160 of the enrolled patients. Comparable OS and PFS were observed in the EGFR wild-type subgroup under chemotherapy or erlotinib.

These results were partly confirmed by the DELTA and the HORG studies (**Table 2**). The primary objective of the DELTA trial was to show 1-month PFS superiority of erlotinib over docetaxel in unselected second- or third-line 301 Asian NSCLC patients (36). Even though no significant difference was observed in terms of PFS and OS, docetaxel statistically prolonged PFS in the EGFR wild-type subgroup (199 patients out of 255 analyzed). However, this improvement did not translate into longer survival. The HORG study randomized 322 NSCLC patients, previously progressed to one or two chemotherapy lines, between erlotinib and pemetrexed (39). Squamous histology was not an exclusion criterion and the primary end-point was TTP. There was no difference in terms of TTP, ORR, or OS between the two treatment

TABLE 2 | Clinical trials exploring epidermal growth factor receptor gene–tyrosine kinase inhibitors with second-line chemotherapy.

Study	Treatment	*N* of pts	Major toxicities	Progression-free survival HR (95%CI)	Overall survival HR (95% CI)
BR.21 (30)	Erlotinib vs best supportive care	731	Skin rash, diarrhea	HR 0.61 (0.51–0.74) $p < 0.001$	HR 0.70 (0.58–0.85) $p < 0.001$
TITAN (35)	Erlotinib vs docetaxel or pemetrexed	424	Skin rash, diarrhea for erlotinib	–	HR 0.96 (0.78–1.19) $p = 0.73$
DELTA (36)	Erlotinib vs docetaxel	301		HR 1.22 (0.97–1.55) $p = 0.09$	HR 0.91 (0.68–1.22) $p = 0.53$
TAILOR (37)	Docetaxel vs erlotinib	222	Leukopenia, neutropenia, hair loss for docetaxel	HR 0.71 (0.53–0.95) $p = 0.02$	HR 0.73 (0.53–1.0) $p = 0.05$
PROSE (38)	Docetaxel or pemetrexed vs erlotinib	285		HR = 1.35 (1.05–1.73) $p = 0.020$	HR = 1.22 (0.93–1.59) $p = 0.148$
HORG (39)	Erlotinib vs pemetrexed	322	Skin rash, diarrhea for erlotinib	$p = 0.136$	$p = 0.986$
LUX-LUNG 8 (40)	Afatinib vs erlotinib	795	Skin rash, diarrhea	HR 0.82 (0.68–1.0) $p = 0.04$	HR 0.81 (0.69–0.95) $p = 0.01$

arms. EGFR mutations were analyzed in 123 patients, and no OS, TTP, or ORR difference was observed, but EGFR wild-type patients had higher disease control rate under pemetrexed over erlotinib.

In contrast with the other studies, significantly longer PFS and OS (at the adjusted multivariate analysis) were found in favor of docetaxel in the 222 EGFR wild-type patients, enrolled in the TAILOR trial (**Table 2**), whose primary objective was to show 14% OS improvement at 1 year of docetaxel over erlotinib in EGFR wild-type NSCLC patients (37). The cross-over treatment in further lines was not allowed and only taxane-naïve patients were included. These differences might have influenced the OS results.

Finally, the PROSE study (**Table 2**) randomized 285 unselected second-line NSCLC patients, who were blinded classified according to a serum proteomic algorithm (the VeriStrat® test), previously developed, with the aim to identify patients who could benefit from EGFR–TKIs (38, 41). Patients were stratified by the proteomic algorithm, ECOG PS, and smoking history. The primary end-point was OS, and the primary hypothesis was to demonstrate the existence of a significant interaction between the proteomic classification and treatment efficacy. The VeriStrat® test is a multivariate biomarker, developed using eight *m/z* ratio mass spectrometric peaks. It classifies patients into two groups (*good* and *poor*), according to the clinical outcome observed under treatment with EGFR–TKIs. The results from the PROSE study were comparable to previous reports and showed that the PFS was longer in patients receiving chemotherapy, while no OS difference was found in the intent to treat analysis. The VeriStrat® test was prognostic, since g*ood* classified patients had better OS and PFS than *poor* classified ones. Furthermore, while *good*-classified patients derived similar OS benefit from erlotinib and chemotherapy, VeriStrat *poor*-patients had significantly shorter OS under erlotinib, suggesting that the algorithm was also predictive of differential OS benefit between erlotinib and chemotherapy. EGFR mutations were analyzed in 176 patients included in the primary analysis, 14 of whom carried EGFR mutations. No statistical significant interaction was observed between VeriStrat classification and the EGFR mutation status, and comparable PFS and OS results were found in the EGFR wild-type subgroup. The prognostic and predictive role of the VeriStrat® test was also retrospectively evaluated in 441 patients from the BR.21 trial (42). VeriStrat results demonstrated prognostic for OS and PFS, and predictive of response, but not predictive of differential benefit from erlotinib vs placebo.

Several meta-analyses have been performed to address the issue about the efficacy of EGFR–TKIs or chemotherapy in the second-line setting for the treatment of EGFR wild-type patients or patients with unknown molecular status. Recently, a meta-analysis, including 10 randomized trials and 1,119 EGFR wild-type patients, showed a significant PFS improvement for chemotherapy compared with EGFR–TKI therapy, with no OS difference (43). These results were confirmed by an individual patient data analysis, not yet published, and presented at ASCO in 2015, including 587 EGFR wild-type patients, enrolled in TAIOLR, DELTA, and PROSE studies. Chemotherapy determined longer PFS, which did not translate into longer OS.

Based on these findings, there are sufficient evidences suggesting that, in EGFR wild-type patients with good ECOG PS, chemotherapy determines a greater disease control, although with more toxicity and without increasing survival.

Results from PROSE might partly explain as to which factors can contribute to the discrepancy observed between PFS and OS. One possible explanation is that, since *poor* classified patients have a detrimental effect under erlotinib, they do not benefit from third-line chemotherapy, and this determines shorter OS. Conversely, in *good* classified patients, erlotinib does not worsen their clinical conditions, allowing them to take advantage from further lines, thus influencing survival. Considering that 30% of NSCLC patients are classified as *poor*, it is possible that in an unselected population, the OS difference between chemotherapy and erlotinib does not emerge. The biological rationale behind the proteomic status is currently the subject of research. Four out of the eight *m/z* peaks composing the VeriStrat *poor* profile are generated by Serum Amiloid A1 (SAA-1) and its two truncated forms (44). Moreover, in VeriStrat *poor* classified patients, higher level of a panel of anti-inflammatory proteins (haptoglobin, SAA2, SAA3, α1-antitripsyn, and α1-antichimotrypsin) was observed. SAA1 is an acute-phase protein, and it is a non-specific tumor prognostic marker (45, 46). It is induced by interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNFα) (47). Data from literature showed that IL-6 reduced the sensitivity to erlotinib in NSCLC cells harboring EGFR mutations, due to an increased autocrine stimulation of the IL-6/gp130/signal transducer and activator of transcription 3 (STAT3) pathway (46). IL-6 activates the janus (JAK) and the Src kinases, which are responsible for the phosphorylation on the tyrosine 705 of the STAT3. Once phosphorylated, STAT3 translocates to the nucleus and activates the transcription of genes involved in cell cycle progression (cyclin D1, survivin), cell survival (B-cell lymphoma 2), angiogenesis (vascular endothelia growth factor a), and immune suppression [programed death ligand 1 (PD-L1)] (48, 49). These data suggest that the immune cells infiltrating tumor microenvironment might be the crucial determinants for influencing tumor biology, and the clinical outcome observed in VeriStrat *poor* classified patients. While erlotinib has no inhibitory effect on the stromal elements infiltrating tumor microenvironment, chemotherapy inhibits these cells, thus reducing tumor aggressiveness and prolonging survival.

Combinatorial strategies, including second-line docetaxel chemotherapy with the EGFR monoclonal antibody cetuximab, have been evaluated, with poor results. The greatest benefit was observed in those who continued previous EGFR-TKIs for ≥ 6 months (50).

THE ROLE OF EGFR–TKIs IN PATIENTS WITH SQUAMOUS HISTOLOGY

In the field of lung squamous cell carcinoma (LSCC), less progress has been made. Although molecular alterations in LSCC have been described, effective targeted therapies have not yet been developed (51). These potentially targetable molecular alterations include phosphoinositide 3-kinase (PIK3CA), fibroblast

growth factor receptor 1 (FGFR1), or c-MET amplification and discoidin domain receptor tyrosine kinase 2 mutations, though none of these biomarkers have been validated in the clinical setting (52). The EGFR gene is commonly overexpressed in patients with LSCC (53), and two monoclonal anti-EGFR antibodies, cetuximab and necitumumab, in combination with platinum-based chemotherapy in the first-line setting, have demonstrated improved survival in phase III studies (54, 55).

Based on these data, recently, the irreversible ErbB-family inhibitor afatinib has been compared with erlotinib in the phase III Lux-Lung 8 trial, enrolling 795 squamous patients, previously progressed on platinum-based chemotherapy (**Table 2**) (40). The primary end-point was PFS and the primary objective was to demonstrate a 29% reduction in the risk of progression with afatinib over erlotinib. Afatinib significantly prolonged PFS and OS, health-related QoL outcomes, and symptoms control. Archived tumor tissue was collected. Six percent of the patients carried EGFR activating mutations, and another six percent harbored EGFR amplification.

Even though, based on these results, afatinib may represent an additional option for the treatment of LSCC, and it has been approved by the Food and Drug Administration (FDA) for the treatment of squamous NSCLC progressing after platinum-based chemotherapy, the new programed death 1 (PD-1) and PD-L1 inhibitors have dramatically changed the therapeutic algorithm of patients with squamous histology and represent the first therapeutic choice for second-line treatment.

COMMENTS AND FUTURE PERSPECTIVES

New options have emerged in the second-line armamentarium for the treatment of patients with NSCLC, including immune checkpoint inhibitors and antiangiogenic agents. The genome instability of cancer cells (56) favors the development of immunogenic clones (57). The antigen presenting cells (APC) or the dendritic cells recognize the tumor antigens, which are presented to the T cell receptors, that once activated on CD8+ T cells induce the killing of tumor cells. Inhibitory pathways have been selected to switch off the duration of the immune responses and prevent the tissue damage. Tumor cells take advantage of these inhibitory pathways to escape immune recognition and continue to proliferate. The binding of PD-1, expressed on activated T cells, tumor-infiltrating lymphocytes, and T regulatory cells, with PD-L1 or PD-L2, located on APC or tumor cells favors the T cells apoptosis and decrease cytokines production, thus modulating the immune system activation (58). Agents targeting the PD-1 axis suppress the inhibitory pathways responsible for the induction of the immune tolerance, resulting in the restoration of T cells antitumor activity. Based on the results from the phase III CheckMate-017 and CheckMate 057 trials, showing the OS improvement of the *PD-1 inhibitor* nivolumab over docetaxel in squamous and non-squamous patients, respectively, nivolumab was granted approval by the FDA and the European Medicine Agency (EMA) (59, 60). Moreover, recently, the phase III OAK study, comparing docetaxel with the *PD-L1 inhibitor* atezolizumab, showed a significant survival improvement of 27% in patients receiving atezolizumab, leading to atezolizumab FDA approval for the treatment of second-line NSCLC patients. Similarly, the phase II–III KEYNOTE-010 study, comparing the *PD-1 inhibitor* pembrolizumab with docetaxel in NSCLC patients with PD-L1 expression on at least 1% of tumor cells, showed that OS was significantly longer for pembrolizumab vs docetaxel (61). Among patients with at least 50% of tumor cells expressing PD-L1, both OS and PFS were significantly longer with pembrolizumab than docetaxel, thus determining the approval of pembrolizumab by EMA for the treatment of second-line NSCLC patients, positive for PD-L1 expression.

Another attractive therapeutic target is represented by angiogenesis, involved in the development and progression of NSCLC. Angiogenesis acts as one of the essential alterations occurring in cells during malignant transformation (56), since the delivery of oxygen and nutrients, provided by blood vessels, is required for cell survival and proliferation. Different molecules, inhibiting the angiogenic regulators, have been tested in combination with second-line chemotherapy (pemetrexed and docetaxel) in patients with NSCLC, but with disappointing results (62). Only recently, two drugs, interfering with the angiogenic pathways, nintedaninb and ramucirumab, have received the regulatory approval in association with docetaxel in the second-line setting.

Nintedanib is an oral triple angiokinase inhibitor, hindering the vascular endothelial growth factor receptor (VEGFR1–3), the FGFR1-3, the platelet-derived growth factor receptors (PDGFRα/β), fms-like tyrosine kinase 3 and members of the Src family (Src, Lyn, Lck) (63). Based on the results from the LUME Lung 1 study (64), showing a PFS improvement in patients receiving nintedanib in combination with docetaxel and a significantly prolonged OS in the subgroup of patients with adenocarcinoma, who had progressed within 9 months from the beginning of first-line treatment, the EMA approved the use of nintedanib for the treatment of locally advanced or metastatic patients with lung adenocarcinoma after platinum recurrence.

Ramucirumab is an IgG1 monoclonal antibody, targeting the extracellular domain of the VEGFR-2, thus preventing the binding of VEGF ligands and hindering receptor activation (65). When associated with docetaxel, it improves both PFS and OS (66). These clinically meaningful findings led FDA and EMA to expand the indication of ramucirumab, previously approved for the treatment of gastric cancer, to include the treatment of metastatic NSCLC.

Emerging evidence that pro-angiogenic factors have immunosuppressive activity has suggested that agents targeting angiogenesis may be potentially synergistic with immunotherapy (67–69). Data from literature indicate that VEGF influences lymphocyte trafficking, stimulates T regulatory cells and myeloid-derived suppressor cells, and inhibits T-cell development, thus favoring tumor immune escape (70–72). Moreover, it has been reported that immunotherapies can also be antiangiogenic. Different phase I trials exploring the safety and efficacy of combination regimens are currently ongoing in different types of tumors, including NSCLC.

However, based on the recent results from the Phase III KEYNOTE-024 study, showing doubling PFS and ORR in favor

of pembrolizumab- vs cisplatin-based first-line chemotherapy in patients with PD-L1 expression on at least 50% of tumor cells (73), and the Phase II KEYNOTE-021 trial, demonstrating a significant PFS and ORR improvement when pembrolizumab was combined with carboplatin pemetrexed chemotehrapy, compared with chemotherapy alone (74), it is supposed that PD-1 or PD-L1 inhibitors alone or in combination with chemotherapy will become the standard of care for first-line treatment of NSCLC patients. As a consequence, clinicians will deal with new challenges for the definition of the second-line treatment algorithm.

Our knowledge on cancer immunology is not fully complete, and it is still not clear how to select those patients who benefit more from therapy with immune checkpoint inhibitors. Different studies are ongoing, and the predictive role of PD-L1 expression, evaluated by immunohistochemistry, is the focus of much research. Different PD-L1 antibodies, with different cutoff levels, have been selected according to the different PD-1 or PD-L1 inhibitors evaluated in the clinical trials. Recently, thanks to the collaboration between academy, pharmaceutical, and diagnostic companies, there has been an attempt to compare and explore the differences and the similarities between the PD-L1 diagnostic assays (75). A weak correlation was found. Other markers are under evaluation. Data from retrospective analyses indicate that tumors with a high mutational burden, abundant neoantigens, and micro-satellite high status are associated with a good response to anti-PD-1/PD-L1 therapy, but additional studies are warranted (76–79).

In conclusion, new agents have been developed and approved for the treatment of NSCLC patients without oncogene-addicted tumors, after platinum-based chemotherapy progression, thus improving the number and efficacy of therapeutic opportunities, but increasing the complexity of the therapeutic selection. Currently, the most remarkable challenge remains the lack of predictive biomarkers, able to identify which patients might gain most benefit from these agents.

AUTHOR CONTRIBUTIONS

The authors CL, VG, AB, MD, MV, EB, and LG have contributed equally to write this paper.

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MINI REVIEW
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Second-Line Treatment of NSCLC—The Pan-ErbB Inhibitor Afatinib in Times of Shifting Paradigms

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In contrast to the established role of epidermal growth factor receptor (EGFR) inhibitors for the first-line treatment of patients with non-small cell lung cancer (NSCLC) harboring activating *EGFR* mutations, the role of EGFR blockade and of *EGFR* molecular testing in the second-line treatment remains less clear. The irreversible pan-ErbB family inhibitor afatinib (Gi(l)otrif®) was recently FDA- and EMA-approved for the second-line treatment of NSCLC with squamous cell histology irrespective of the *EGFR* mutational status (LUX-Lung 8). Contrariwise, results from the TAILOR and DELTA trials among retrospective biomarker analyses show the predictive value of the *EGFR* mutational status for efficacy of reversible EGFR inhibitors also as a second-line therapy. This mini review critically summarizes the current role of EGFR-targeting strategies in the second-line treatment of NSCLC with special respect to afatinib in light of emerging *T790M*-specific EGFR and immune check point inhibitors. The review also emphasizes the urgent need for reliable biomarkers to guide therapeutic decision-making and outlines prospective changes to the second-line landscape with some of the current second-line treatment concepts likely to be moved to the first-line.

Keywords: afatinib, EGFR mutation, TKI, second-line treatment, NSCLC, squamous cell carcinoma, T790M-specific inhibitors, checkpoint blockade

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INTRODUCTION

Over the past decade, various genomic alterations relevant for non-small cell lung cancer (NSCLC) biology ("oncogene addiction") were discovered and have subsequently changed the treatment paradigm from a histology-oriented to a biomarker-driven approach [reviewed by Thomas et al. (1)]. Historically, docetaxel was the gold standard second-line treatment (2) until erlotinib (Tarceva®), a first-generation tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR) was FDA-approved in 2004 as maintenance therapy and for second and subsequent line treatment, after failure of chemotherapy in unselected patients (3). In the meantime, several phase-III trials compared EGFR TKIs with chemotherapy and have established EGFR TKIs as the standard first-line treatment for patients with *EGFR*-mutant NSCLC (4–7). Nowadays, not less than three EGFR TKIs—erlotinib, gefitinib (Iressa®) and the pan-ErbB family inhibitor afatinib (Giotrif®)—are licensed for the first-line treatment. Drug reimbursement is bound to the presence of a common activating *EGFR* mutation (i.e., exon 19 deletions and L858R point mutations) detected by FDA-approved tests [erlotinib—cobas®; gefitinib (Iressa®) and afatinib (Gi(l)otrif®)—therascreen

EGFR RGQ]. However, the relevance of *EGFR* mutations for the second-line decision-making process remained less clear, and erlotinib (for all NSCLC) as well as afatinib (for squamous cell histology only) have initially been FDA-approved irrespective of *EGFR* mutational status or other predictive markers (3, 8). Several recent prospective clinical trials (TAILOR, DELTA) and retrospective biomarker analyses challenge this broad approval and emphasize the need for *EGFR* mutational re-testing ahead of the second-line therapy if not performed at diagnosis (9, 10).

EVIDENCE FOR CLINICAL EFFICACY OF EGFR TKIs IN THE SECOND LINE

The use of first-generation EGFR TKIs like erlotinib and gefitinib in the second-line treatment of patients with *EGFR*-mutant NSCLC is supported by prospective single-arm studies, retrospective biomarker analyses of phase-II studies and subgroup analyses from phase-III studies (11–20). Several randomized trials have compared single-agent EGFR TKIs with single-agent chemotherapy and showed an improvement in progression-free survival (PFS) but mostly not in overall survival (OS) with chemotherapy compared with EGFR TKIs in an *EGFR* wild-type population (9, 10, 19, 21–28). Afatinib has been tested in the worldwide LUX-Lung trial program and second-line studies included LUX-Lung 2 (first- or second-line, single-arm), LUX-Lung 4 (second-line or beyond), and the head-to-head comparison with erlotinib in LUX-Lung 8 (second-line) (8, 29). Afatinib like dacomitinib (the latter is not FDA-approved yet) irreversibly inhibits all ErbB family members and was supposed to overcome resistance mediated by secondary *EGFR T790M* mutations (30) which occur in ~50–60% of cases upon progression with reversible EGFR TKIs (31). Both drugs demonstrated promising activity against T790M in preclinical models but failed to overcome T790M-mediated resistance in patients due to dose-limiting toxicity resulting from inhibition of wild-type EGFR (32). Furthermore, analyses of small numbers of re-biopsy samples suggest that treatment with afatinib in the first-line results in similar rates (~50–60%) of secondary *T790M* mutations upon progression compared to reversible EGFR TKIs (31, 33). This may be due to the high frequency (up to 80%) of pretreatment *EGFR T790M* mutations (34). However, the results from LUX-Lung 4 and 5 suggested that some patients not only may benefit from afatinib after acquired resistance to gefitinib/erlotinib but also from continued ErbB inhibition during chemotherapy versus switching to single-agent chemotherapy after progression with EGFR TKIs (35). The LUX-Lung 5 results have yet not let to changes in second-line treatment recommendations in terms of combining EGFR inhibition with cytotoxic chemotherapy post-progression in patients with *EGFR*-mutant NSCLC who initially responded to EGFR TKI treatment.

AFATINIB IN NSCLC WITH SQUAMOUS CELL HISTOLOGY

Currently, treatment paradigms are most dramatically changing in tumors with squamous cell histology. This entity has unmet medical needs even though the incidence in Western countries is decreasing (25% of all lung cancer cases). Reflecting the tobacco carcinogenesis, tumors are genomically complex yet *EGFR* mutations are sporadic, and *EGFR* molecular testing is not routinely performed in this subgroup (36). Molecular analyses indicated that pan-ErbB blockade could be of therapeutic benefit in squamous cell tumors due to multiple genetic aberrations in ErbB receptors (*HER2*: 4%, *HER3:* 2%) and in downstream signaling molecules (*KRAS:* 3%, *HRAS*: 3%, *BRAF*: 4%, *NF1*: 11%, *NRG1*) (36). Furthermore, 20–30% of tumors overexpress HER2 and HER3. Whereas erlotinib was the only approved second-line TKI in squamous cell lung cancer since 2004, afatinib received FDA- and EMA-approval for the second-line treatment of squamous cell NSCLC in 2016 based on results of the head-to-head (against erlotinib) study LUX-Lung 8 (8). This approval is irrespective of the intratumoral *EGFR* mutational status. Supposedly, the improved OS [median 7.9 months (95% CI 7.2–8.7) versus 6.8 months (5.9–7.8); HR 0.81 (95% CI 0.69–0.95), $p = 0.0077$] is unlikely driven by the inhibition of mutant *EGFR* which was found in only 6% of the patients but rather by the broader irreversible pan-ErbB blockade with afatinib compared to erlotinib.

NEWLY EMERGING THIRD-GENERATION EGFR INHIBITORS AND IMMUNE CHECKPOINT BLOCKADE IN THE SECOND-LINE TREATMENT

After second-generation EGFR TKIs failed to effectively overcome T790M-mediated resistance in the clinical setting, drugs that specifically inhibit EGFR T790M without affecting wild-type EGFR were developed subsequently. Osimertinib (Tagrisso®), a EGFR T790M-specific kinase inhibitor, inhibits EGFR exon 18, 19, and 21 mutations and the drug-resistant *T790M* mutation and received accelerated FDA approval in 2015. Response rates to osimertinib in patients with *T790M*-positive tumors after first-generation EGFR TKI are comparable to those with first-line EGFR TKI (58–61%) and the median PFS reached 9.6 months compared to 2.8 months in EGFR T790M-negative patients. Osimertinib has a better toxicity profile than first- and second-generation EGFR TKI due to the reduced wild-type EGFR inhibition. Common adverse events are class-specific (i.e., diarrhea, rash, nail toxicity) but were generally mild to moderate (37).

Other promising therapeutic concepts that experienced a tremendous renaissance especially in squamous cell NSCLC include the modulation of the tumor vasculature [anti-VEGFR-2 antibody ramucirumab (Cyramza®), REVEL trial] (38) and of the immune environment. The latter strategy enhances the patient's natural immune response to cancer mainly *via* CD8+ cells. Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death protein (PD-1) have been identified as important targets which are expressed on activated T cells and interact with ligands on antigen-presenting cells thereby limiting the immune response. Both, the anti-PD-1 monoclonal antibody Nivolumab (Opdivo®) (39, 40) and pembrolizumab (Keytruda®) (41) have

been FDA-approved for PD-L1-positive (defined as a tumor proportion score ≥ 50%) metastatic squamous (nivolumab) or squamous and non-squamous (pembrolizumab) NSCLC lacking *EGFR* or *ALK* mutations with progression to platinum-based chemotherapy. Other antibodies targeting PD-L1 like atezolizumab (MPDL3280A) confirm the efficacy of this innovative concept of immune checkpoint blockade (42).

CONCLUSION AND OUTLOOK

Compared to the their role in the first-line, reversible (erlotinib, gefitinib) and irreversible (afatinib) EGFR TKIs have relatively less impact on the second-line treatment of patients with advanced NSCLC. Afatinib, however, was recently approved for patients with squamous cell NSCLC irrespective of the *EGFR* mutational status. With the advent of innovative treatment concepts as e.g., immune checkpoint blockade or T790M-specific EGFR inhibition, it is likely that EGFR TKIs will be further pushed into the first-line where they already today face ongoing head-to-head comparisons with the EGFR T790M-specific inhibitor osimertinib to identify the most effective upfront treatment option for patients with *EGFR*-mutant NSCLC (e.g., FLAURA trial: osimertinib versus gefitinib or erlotinib).

Currently, patients with *EGFR*-mutant tumors should be treated with EGFR TKIs as soon as possible, ideally in the first-line setting. This is supported by several first-line phase-III clinical trials, which showed higher response rates (>70%) (5, 43) as if the EGFR TKI was given in the second-line (27–67.4%) even though some of the reported data on response rates have been conflicting (5, 18, 19, 43, 44). Apart from the pooled LUX-Lung 3 and 6 analyses, all EGFR TKI first-line trials failed to show an OS benefit (45). This is likely confounded by crossover of patients to EGFR TKI post-progression to first-line chemotherapy. From the only prospective randomized TORCH trial which compared first-line EGFR TKI followed by chemotherapy with first-line chemotherapy followed by second-line EGFR TKI, the authors concluded, that patients with *EGFR* mutations would experience greater benefit from first-line EGFR TKI followed by second-line chemotherapy. However, patients in this study were not selected by *EGFR* mutational status (only 14.2% were *EGFR* mutation positive) and the small sample size as well as the fact that only 60% of patients in both arms received second-line treatment furthermore confounded the result (46). Numerous arguments yet support the application of EGFR TKI in the first-line over second-line: quality of life during EGFR TKI treatment is better compared to first-line chemotherapy especially in patients with poor performance status, whole-brain irradiation with its detrimental consequences on cognitive functions for patients with brain metastasis may be delayed by EGFR TKIs (47–49) and giving EGFR TKIs upfront increases the chance of TKI exposure for those patients whose tumors harbor the target. This is supported by the fact that about 1/3 of patients with *EGFR* mutations assigned to first-line chemotherapy did not receive EGFR TKI as salvage therapy in IPASS, WJTOG 3405, and OPTIMAL (4, 6, 50). LUX-Lung 6 reported the longest PFS (13.7 months) of all first-line EGFR TKIs and two head-to-head comparison studies [LUX-Lung 7 (first-line) and 8 (second-line)] were slightly in favor of afatinib over erlotinib and gefitinib even though there was some criticism about the interpretation of results and the publication strategy (26). Nevertheless, these trials indicate that afatinib is a highly effective drug in this setting but comes with numerically higher side effect rates compared to erlotinib and gefitinib (8, 51, 52). These toxicities are effectively manageable by supportive measures (53, 54) and tolerability-guided dose reductions which do not affect therapeutic efficacy (55). Especially afatinib, however, will be confronted with EGFR T790M-specific inhibitors like osimertinib in the first-line setting as the latter have a more favorable toxicity profile due to less wild-type EGFR inhibition.

If *EGFR* mutational testing has not been performed ahead of the first-line therapy—it is estimated that 15 to 35% of patients have insufficient tumor tissue for genotyping (56, 57), patients should be considered for repeated testing before starting second-line therapy. Plasma-genotyping, a technique that uses cell-free (cf)DNA, may be an important alternative to the classical biopsy approach in this scenario (58, 59) and it is highly likely that "liquid biopsies" will become available for many known oncogenic and resistance mutations in the near future. This may substantially change the decision-making process as liquid biopsies will enable the physician to monitor development of resistance more promptly and to decide more accurately on therapeutic consequences (60). TAILOR, DELTA, and other trials indicate the predictive value of *EGFR* mutational status on EGFR TKIs in the second-line (9, 10). In particular, the TAILOR study clearly suggests that second-line docetaxel is superior to erlotinib in terms of survival in all patients with *EGFR* wild-type NSCLC who are able to tolerate toxicities of chemotherapy. DELTA and other trials (CTONG0806 (28) and NCT01783834 (61): pemetrexed versus gefitinib) as well as a meta-analysis by Li et al. (62) point into the same direction with a better PFS for second-line chemotherapy in *EGFR* wild-type patients. On the contrary, there is evidence to suggest that patients with *EGFR*-mutant NSCLC who are still TKI naive perform better with EGFR TKIs (9, 10, 19, 21–28). In this context, reacting to the JUNO trial results (not fully published yet), FDA restricted the indication for erlotinib as maintenance or second or greater line treatment to those NSCLC patients whose tumors harbor common *EGFR* mutations in October 2016.

If the *EGFR* mutation status remains unknown for the second-line treatment decision, a preferred strategy would be to offer nivolumab for squamous NSCLC or pembrolizumab for squamous and non-squamous histology (after platinum-based chemotherapy if PD-L1 expression ≥ 50%). The approval of immune checkpoint inhibitors will consequently push docetaxel—long the standard of care treatment in the second-line—to the third-line or even beyond. Especially for squamous cell NSCLC, based on the positive survival results of the SQUIRE study which tested the human EGFR monoclonal antibody necitumumab in combination with cisplatin-gemcitabine chemotherapy, the treatment might soon change even in the first-line setting (63). Big efforts are furthermore ongoing to advance biomarker-driven therapies for patients with squamous cell carcinoma of the lung within the Lung-MAP studies (64) and it is also not a far-fetched vision that immune checkpoint inhibitors will have a role in untreated

advanced lung cancer. Currently, more than 10 randomized trials (among them KEYNOTE, CHECKMATE, IMPOWER) are ongoing and the question will rather be how checkpoint inhibitors will integrate into the upfront setting, as monotherapy or in concurrent or sequential combination with chemotherapy.

Other important questions remain that may open up new indications for afatinib, but also other EGFR TKIs as to which drug is most effective in controlling brain metastases and rare *EGFR* mutations. It is known, that patients with *EGFR* mutations have an increased risk especially for leptomeningeal tumor dissemination (65, 66). Penetration of the blood-brain barrier as well as clinical efficacy have been described for both afatinib (47–49) and osimertinib (67). Other EGFR inhibitors with high *in vivo* CNS penetration (e.g., AZD3759) are currently under early clinical phase evaluation. To determine the most effective drug for CNS disease, also more systematic investigation of the mutational spectrum in brain metastases is required. In this context, surprisingly, a restrospective study found the majority of CNS and leptomeningeal metastases to be negative for EGFR T790M despite of T790M positivity in the extracranial tumor (spatiotemporal heterogeneity) (68). This may argue against T790M-specific and rather for first- or second-generation EGFR TKIs.

Another field of current interest are less common *EGFR* mutations which together represent about 10% of all *EGFR* mutations (69). Especially afatinib may be a good option for these rare *EGFR* mutations that include exon 18-21 duplications, G719X, Del18, E709K, insertions in exon 19, S768I, or L861Q as erlotinib, osimertinib and gefitinib showed only moderate activities in these mutations (70, 71, 72). Osimertinib contrariwise may be effective in rare exon 20 insertions whereas nazartinib (EGF816) shows promising efficacy in the majority of exon 20 mutations. The quinazoline-based EGFR inhibitors, gefitinib and afatinib finally proofed efficacy in tumors containing a common *EGFR* mutation (i.e., Del19 or L858R), in conjunction with L718Q, L844V, or C797S (73, 74).

To summarize, treatment paradigms for NSCLC patients in the second-line are currently experiencing dramatic changes. Many of the currently tested innovative concepts will likely move forward to the first-line treatment, whereas other strategies and possibly indications for EGFR TKIs (as, e.g., continued ErbB blockade post-progression, TKI-specific efficacy in rare mutations) may be established in the second-line. One necessity that all therapeutic concepts and treatment lines share in common is the urgent need for reliable predictive factors in times of increasing treatment costs. These are still not available for anti-angiogenic agents like ramucirumab and it remains unclear, if any predictive biomarker will help to select patients with squamous cell NSCLC for afatinib treatment in the future.

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REVIEW
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Second-Line Treatment of Non-Small Cell Lung Cancer: Clinical, Pathological, and Molecular Aspects of Nintedanib

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Lung carcinoma is the leading cause of death by cancer in the world. Nowadays, most patients will experience disease progression during or after first-line chemotherapy demonstrating the need for new, effective second-line treatments. The only approved second-line therapies for patients without targetable oncogenic drivers are docetaxel, gemcitabine, pemetrexed, and erlotinib and for patients with target-specific oncogenes afatinib, osimertinib, crizotinib, alectinib, and ceritinib. In recent years, evidence on the role of antiangiogenic agents have been established as important and effective therapeutic targets in non-small cell lung cancer (NSCLC). Nintedanib is a tyrosine kinase inhibitor targeting three angiogenesis-related transmembrane receptors (vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor). Several preclinical and clinical studies have proven the usefulness of nintedanib as an anticancer agent for NSCLC. The most important study was the phase III LUME-Lung 1 trial, which investigated the combination of nintedanib with docetaxel for second-line treatment in advanced NSCLC patients. The significant improvement in overall survival and the manageable safety profile led to the approval of this new treatment in Europe. This review focuses on the preclinical and clinical studies with nintedanib in NSCLC.

Keywords: non-small cell lung cancer, angiogenesis, target therapy, nintedanib, second-line treatment, clinical trials

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INTRODUCTION

Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer-related deaths worldwide, accounting for 1.59 million deaths yearly. In the United States alone, an estimated 221,200 new cases of lung cancer were diagnosed in 2015, and 158,040 people will die of this disease (1–3). Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer, accounting for more than 80% of all cases, whereas small cell lung cancer represents 15–20% (4, 5). Most patients will experience disease progression during or after first-line chemotherapy, and

there is a significant unmet need for new, effective second-line treatments. Currently, the only approved second-line therapies for patients who do not harbor identifiable driver oncogenes, such as epidermal growth factor receptor (*EGFR*) gene mutations or anaplastic lymphoma kinase (*ALK*) gene translocations, are docetaxel, gemcitabine, pemetrexed (limited for non-squamous NSCLC), and erlotinib (6–9).

The majority of patients with NSCLC do not achieve prolonged disease control, and the 5-year survival rate remains poor at 18.7% (1). Growing knowledge of NSCLC molecular pathobiology has led to the development of new treatments that target specific oncogenes (10) and have changed the natural history of the disease with a clear improvement of patient's survival (11). However, it is still characterized by a significantly low survival for second-line treatment (12, 13) with a median progression-free survival (PFS) from 2 to 3 months and a median survival rarely exceeding 8 months (14). The recognition of patients harboring EGFR mutations (EGFRm) or EML4-ALK translocation and displaying tyrosine kinase inhibitors (TKI) response rates of approximately 70% account an essential treatment. With the use of molecularly targeted therapies, such as erlotinib (15), afatinib (16) for EGFRm, osimertinib (17) for EGFRm T790, and crizotinib (18), alectinib, and ceritinib (19, 20) for ALK positive (**Table 1**), a higher response rates and prolonged PFS have been obtained when compared to chemotherapy in the first- and second-line setting (21).

Antiangiogenic agents have been established as important and effective therapeutic targets in many cancers, including NSCLC. Angiogenesis is one of the hallmarks of cancer and is critical for the growth, progression, and metastasis of many solid tumor types (22–24). Mechanisms that support the formation of neovasculature include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) signaling pathways (22, 25–27). To date, first-line bevacizumab remains the only approved antiangiogenic treatment in the therapeutic armamentarium for advanced NSCLC. Its use is restricted to patients with tumors with a non-squamous histology (28, 29).

In the Eastern Cooperative Oncology Group, 878 patients with recurrent or advanced NSCLC were recruited and assigned to paclitaxel/carboplatin chemotherapy alone or paclitaxel/carboplatin and bevacizumab. The addition of the anti-VEGF to a standard, platinum-based doublet regimen conferred a significant prolongation in overall survival (OS), PFS, and response rate in patients with NSCLC (28) (**Table 1**). Also, bevacizumab administered with paclitaxel showed a median PFS longer compared to docetaxel in second-third line of treatment (30) In the AVAiL trial, patients with non-squamous NSCLC were randomized to receive cisplatin/gemcitabine with or without bevacizumab and in a similar way, the results in this trial demonstrated an improvement in PFS versus placebo (31).

Furthermore, ramucirumab, a vascular endothelial growth factor receptor-2 (VEGFR2) inhibitor, was investigated as second-line therapy with docetaxel for stage IV NSCLC. Median OS and PFS were longer in the ramucirumab arm compared with the placebo arm (32) (**Table 1**). Even though VEGF is the most potent angiogenic molecule, the inhibition of the VEGF pathway with TKI or monoclonal antibodies is associated with a modest survival benefit.

The multikinases inhibitor sorafenib targets VEGFR2–3, PDGFR-β, c-kit, RAF, and FLT-3. In two phase II studies, it was determined an improvement in PFS and in OS when used as a single agent with respect to placebo (33) (**Table 1**). Furthermore, a phase I/II trial studied the effect of sorafenib combined with carboplatin/paclitaxel and showed a median PFS of 34 weeks with a good toxicity profile (34). However, two Phase III trials, ESCAPE and NEXUS trials, were conducted to confirm the efficacy and feasibility of the combination treatment. Unfortunately, neither of the trials met their primary endpoints (35, 36).

Sunitinib, an orally selective multitargeted TKI that inhibits PDGFR, KIT, FLT-3, and VEGFR, has also been evaluated in combination with both chemotherapy and erlotinib after failure of first-line platinum-based chemotherapy. CALGB 30704 randomized patients to pemetrexed alone, sunitinib alone, or the combination of pemetrexed/sunitinib as second-line therapy for advanced NSCLC (37) (**Table 1**). The results demonstrated a non-statistically significant higher response rate in patients receiving pemetrexed/sunitinib and a better PFS and OS in the single agent pemetrexed arm. Also, two trials evaluated the combination of erlotinib and sunitinib, and no differences in PFS or OS were observed (38, 39).

Unfortunately, the activation of other angiogenic pathways has also developed drug resistance by the tumor. Molecules, such as FGF and PDGF, have been found upregulated in patients exhibiting acquired resistance to anti-VEGF treatment. The use of multitargeted anti-angiogenesis tyrosine kinase inhibitors (MATKIs) to achieve simultaneous inhibition of two or three angiogenic pathways has been proposed as a promising strategy for improved outcomes in NSCLC patients (40).

Nintedanib (Vargatef®; BIBF 1120) is a novel, potent, oral, triple angiokinase inhibitor that targets VEGF receptors 1 to 3, PDGF receptors alpha and beta, and FGF receptors 1 to 3 (41–43), as well as members of the Src family and FLT-3 (43) (**Figure 1**).

PRECLINICAL DEVELOPMENT

Nintedanib was identified during a program for small molecule inhibitors of angiogenesis, and studies were extended to various solid tumors (43). Recent evidence shows that nintedanib is a potent endothelial cell proliferation inhibitor with a good safety profile, proven in both *in vitro* and *in vivo* studies.

This molecule, an indolinone derivative, occupies the adenosine triphosphate-binding sites in the kinase domain of pro-angiogenic receptors previously mentioned, inhibiting the downstream signaling pathways. Overall, the spectrum is fairly restricted (VEGFR-1, VEGFR-2, VEGFR-3, FGFR-1, 2, 3, PDGFR-α and β, FLT3, and SRC family member) and has shown low cross-reactivity with other human kinases (41, 43, 44). Peak plasma concentrations of nintedanib are reached 2–4 h after oral administration and have a terminal half-life of 10–15 h. Also, it is metabolized largely *via* hydrolytic cleavage by esterases; cytochrome P450 pathways have a minor role in the metabolism of the MATKI. The major route of elimination is fecal/biliary excretion (45).

TABLE 1 | Early development of Target therapy in non-small cell lung cancer (NSCLC).

Drug mechanism	Reference	*N* total	Drug	Comparator	Median overall survival (OS)	Median OS regarding sequential combination of EGFR–TKI and chemotherapy	Median progression-free survival (PFS)
Tyrosine kinase inhibitors	Zhou et al. (15)	154	Erlotinib	Gemcitabine + carboplatin	22.8 versus 27.2 months[a]	29.7 versus 20.7 or 11.2 months, respectively ($p < 0.0001$)	NA
	Yang et al. (16)	631	Afatinib	Cisplatin/pemetrexed OR Gemcitabine/cisplatin	27.3 versus 24.3 months[a]	NA	NA
	ELCC (17)	60	Osimertinib	platinum-pemetrexed	NA	NA	19.3 months
	Noonan and Camidge (18)	343	Crizotinib	platinum-pemetrexed	[a]	NA	10.9 versus 7.0 months
	Shaw et al. (19)	130	Ceritinib	NA	NA	NA	7 months
Antiangiogenic agents	Sandler et al. (28)	878	Bevacizumab + Paclitaxel + carboplatin	Paclitaxel + carboplatin	12.3 versus 10.3 months ($p = 0.003$)	NA	6.2 versus 4.5 months ($p < 0.001$)
	Garon et al (32)	1,253	Ramucirumab + Docetaxel	Docetaxel + Placebo	10.5 versus 9.1 months ($p = 0.023$)	NA	4.5 versus 3.0 months ($p < 0.0001$)
	Blumenschein et al. (33)	52	Sorafenib	NA	6.7 months	NA	2.7 months
	Heist et al. (37)	125	Sunitinib	Sunitinib + Pemetrexed OR Pemetrexed	8.0 versus 6.7 versus 10.5 months	NA	3.3 versus 3.7 versus 4.9 months

[a]*not statistically significantly different.*
NA, non-applicable.

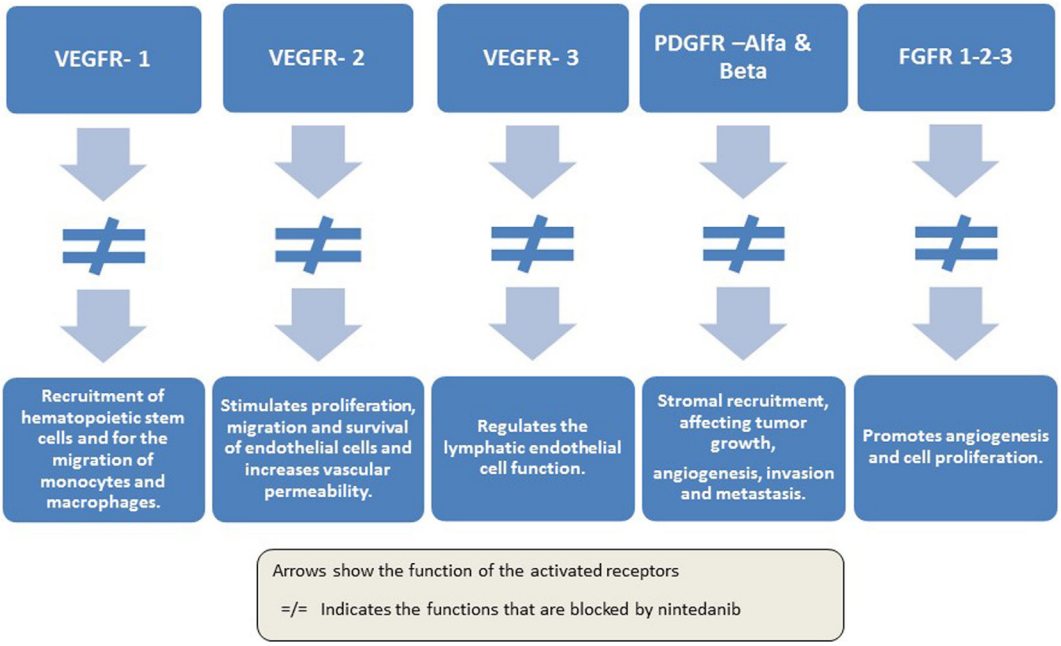

FIGURE 1 | **Mechanism of action of nintedanib in lung cancer treatment.**

In vitro studies showed that treatment with nintedanib induced proliferation arrest and apoptosis in endothelial cells, smooth muscle cells, and pericytes, cell types involved in angiogenesis, through the inhibition of both AKT and mitogen-activated protein kinases signaling pathways, resulting in an overexpression of the apoptosis marker cleaved caspase-3 (43).

Moreover, *in vivo* studies performed in human NSCLC xenografts have confirmed these results. One of the studies showed that at well-tolerated doses, nintedanib was highly active and demonstrated additive effects in combination with the cytotoxic drugs docetaxel or pemetrexed (42). In addition, in another study, nintedanib alone and in combination with standard chemotherapy showed a potent inhibition of proliferation and increased apoptosis of tumor cells in NSCLC xenografts that were poor responders to bevacizumab and resistant to platinum doublet chemotherapy (46). It demonstrated rapid changes in tumor vessel architecture, such as reduction of vessel permeability and perfusion, and microvessel density. Intracellularly, the inhibitory effect of nintedanib was found to be markedly sustained, with inhibition of VEGF receptor activation for at least 32 h after being treated for 1 h with nintedanib, suggesting slow receptor dissociation kinetics and sustained inhibition (43). There was no association with an increased expression of the epithelial mesenchymal transition (EMT) markers, a common mechanism of resistance to antiangiogenic therapies (46).

Another recent study evaluating the co-treatment of nintedanib with small interfering RNAs against six specific genes involved in EMT has shown that this molecule is able to do a downregulation of SYDE1 and ZEB1, and this sensitizes the cell's response to the drug in terms of EMT reversal (47). Additionally, *in vitro* and *in vivo* studies have evaluated the toxic potential of nintedanib, showing a tolerable safety profile of this compound, excluding any severe cardiovascular, respiratory, or neurological adverse effects, as well as any mutagenic potential of nintedanib (48).

Furthermore, the combination potential of nintedanib with PD-1 antagonists was explored in an *in vivo* combination experiments in two syngeneic murine tumor models. The murine tumor cell lines CT-26 and 4T1 were injected subcutaneously into female mice and subsequently treated with RMP1-14, a murine anti PD-1, nintedanib, or RMP1-14/nintedanib. Single agent treatment of CT-26 subcutaneous tumors with RMP1-14 resulted in antitumor effect with treated to control values of 45% and nintedanib resulted in a 63%. The combination treatment group after 24 days showed a value of 34%. Additionally, the use of nintedanib in the anti PD-1 refractory model 4T1 showed a synergistic combinatorial antitumor effect. The combination of angiogenic and immune checkpoint inhibition is an attractive opportunity to improve overall response rates and efficacy based on the dual roles of angiogenic factors in blood vessel formation and immune regulation (49).

PHASE I AND PHASE II CLINICAL TRIALS

The tolerability of nintedanib has been studied in different kinds of neoplasm, such as ovarian cancer, NSCLC, breast cancer, colorectal cancer, urothelial carcinoma, and head and neck cancer (50). In a phase I open-label, dose-escalation trial, Doebele et al. studied the combination of this MATKI with paclitaxel and carboplatin in chemotherapy-naïve advanced NSCLC (51). Twenty-six patients enrolled and received nintedanib at the starting dose of 50 mg twice daily on days 2–21 in association with 200 mg/m^2 paclitaxel

and area under the curve 5 of carboplatin on day 1 of each 21-day cycle. Overall, 84.6% ($n = 22$) experienced a partial response or stable disease without confirmation, and 26.9% ($n = 7$) achieved a confirmed partial response. The treatment was well tolerated with liver enzyme elevations, thrombocytopenia, abdominal pain, and rash being the dose-limiting toxicities (DLT) (**Table 2**).

In another dose-escalation phase I/II trial, 26 patients with advanced NSCLC previously treated with first-line platinum-based chemotherapy, received nintedanib in association with pemetrexed. Patients received a starting dose of nintedanib of 100 mg twice daily on days 2–21 in association with 500 mg/m^2 of pemetrexed on day 1 of a 21-day cycle. Similar to the previous studies, the resultant maximum tolerated dose (MTD) of nintedanib was established at 200 mg twice daily. Moreover, of the enrolled patients, 1 had a complete response, 13 had stable response, and 8 patients showed progressive disease. The median PFS was approximately 5.4 months. A good safety profile was confirmed, with fatigue, anorexia, and ALT increase being the most frequent grade 3 drug-related adverse events (52) (**Table 2**). Moreover, in a Japanese trial, the same MTD of nintedanib (200 mg twice daily) was established and a manageable safety profile and similar efficacy results as the previous studies were found (53).

Okamoto et al. evaluated in a phase I trial, the combination of nintedanib with docetaxel in advanced NSCLC patients who had been previously treated. Forty-two patients (17 BSA < 1.5, 25 BSA ≥ 1.5) were treated. The MTD of nintedanib was 150 and 200 mg twice daily in patients with BSA less than 1.5 and BSA greater than or equal to 1.5, respectively, in combination with 75 mg/m^2 of docetaxel. They found encouraging efficacy results, yielding a 73.7% of disease control rate. Furthermore, DLT, all grade 3 hepatic enzymes elevations, occurred in only one-third of the enrolled patients. All hepatic enzyme elevations were reversible and manageable with dose reduction or discontinuation. The main drug-related adverse events included neutropenia (95%), leukopenia (83%), fatigue (76%), alopecia (71%), decreased appetite (67%), and elevations in alanine aminotransferase and aspartate aminotransferase (64%) (40) (**Table 2**).

Also, a phase II double-blind study assessed the efficacy, safety, and tolerability of nintedanib in stage IIIB/IV NSCLC. The 73 patients recruited tolerated the continuous treatment and had no significant difference in efficacy between treatment arms (nindetanib 250 mg twice a day versus 150 mg twice a day). The median PFS was 6.9 weeks and the median OS was 21.9 weeks with no significant difference between the two groups; the disease control rate was 59% (54) (**Table 2**).

PHASE III TRIALS

The LUME-Lung 1 trial (NCT00805194) is a multinational, randomized, placebo-controlled phase 3 trial that assessed the efficacy and safety of the combination of nintedanib and docetaxel in patients with stage IIIB/IV NSCLC progressing after first-line chemotherapy. Patients were assigned to docetaxel 75 mg/m^2 by intravenous infusion on day 1 in addition to nintedanib 200 mg twice daily orally or matching placebo on days 2–21, every 3 weeks. The primary endpoint was PFS, which was assessed by an independent central review, analyzed by intention to treat after 714 events in all patients. As key secondary outcome, OS was predefined and analyzed on an intention-to-treat basis in a prespecified, stepwise, fixed-sequence order: first, in a predefined group of patients with adenocarcinoma and poor prognosis (i.e., time elapsed since start of first-line therapy of less than 9 months until randomization into the trial); second, in patients with adenocarcinoma; and finally, in all patients regardless of histology. Other secondary outcomes were investigator-assessed PFS, tumor response by central review and investigator assessment, safety, and tolerability.

The study met its primary endpoint demonstrating a statistically significant improvement in PFS that translated into a 21% reduction in risk of progression (55). The PFS according to central independent review was significantly longer in nintedanib plus docetaxel group than in docetaxel plus placebo group (median PFS 3.4 versus 2.7 months; HR 0.79; 95% CI 0.68–0.92; $p = 0.0019$), with a more pronounced benefit in patients with adenocarcinoma histology (median PFS 4.2 versus 1.5 months; HR 0.68; 95% CI 0.54–0.84; $p = 0.0005$). Also, the subset of patients with adenocarcinoma and poor prognosis had a median PFS of 4.2 months in the docetaxel plus nintedanib group versus 1.6 months in the docetaxel plus placebo group (HR 0.67; 95% CI 0.43–1.04, $p = 0.0725$) (56).

Even though, in the total population of patients there was only a trend in favoring the combination of docetaxel and nintedanib (median OS 10.1 versus 9.1 months; HR 0.94; 95% CI 0.83–1.05; $p = 0.2720$), in adenocarcinoma subgroup there was a significant difference in OS (median OS 12.6 versus 10.3 months; HR 0.83; 95% CI 0.70–0.99; $p = 0.0359$). Improvement was also observed in patients with adenocarcinoma histology and poor prognosis; the median OS was longer in the docetaxel plus nintedanib group compared with the docetaxel plus placebo group (median OS 10.9 months versus 7.9 months; HR 0.75; 95% CI 0.60–0.92; $p = 0.0073$) (56). The intent-to-treat analysis of OS in all studied patients showed a 1-month improvement that did not reach statistical significance; however, when adjusted to the sum of longest diameters of target lesions, a significant OS benefit was seen (55).

The tolerability profile was similar to that shown in phase I/II clinical trials. The adverse events that were more common in the docetaxel plus nintedanib group than the docetaxel plus placebo group were: diarrhea, increases of transaminases, nausea, decreased appetite, and vomiting, with only a 18.6% requiring dose reduction (56). Also, a study determined the impact on tumor growth over time as a treatment effect, with a specific focus on patients with poor prognosis (i.e., time of progression less than 9 months and who had progressive disease as best response to first-line treatment). The use of nintedanib and docetaxel showed a significant reduction in tumor burden and tumor growth over time compared to docetaxel in patients with adenocarcinoma histology and in the group of patients with the poorest prognosis (57) (**Table 2**).

Furthermore, Heigener et al. performed an analysis of adenocarcinoma population in the LUME-Lung 1 to determine if first-line treatment could influence subsequent outcomes for nintedanib and docetaxel arm. In the study, the efficacy outcomes,

TABLE 2 | From phase I to phase III clinical trials on nintedanib.

Clinical trial (phase)	Reference	Patient characteristics	*n*	Drug combination	*N* dose/ frequency	Response *n* (%)			Stable disease	Progression	Median PFS	Median OS
						Stable disease or partial response	Partial response	Complete response				
I	Doebele et al. (51)	Chemotherapy-naïve advanced NSCLC	26	Paclitaxel + carboplatin + N	50 mg/2 id	22 (84.6)	7 (26.9)	0	15 (57.7)	NA	NA	NA
I/II	Ellis et al. (52)	Advanced NSCLC preciously treated with first-line platinum-based chemotherapy	26	Pemetrexed + N	100 mg[a]/2 id	NA	NA	1 (3.8)	13 (50)	8 (30.8)	5.4 months	NA
I	Okamoto et al. (40)	Advanced NSCLC previously treated	42	Docetaxel + N	150–200 mg/2 id	31 (73.7)	NA	NA	NA	NA	NA	NA
II	Reck et al. (54)	Stage IIIB/IV NSCLC	73	N	150 or 250 mg/2 id	43 (59)					6.9 weeks	21.9 weeks
III	LUME-Lung 1 Trial (55–57)	Stage IIIB/IV NSCLC progressing after first-line chemotherapy	1,314	Docetaxel + N	200 mg/2 id	NA	NA	NA	NA	NA	3.4 versus 2.7 months+	10.1 versus 9.1 months++
	Campos-Gomez and Campos-Gomez (61)	Advanced NSCLC progressing after one line of chemotherapy	17	Docetaxel + N	200 mg/2 id	NA	13 (81.25)	NA	3 (18.75)	NA	NA	42 months
	Garcia Montes (62)	Advanced lung adenocarcinoma who progressed to first-line treatment + bevacizumab	99	Docetaxel + N	200 mg/2 id	79 (79.6)	52 (53)	NA	26 (26.5)	16 (16.3)	NA	NA
	LUME-Lung 2 Trial (63)	Advanced non-squamous NSCLC previously treated with chemotherapy	713	Pemetrexed + N	200 mg/2 id	435 (61)	NA	NA	NA	NA	4.4 versus 3.6 months	12.2 versus 12.7 months

N, nintedanib; n, number of patients enrolled; NA, non-applicable; +, 4.2 months when considering group of patients with adenocarcinoma; ++, 12.6 versus 10.3 months when considering group of patients with adenocarcinoma, p = 0.0359.

[a]*Initial dose.*

the OS benefit, and the frequency of adverse events were similar regardless of prior treatments with taxanes, pemetrexed, or bevacizumab (58).

Popat et al. confirmed LUME-Lung-1 findings in a meta-analysis of nine studies. They estimated a probability of 70% for nintedanib plus docetaxel being the best second-line treatment with regard to OS and PFS (59). Based on these findings, the European Medicines Agency approved in November 2014 the combination of nintedanib with docetaxel for the second-line treatment of adenocarcinoma patients (60). Furthermore, using patient-reported outcomes [i.e., 30-item European Organisation for Research and Treatment of Cancer Core Quality of Life (QoL) Questionnaire and its 13-item lung cancer-specific supplement] to complement the objective measures of efficacy and safety, this trial allowed the assessment of patients' subjective perception of their symptom burden and health-related QoL. This analysis demonstrated that the survival benefits achieved in the LUME-Lung 1 trial were not at the expense of patients' QoL. No significant differences in the PRO composites for cough, dyspnea, or pain were observed between the treatment groups (56).

Moreover, a cohort of NSCLC Mexican patients receiving nintedanib with docetaxel demonstrated efficacy and that was well tolerated; 81.25% had a partial response and 18.75% had stable disease (61). Also, a descriptive trial used the clinical data collection of patients with advanced lung adenocarcinoma who progressed to first-line treatment plus bevacizumab included in the compassionate-use program of nintedanib. The primary objective of the study was to describe the characteristics of the patients and their tumors, including previous therapies. The secondary objectives were to estimate the time under nintedanib treatment and the response rate and to evaluate the safety of this new treatment in daily clinical practice. From the 99 patients who were included, the objective response rate was 53%, stable disease 26.5%, disease progression 16.3%, and 4% were non-evaluable. Also, the disease control rate was 79.6%. The majority of patients had adequate tolerance, similar to the results obtained in LUME-Lung 1, mostly toxicities grades 1–2. However, the retrospective design of the study and the biased criteria of the investigator could have influenced in the overestimated responses (62).

Another phase III controlled randomized trial, LUME-Lung 2 (NCT00806819) evaluated the use of nintedanib in combination with pemetrexed (500 mg/m^2) and compared with pemetrexed (500 mg/m^2) plus placebo in patients with advanced, non-squamous NSCLC previously treated with chemotherapy (63). The primary endpoint was the same as LUME-Lung 1, while the secondary endpoints included OS, investigator-assessed PFS, response rate, safety, and QoL. Even though the enrollment was halted after randomizing 713 patients based on a planned futility analysis, the study met its primary endpoint. The nintedanib arm had a significant better PFS (median PFS 4.4 versus 3.6 months compared with placebo; HR 0.83; 95% CI 0.70–0.99; $p = 0.0435$); however, this difference was not translated into an OS benefit (12.2 versus 12.7 months; HR 1.03; 95% CI 0.85–1.24; $p = 0.7921$). Moreover, disease control was also significantly improved in the nintedanib arm (61 versus 53%, odds ratio 1.37, $p = 0.039$). Also, in this study, nintedanib showed a higher incidence of grade 3 increases in liver enzymes and gastrointestinal events, which resolved with dose reduction and supportive treatment (56). In contrast to other antiangiogenic agents, no grade 3/4 hypertension or hand-foot syndrome was reported (54) (**Table 1**).

Additionally, the association between plasma levels of VEGF, FGF, and PDGF was evaluated, both baseline and after treatment with nintedanib plus docetaxel, as well as disease control rate, PFS, and OS, among 38 patients with NSCLC. A higher percentage change reduction in PDGF after treatment was associated with a longer PFS and a higher percentage change in FGF was associated with a longer OS. Also, a higher reduction of plasma levels of FGF and PDGF was associated with better clinical outcomes (64).

Several clinical trials involving nintedanib are ongoing, including a phase III study (NCT02299141), that will evaluate the effectiveness of nintedanib in molecularly selected NSCLC patients and investigate the potential role of some genes (VEGFR1-3, PDGFR-A, PDGFR-B, and FGFR1-3) that might be involved in the regulation of mechanisms of acquired resistance to antiangiogenic agents. Results are expected by June 2017.

CONCLUSION

Nintedanib might be a good treatment option that fulfils the unmet need for effective, well-tolerated treatment options in advanced NSCLC and alleviate the disease burden for a broad selection of patients. The significant improvement in PFS in the overall population and the subgroup of patients with adenocarcinoma observed with the addition of nintedanib to cytotoxic drug therapy represents an attractive second-line treatment option. Moreover, the safety profile of this MATKI is manageable, giving this new treatment option great potential as an emerging combination for the management of NSCLC.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this paper. All authors agreed to be accountable for the content of the work.

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REVIEW
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Focus on Nintedanib in NSCLC and Other Tumors

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Nintedanib is a new triple angiokinase inhibitor that potently blocks the proangiogenic pathways mediated by vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and fibroblast growth factor receptors. Evidence about its efficacy in addition to second-line chemotherapy in non-small cell lung cancer (NSCLC) has been produced by two large randomized phase III clinical trials (LUME-Lung 1 and LUME-Lung 2), conducted in patients with pretreated NSCLC, without major risk factors for bleeding. In the LUME-Lung 1, the addition of nintedanib to docetaxel significantly improved progression-free survival, which was the primary end point of the trial (3.4 vs. 2.7 months, hazard ratio: 0.79; $p = 0.0019$). Furthermore, a significant improvement in median overall survival (from 10.3 to 12.6 months) was observed in patients with adenocarcinoma histology, with a greater advantage in patients who progressed within 9 months after start of first-line treatment (from 7.9 to 10.9 months) and in patients who were most refractory to first-line chemotherapy (from 6.3 to 9.8 months). Adverse events were more common in the docetaxel plus nintedanib group, and they included diarrhea and increased liver enzymes, while no statistically significant increase in the incidence of bleeding and hypertension events by the addition of nintedanib was observed. On these bases, the combination of docetaxel and nintedanib can be considered a new option for the second-line treatment for patients with advanced NSCLC with adenocarcinoma histology. Future challenges are the identification of predictive factors to help the decision of using nintedanib in eligible patients.

Keywords: nintedanib, angiogenesis inhibitors, VEGF, NSCLC, review

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INTRODUCTION

In recent years, a better understanding of the biology of cancer led to the development of molecular targeted therapies that have radically changed the treatment of many solid tumors, including non-small cell lung cancer (NSCLC). The new tailored agents, such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) and anaplastic lymphoma kinase inhibitors, are able to inactivate specific molecular alterations that occur in specific oncogenes, which cause cancer cell survival strictly dependent on such aberrant genes, as explained by the "oncogene addiction theory" (1). However, only a minority of tumors are oncogene addicted, and chemotherapy remains the only treatment available for the majority of cancer patients.

In this setting, targeting the angiogenesis pathways represents an alternative and attractive strategy, inasmuch as tumor development, progression, and metastasis are demonstrated strongly linked to angiogenesis. Angiogenesis is a very complex process, which is highly regulated by many molecules with both proangiogenic and antiangiogenic activity. The tumor microenvironment is composed of hyperproliferating cells that need large amounts of oxygen and nutrients. Such cells are able to deregulate the angiogenic process inducing an abnormal secretion of proangiogenic factors and the consequent development of disorganized, tortuous, enlarged, high permeable blood vessels, which are needed for both tumor growth and its metastatic potential (2). Therefore, angiogenic pathways have been investigated as potential therapeutic targets in patients with NSCLC (3). Several antiangiogenic agents have been developed, including monoclonal antibody anti-vascular endothelial growth factor (VEGF) such as bevacizumab or vascular endothelial growth factor receptor (VEGFR) TKIs, such as sorafenib and sunitinib. In particular, bevacizumab in combination with platinum-based chemotherapy has demonstrated superior efficacy compared with chemotherapy alone as first-line treatment in patients with non-squamous NSCLC, reaching the approval for use in this setting (4). However, because of substantial redundancy of proangiogenic pathways, patients treated with bevacizumab inevitably develop resistance to this agent (3).

One strategy for overcoming acquired resistance to bevacizumab is to target simultaneously multiple angiogenic receptors. Nintedanib is a new triple angiokinase inhibitor that potently blocks the proangiogenic pathways mediated by VEGF receptors, platelet-derived growth factor (PDGF) receptors, and fibroblast growth factor (FGF) receptors. This review summarizes the clinical data emerging from phase I–III clinical studies with nintedanib in NSCLC and in other tumors, focusing on the data that led to the recent approval by the European Medicines Agency as a second-line treatment in association with docetaxel in patients with advanced NSCLC.

PRECLINICAL EVIDENCE

Nintedanib (BIBF 1120; methyl (3Z)-3-[[4-[methyl-[2-(4-methylpiperazin-1-yl)acetyl]amino]anilino]-phenylmethylidene]-2-oxo-1H-indole-6-carboxylate) is a potent, oral angiokinase inhibitor that targets the proangiogenic pathways (**Figure 1**). This molecule is an indolinone derivative that blocks adenosine triphosphate-binding sites in the kinase domain of proangiogenic receptors inhibiting the downstream signaling pathways related to neoangiogenesis. Nintedanib is a TKI targeting VEGFR1–3, platelet-derived growth factor receptor α (alpha) and β (beta), and fibroblast growth factor receptors (FGFR) 1–3 and, in addition, it also inhibits the Src family, RET, and FLT3 (5, 6) (**Figure 2**). The three VEGF receptors have different functions, but all take part in tumorigenesis, directly stimulating cancer stem cell proliferation (6). Moreover, VEGFR-2 is considered the crucial receptor involved in initiation of the formation as well as the maintenance of tumor vasculature. Preclinical studies with nintedanib have shown sustained (>30 h) blockade of VEGFR2 *in vitro* and delay or arrest of tumor growth in xenograft models of human solid tumors, including lung cancer models (7). The specific and simultaneous abrogation of all the pathways targeted by nintedanib results in effective growth inhibition of both endothelial and perivascular cells, which may be more effective than inhibition of endothelial cell growth alone.

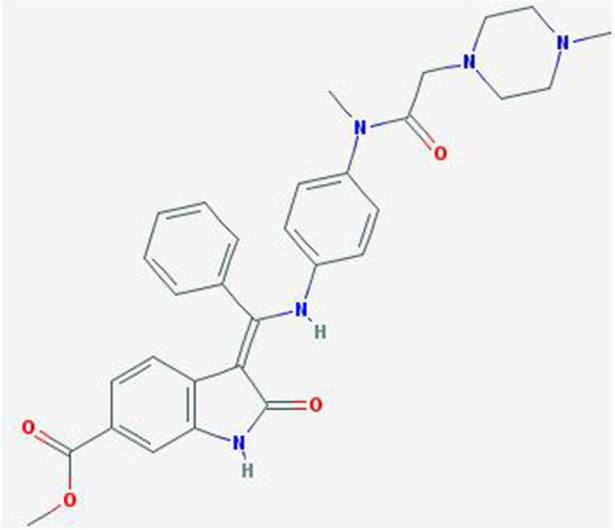

FIGURE 1 | Chemical structure of Nintedanib.

Furthermore, signaling by FGF receptors has been identified as a possible escape mechanism for tumor angiogenesis when the VEGF pathway is disrupted (8). Nintedanib leads to an important decrease of microvessel density and pericyte coverage, and this leads to a diminished perfusion and thereby to the death of tumor cells. In addition, a therapeutic effect may also result from inhibition of tumor autocrine and paracrine growth factor loops involving VEGF, PDGF, and bFGF.

In a preclinical study with models of lung and pancreatic cancer, it has been described that nintedanib does not increase the markers of epithelial to mesenchymal transition that usually allow tumor cells to switch from one pathway to another. This evidence is very important and could explain why this drug does not promote the change to a more aggressive tumor subtype and does not induce chemotherapy resistance (9).

Following oral administration, nintedanib is rapidly absorbed, with a median time to maximum plasma concentration of 1.3 h and a terminal half-life of 13.7 h (10). The major route of elimination of nintedanib is through metabolism, and its metabolites are excreted *via* the biliary system into the feces; urinary excretion is minor (1%). Nintedanib metabolism in healthy humans occurs predominantly by cleavage of the methyl ester moiety, yielding the carboxylate BIBF 1202 (metabolite 1). BIBF 1202 is then conjugated to glucuronic acid, yielding 1-*O*-acylglucuronide (metabolite 2). Thus, metabolism of nintedanib is predominantly cytochrome P450 enzyme independent, which facilitates the combination of nintedanib with cytotoxic chemotherapies, such as docetaxel, that are metabolized *via* cytochrome P450 enzymes (10).

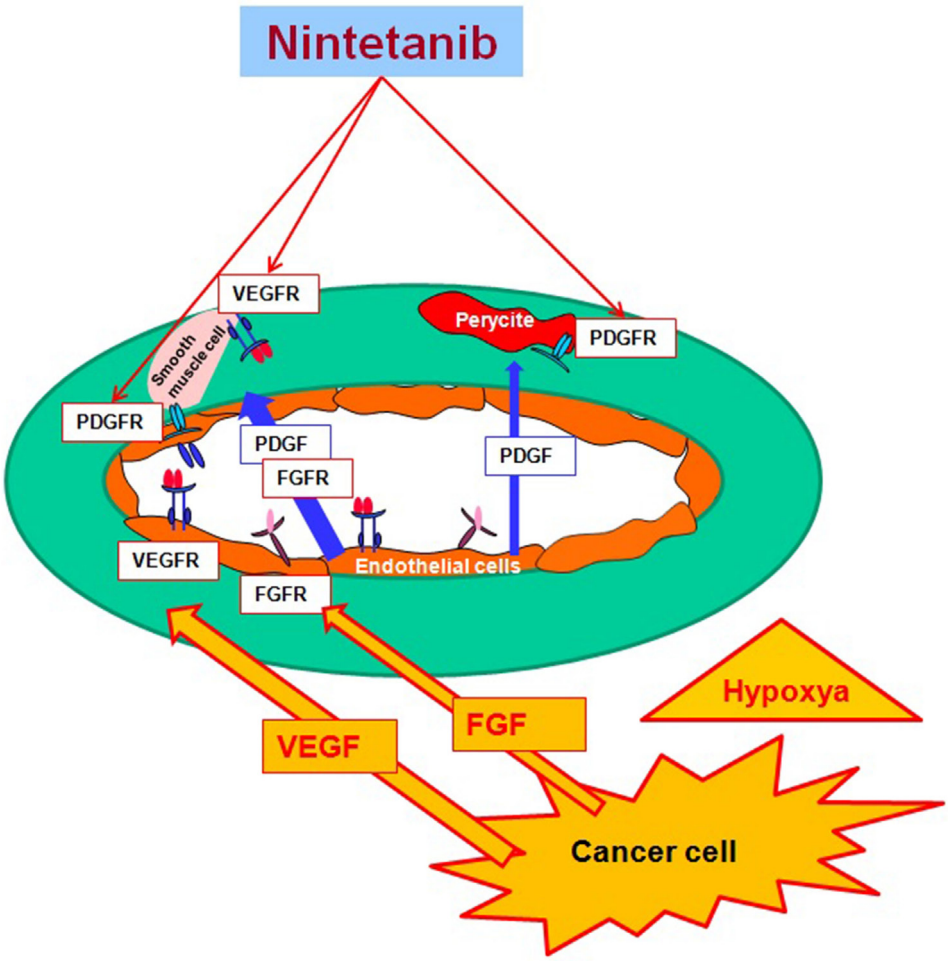

FIGURE 2 | **Nintedanib and targeted proangiogenic pathway**.

Phase I, II, and III clinical trials have been conducted in NSCLC to investigate the pharmacokinetics, tolerability, and efficacy of this triple angiokinase inhibitor (**Table 1**).

PHASE I STUDIES

Nintedanib showed a manageable safety profile and antitumor activity in patients with solid tumors, including NSCLC (13, 16). Based on several phase I dose-escalation trials of nintedanib as monotherapy, the maximum tolerated dose (MTD) of nintedanib was defined as 250 mg twice a day (b.i.d.) in Caucasian patients and 200 mg b.i.d. in Japanese patients (17, 18).

In a phase I accelerated titration study, Mross et al. investigated the MTD and tolerability of nintedanib in 61 patients with advanced cancers (16). Nintedanib showed a favorable safety profile in this advanced cancer patient population. Twice-daily dosing permitted an increase in total dose without additional toxicity. Because of its pharmacokinetic profile and absence of interaction with CYP450 enzymes, nintedanib was investigated in combination with standard cytotoxic chemotherapies, such as docetaxel or pemetrexed (11, 19, 20).

Ellis et al. investigated the MTD of continuous oral treatment with nintedanib in combination with standard-dose pemetrexed (500 mg/m^2) (11). Doebele et al. have also investigated the safety, tolerability, and MTD of nintedanib (starting dose 50 mg b.i.d.) on days 2–21 in combination with carboplatin [area under the curve (AUC) 6 mg/ml/min] and paclitaxel (200 mg/m^2) on day 1 of each 21-day cycle, in first-line setting in 26 patients with advanced NSCLC (12). The MTD of nintedanib was 200 mg/mq b.i.d. in combination with full doses of paclitaxel and carboplatin, and dose-limiting toxicities were liver enzyme elevations, thrombocytopenia, abdominal pain, and rash. Partial responses were observed in 26.9% of patients, and stable disease was observed in 38.5% of patients.

These trials confirm that splitting the total daily dose into two daily administrations increases the total daily exposure without additional toxicity. They also showed that 200 mg b.i.d. of nintedanib is the recommended dose for continuous daily treatment

TABLE 1 | Randomized clinical studies with nintedanib in non-small cell lung cancer (NSCLC).

Phase and reference	Line of treatment	Setting	#Patients	Treatment	Results
Systemic treatment					
I; Ellis et al. (11)	>1st	Advanced NSCLC	26	Nintedanib (starting dose 100 bid) days 2–21 + pemetrexed 500 mg/mq q 21	Maximum tolerated dose (MTD) 200 mg bid SD 50%
I; Doebele et al. (12)	1st	Advanced NSCLC	26	Nintedanib (starting 50 mg bid) days 2–21 + carboplatin AUC6 + paclitaxel 200 mg/mq q 21	MTD 200 mg bid PR 26.9%; SD 38.5%
II; Reck et al. (13)	≥2nd	Advanced NSCLC, any histology	73	Nintedanib 250 mg × bid or nintedanib 150 mg bid	mPFS (all patients) 6.9 weeks mOS: 21.9 weeks Overall survival (OS) 150 vs. 250 mg b.i.d., 20.6 vs. 29.7 weeks; hazard ratio (HR): 0.693; $p = 0.21$
III, LUME-Lung 1; Reck et al. (14)	2nd	Advanced NSCLC, any histology	1,314	Docetaxel 75 mg/mq q 21 + nintedanib 200 mg bid, days 2–21 vs. docetaxel 75 mg/mq q 21	RR%: 4.7 vs. 3.6 Disease control rate%: 60.2 vs. 44, $p < 0.0001$ Progression-free survival (PFS): 3.4 vs. 2.7 months, HR: 0.79, $p = 0.0019$ OS:10.1 vs. 9.1 months, [a]HR: 0.94, $p = 0.27$
III, LUME-Lung 2; Hanna et al. (15)	2nd	Advanced NSCLC non-squamous histology	713	Docetaxel 75 mg/mq q 21 + nintedanib 200 mg bid, days 2–21 vs. docetaxel 75 mg/mq	RR%: 9.1 vs. 8.3 Disease control rate%: 60.9 vs. 53.3, $p = 0.039$ PFS: 4.4 vs. 3.6 months, HR: 0.83, $p = 0.04$ OS:12.2 vs. 12.7 months, HR: 1.03, $p = 0.79$

[a]*OS not statistically different for all histology, but for subgroup non-squamous histology, OS is 12.6 vs. 10.3 months.*

in combination with standard-dose pemetrexed or carboplatin and paclitaxel for patients with advanced or metastatic NSCLC (11, 12).

In all these phase I studies, nintedanib revealed a similar adverse event profile with respect to fatigue and gastrointestinal adverse events as compared with other VEGFR TKIs. The predominant adverse events were nausea, diarrhea, vomiting, abdominal pain, and fatigue of low to moderate intensity during the first 2 months of therapy. Dose-limiting toxicities were dose-dependent hepatic enzyme elevations that were reversible after discontinuation of nintedanib treatment. Only in few patients, liver enzyme elevations were accompanied by a simultaneous increase in bilirubin. In general, common terminology criteria for adverse events (version 3.0) grade 3 liver enzyme increases were reported in the dose groups of 250 mg twice daily or higher. Severe grade 4 liver enzyme elevations were observed only occasionally, and they were fully reversible within 2 weeks to treatment discontinuation or dose reduction. Fatigue was also reported of a mild-to-moderate intensity, instead in the trial of nintedanib with pemetrexed it was reported as the most relevant dose-limiting toxicity. There were no drug-related bleeding events. Hypertension or thromboembolic events were rare and did not suggest an increased frequency as a consequence of therapy with nintedanib. There was no increase in hematologic toxicity observed when nintedanib was combined with chemotherapy. Unlike some other oral angiogenesis inhibitors, nintedanib did not seem to cause relevant skin abnormalities and no hand-foot syndrome was observed.

PHASE II STUDIES

Reck et al. conducted a phase II double-blinded, two-arm, randomized monotherapy trial with nintedanib (13). Patients with locally advanced or metastatic relapsed NSCLC of any histology after failure of first- or second-line chemotherapy with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–2 were randomized to continuous 150 or 250 mg b.i.d. nintedanib treatment until disease progression. Progression-free survival (PFS) and overall response rate were primary end points. Secondary end points included pharmacokinetic profiles of nintedanib, safety, and overall survival (OS). There was no significant difference in the PFS and the OS between the two groups. The results of this trial demonstrate that nintedanib in patients with ECOG 0–1 reaches effectiveness comparable to historical phase II data of other VEGFR inhibitors in a similar patient population: median PFS was 2.9 months with nintedanib, 2.8 months with sunitinib (21), 2.8 months with sorafenib (22), 2.6 months with vandetanib (23), and 3.5 months with vatalanib (24). The toxicity profile in this study was similar to that seen in phase I trials (17, 18). The majority of the adverse events were mild-to-moderate gastrointestinal symptoms with reversible hepatic toxicity. Tolerability was comparable between the two doses, with the exception of a higher frequency of liver enzyme elevations in the higher dose group.

PHASE III STUDIES

Two randomized prospective clinical trials have been conducted to evaluate the efficacy of nintedanib in patients with advanced NSCLC. The LUME-Lung 1 was a large multicenter double-blind, placebo-controlled, phase III trial randomizing patients with NSCLC to second-line docetaxel plus placebo ($n = 659$) or docetaxel plus nintedanib ($n = 655$) (14). The primary end point was PFS by central independent review, and the secondary end point was OS; additional secondary end points

included investigator-assessed PFS, tumor response by central review and investigator assessment, safety, and patient-reported quality of life (QoL). Patients were randomized in a 1:1 ratio to investigational arm of nintedanib 200 mg b.i.d. plus standard docetaxel therapy 75 mg/m^2 vs. placebo plus standard docetaxel therapy. A total of 1,314 patients were randomized: 655 assigned to experimental arm and 659 to standard arm. Patients were stratified by histology, ECOG PS, prior bevacizumab treatment, and the presence of brain metastases allowed if stable. Exclusion criteria were as follows: previous treatment with docetaxel or other VEGF inhibitors therapy (with the exception of bevacizumab), active and unstable brain metastasis or radiographic evidence of cavitary or necrotic tumors. Baseline demographics were well balanced between both arms. In this trial, the addition of nintedanib to docetaxel significantly improved PFS in the total study population (median 3.4 months [95% CI: 2.9–3.9] vs. 2.7 months [2.6–2.8]; hazard ratio (HR): 0.79 [95% CI: 0.68–0.92], $p = 0.0019$). The benefit in PFS was consistent, regardless of gender, age, ethnicity, or PS.

Moreover, the addition of nintedanib improved median OS in patients with adenocarcinoma (12.6 months [95% CI: 10.6–15.1] vs. 10.3 months [95% CI: 8.6–12.2]; HR: 0.83 [95% CI: 0.70–0.99], $p = 0.0359$). The prolongation of OS was consistent with the improvement of 1-year survival rate from 45 up to 53% and 2-year survival rate from 19 up to 26%. OS was also increased in patients with adenocarcinoma histology who progressed within 9 months after start of first-line treatment (median OS increased from 7.9 to 10.9 months corresponding to a HR of 0.75 and p value of 0.0073) and in patients refractory to first-line chemotherapy. In this group of poor prognosis patients, an advantage of more than 3 months was observed with the addition of nintedanib to docetaxel compared to docetaxel alone (9.8 vs. 6.3 months, HR of 0.62, $p = 0.0246$). There was no difference in OS in the total study population (median 10.1 months [95% CI: 8.8–11.2] vs. 9.1 months [8.4–10.4]; HR: 0.94 [95% CI: 0.83–1.05], $p = 0.2720$) and in patients with squamous cell carcinoma between both arms. Finally, the investigation of the interaction between treatment and tumor burden showed that a greater tumor burden was associated with a greater treatment effect for docetaxel and nintedanib. In addition, a significant improvement in disease control rate (60.2 vs. 44%) in favor of nintedanib plus docetaxel was observed in adenocarcinoma patients. Adverse events more common in the docetaxel plus nintedanib group than the docetaxel plus placebo group were as follows: diarrhea (all grades: 42.3 vs. 21.8%; grade $\geq$ 3 6.6 vs. 2.6%), increases in alanine aminotransferase (all grades, 28.5 vs. 8.4%; grade $\geq$ 3 7.8 vs. 0.9%), nausea (all grades, 24.2 vs. 18.0%; grade $\geq$ 3, 0.8 vs. 0.9%), increases in aspartate aminotransferase (all grades, 22.5 vs. 6.6%; grade $\geq$ 3, 3.4 vs. 0.5%), decreased appetite (all grades, 22.2 vs. 15.6%; grade $\geq$ 3, 1.4 vs. 1.2%), and vomiting (all grades 16.9 vs. 9.3%; grade $\geq$ 3 0.8 vs. 0.5%). There was no statistically significant increase in the incidence of bleeding and hypertension events by the addition of nintedanib (25). Moreover, the significant OS benefit observed with the addition of nintedanib to docetaxel therapy was achieved with no detrimental effect on patient self-reported QoL, with significant reductions in some pain items with nintedanib vs. placebo (26).

LUME-Lung 2 was a multicenter, randomized, double-blinded phase III study that investigated the efficacy and safety of nintedanib in combination with pemetrexed vs. placebo plus pemetrexed in patients with locally advanced or metastatic non-squamous NSCLC with relapse or failure after chemotherapy (15). A total of 713 patients were randomized 1:1 to experimental arm (353 patients) and to standard arm (360 patients). The primary end point was centrally reviewed PFS, the secondary end points were OS, investigator-assessed PFS, objective response rate (ORR), safety, and QoL. The study enrolled patients with ECOG PS 0–1 without active brain metastases, cavitary or necrotic tumors, and clinically significant hemoptysis, not previously treated with VEGF inhibitors (except bevacizumab). Baseline patient characteristics were balanced between both arms for age, gender, PS, histology type, and prior bevacizumab treatment. All randomized patients were included in the intention-to-treat (ITT) population. The study was designed to have 90% power to demonstrate a significant (27.5%) improvement in PFS with a HR of 0.78 after 713 PFS events.

The analysis suggested that the primary end point of centrally assessed PFS would likely not be met; however, there were no safety concerns. Ongoing patients were unblinded and follow-up was continued per protocol. Analysis of the primary end point PFS by independent central review was conducted after 498 events had occurred, and analysis of the secondary end point OS was conducted after 436 events had occurred. The primary end point of this phase III trial was met even though the study was stopped prematurely. ITT analysis of the primary end point showed that treatment with nintedanib plus pemetrexed resulted in a significant prolongation of PFS compared with placebo plus pemetrexed (4.4 vs. 3.6 months with a HR of 0.83 and a p value of 0.04). Disease control rate was also increased significantly in nintedanib-treated group (61 vs. 53%, with an odds ratio of 1.37 and a p value of 0.039). No difference in OS was seen between the arms. There was no increase in serious side effects in the combination arm. However, there was an increase in the incidence of diarrhea and elevated liver enzymes, each of which were reversible. There was no difference between the arms in terms of the incidence of hypertension, bleeding, thrombosis, mucositis, or neuropathy.

NINTEDANIB IN OTHER TUMORS

Due to the important rule of angiogenesis pathways identified in cancer development, Nintedanib has also been evaluated in other tumors (**Table 2**).

Small Cell Lung Cancer

A phase II study evaluated nintedanib activity in 24 patients with small cell lung cancer (SCLC) relapsed after one or two lines of chemotherapy or chemoradiotherapy (27). Eight patients received only one prior chemotherapy. Nintedanib was administered at 200 mg twice daily until disease progression or toxicity. ORR, the primary end point, was 5% [95% CI: 0.1–22.8]. Median PFS was 1 month and OS was 9.8 months. The most frequent drug-related adverse events included hepatic enzyme elevation (86%), anemia (73%), anorexia (59%), and nausea (50%). Most

TABLE 2 | Studies with nintedanib in other tumors.

Phase and reference	Line of treatment	Setting	#Patients	Treatment	Results
Systemic treatment					
II; Han et al. (27)	≥2nd	Relapsed small cell lung cancer	24	Nintedanib 200 mg × 2/day	Objective response rate = 5% Hepatic enzyme elevation 86%
II; Palmer et al. (28)	1st	Unresectable HCC	93	Nintedanib 200 mg × 2/day vs. sorafenib	Time to progression: 5.5 vs. 3.8 months Overall survival (OS): 11.9 vs. 11.4 months Comparable toxicities
II; Eisen et al. (29)	1st	Advanced RCC	96	Nintedanib 200 mg × 2/day vs. sunitinib	Progression-free survival (PFS) at 9 months 43.1 vs. 45.2% OS: 20.4 vs. 21.2 months Comparable toxicities
II; Norden et al. (30)	≥2nd	Recurrent glioblastoma	36	Nintedanib 200 mg × 2/day	No responses PFS at 3 (prior bevacizumab) and 6 (no prior bevacizumab) months = 0%
II; Droz et al. (31)	≥2nd	Prostate cancer	81	Nintedanib 150 or 250 mg × 2/day	PSA decrease under 50% = 5.6% PFS: 73.5–76 days
II; Van Cutsem et al. (32)	1st	Colorectal cancer	126	mFOLFOX6 + nintedanib 200 mg × 2/day or bevacizumab 5 mg/kg every 14 days	PFS at 9 months: 62.1 vs. 70.2%
II; Ledermann et al. (33)	≥2nd	Ovarian cancer	83	Nintedanib 250 mg × 2/day vs. placebo for up to 9 months as maintenance following chemotherapy	% of patients progression free at 36 weeks: 16.3 vs. 5% Grade 3 or 4 hepatotoxicity 51.2 vs. 7.5%
III, AGO-OVAR 12; du Bois et al. (34)	1st	Ovarian cancer	1,366	Carboplatin (AUC 5/6) + paclitaxel (175 mg/mq) d1 + nintedanib 200 mg × 2/day or placebo days 2–21 q21 × 6 cycles → nintedanib or placebo maintenance for up to 2 years	PFS: 17.2 vs. 16.6 months, hazard ratio: 0.84, $p = 0.024$ G3 diarrhea 21 vs. 2%, G4 neutropenia 22 vs. 16%, G4 thrombocytopenia 6 vs. 2%

PSA, prostate-specific antigen.

toxicities were mild and manageable. Grade 3 hepatic enzyme elevation occurred in five patients (23%). The authors concluded that nintedanib exhibited only a modest activity in relapsed or refractory SCLC.

Hepatocellular Carcinoma

A phase II study was designed to compare safety and activity of nintedanib 200 mg b.i.d. vs. sorafenib 400 mg b.i.d. in 93 patients with unresectable hepatocellular carcinoma and Child-Pugh A score, randomized in a 2:1 ratio (28). Time to progression, the primary objective, was comparable between nintedanib and sorafenib (median 5.5 vs. 3.8 months; HR: 1.05 [95% CI: 0.63–1.76]). Median OS was 11.9 vs. 11.4 months, respectively (HR: 0.88 [95% CI: 0.52–1.47]). More patients treated with sorafenib had grade ≥ 3 adverse events (68 vs. 90%). Toxicities leading to dose reduction were higher with sorafenib (19 vs. 42%), whereas side effects leading to drug discontinuation were higher with nintedanib (45 vs. 23%). Rash was reported in >15% of patients only in the sorafenib arm.

Renal Cell Carcinoma

A phase II study evaluated activity and tolerability of first-line nintedanib 200 mg twice daily vs. standard sunitinib in 96 patients with advanced renal cell carcinoma, randomized in a 2:1 ratio (29). The trial would also test possible electrocardiographic changes, particularly in QTc, during nintedanib assumption. PFS at 9 months, the primary objective, was 43.1 vs. 45.2% ($p = 0.85$) for nintedanib vs. sunitinib. Median OS was 20.4 vs. 21.2 months (HR: 0.92; 95% CI: 0.54–1.56; $p = 0.76$). Toxicities were comparable between the two treatments. Nintedanib was associated with lower incidences of some adverse events typical of antiangiogenic TKIs, such as hypertension, hypothyroidism, hand-foot syndrome, cardiac disorders, and hematological abnormalities.

Glioblastoma

Activity of nintedanib was also explored in patients with recurrent glioblastomas, but the results were disappointing. In a phase II study, 36 patients, stratified based on prior bevacizumab, received nintedanib 200 mg twice daily (30). There were no responses, and PFS at 3 (prior bevacizumab) and 6 (no prior bevacizumab) months was 0%.

Castration-Resistant Prostate Cancer

Modest activity was noted with nintedanib 150 or 250 mg twice daily in 81 castration-resistant prostate cancer patients pretreated with docetaxel chemotherapy (31). Only 5.6% of patients treated with nintedanib 250 mg obtained a prostate-specific antigen (PSA) decrease of at least 50%. Median PFS was 73.5 and 76 days with nindetanib 150 and 250 mg, respectively. Toxicities included gastrointestinal disorders, asthenia,

hypertension, and reversible elevated transaminases. A phase I trial tested nintedanib in association with docetaxel (75 mg/m^2 every 3 weeks) and prednisone in castration-resistant prostate cancer patients (19), suggesting the dose of 200 mg twice daily for future investigations. Among 19 assessable patients, 13 (68.4%) showed a ≥50% reduction in PSA levels from baseline. Pharmacokinetic analysis showed no interactions between nintedanib and docetaxel/prednisone.

Metastatic Colorectal Cancer

A phase I/II study tested nintedanib + mFOLFOX6 or bevacizumab + mFOLFOX6 in the first-line treatment of patients with advanced colorectal cancer (32). In the phase II of the study, nintedanib was given at 200 mg twice daily. Overall, 126 patients were randomized in a 2:1 ratio into the nintedanib vs. bevacizumab arm. PFS at 9 months, the primary objective, was 62.1 vs. 70.2%, while objective response was 63.5 vs. 56.1%. The incidence of serious adverse events was 37.6% with nintedanib and 53.7% with bevacizumab. The pharmacokinetics of nintedanib and the components of mFOLFOX6 were unaffected by their combination.

Ovarian Cancer

A randomized phase II study was conducted with nintedanib in 83 relapsed ovarian cancer patients. In this study, women treated with nintedanib as maintenance therapy at 250 mg twice daily for up to 9 months after chemotherapy were less likely to experience disease progression compared to those treated with placebo (33). At 36 weeks, 16.3% of women taking nintedanib were progression free, compared to 5% of those taking placebo (HR: 0.65; 95% CI: 0.42–1.02; $p = 0.06$). Two patients continued nintedanib for another year or more. More patients on nintedanib experienced diarrhea, nausea, or vomiting (no grade 4). There was a higher rate of grade 3 or 4 hepatotoxicity in patients on nintedanib (51.2%) compared with patients on placebo (7.5%; $p < 0.001$).

LUME-Ovar 1, also named AGO-OVAR 12, is a phase III study testing association of first-line chemotherapy plus nintedanib or placebo in patients with advanced ovarian carcinoma (34). The trial recruited 1,366 patients with FIGO IIB-IV ovarian carcinoma and primary debulking surgery to receive in a 2:1 ratio of six cycles of carboplatin (AUC 5 or 6 mg/dl/min) and paclitaxel (175 mg/m^2) on day 1 every 3 weeks plus nintedanib 200 mg twice daily on days 2–21 of each cycle or placebo. The biological agent or placebo were given for up to 120 weeks. Primary end point was PFS by investigator assessment in the ITT population. As a result, 53% of 911 patients in the nintedanib group experienced disease progression or death compared with 58% of 455 patients in the placebo group. Median PFS was significantly longer with nintedanib than placebo (17.2 months [95% CI: 16.6–19.9] vs. 16.6 months [13.9–19.1]; HR: 0.84 [95% CI: 0.72–0.98]; $p = 0.024$). The most common adverse events were gastrointestinal, such as grade 3 diarrhea in 21% of patients receiving nintedanib vs. 2% in the placebo group, and hematological (neutropenia of grade 3 in 20% and grade 4 in 22% of patients receiving nintedanib vs. 20 and 16% in the placebo group, respectively; thrombocytopenia 12 and 6% vs. 5 and 2%; anemia 12 and 1% vs. 6 and 1%). Serious adverse events were reported in 42% with nintedanib and 34% with placebo; 3% of patients receiving nintedanib experienced serious adverse events associated with death compared with 4% in the placebo group.

ONGOING CLINICAL STUDIES WITH NINTEDANIB

Nintedanib is currently under investigation in various types of tumor (**Table 3**). In neoadjuvant setting, a phase I study is evaluating the safety of nintedanib in combination with cisplatin and docetaxel before surgery in patients with stages I–III NSCLC [http://ClinicalTrials.gov: NCT02225405]. LUME-Meso is a randomized double-blind phase II/III study testing safety and efficacy of nintedanib in 537 naïve patients with unresectable pleural mesothelioma (35). Treatment consists of six courses of chemotherapy with cisplatin 75 mg/m^2 and pemetrexed 500 mg/m^2 on day 1 plus nintedanib 200 mg b.i.d. on days 2–21 of each cycle or placebo. Following maintenance with biologic agent, placebo is given to patients with controlled disease. Primary end point is PSF, and secondary end points include OS, objective response,

TABLE 3 | Ongoing studies with nintedanib.

Phase	Line of treatment	Setting	#Patients	Treatment	Endpoints
Systemic treatment					
I	Neoadjuvant	Resectable non-small cell lung cancer stage IB–IIIA	45	Cisplatin + docetaxel + nintedanib	Major pathologic response rate Toxicity of nintedanib given with cisplatin and docetaxel
II/III	1st	Unresectable pleural mesothelioma	537	Cisplatin-pemetrexed + nintedanib or placebo → nintedanib or placebo maintenance	Progression-free survival (PFS)
III	Advanced	Advanced colorectal cancer	764	Monotherapy with nintedanib 200 mg × 2/day vs. placebo (prior regorafenib allowed)	PFS and overall survival
III	Advanced	Advanced colorectal cancer	100	Nintedanib alone or in combination with capecitabine	PFS
II	1st or 2nd	Advanced HER2-negative breast cancer	252	Docetaxel d1 ± nintedanib 200 mg × 2/day, days 2–21	PFS
I	Advanced	Refractory solid tumors	18	Nintedanib + pembrolizumab	Maximum tolerated dose of nintedanib

and disease control rate. Preliminary results are expected in 2019. LUME-Colon 1 is a double-blind randomized phase III study evaluating monotherapy with nintedanib and best supportive care (BSC) vs. placebo and BSC in patients with refractory advanced colorectal cancer pretreated with standard chemotherapies and biologic agents (36). ECOG PS 0–1 and life expectancy of minimum 12 weeks are required. Estimated accrual is of 764 patients. Prior regorafenib is allowed. Patients are stratified based on previous regorafenib, time from onset of metastatic disease to randomization (less or more than 24 months), and region. Nintedanib is administered at 200 mg twice daily vs. placebo in a 1:1 randomization. Primary outcomes are PFS by central review assessment and OS, with objective tumor response and disease control as secondary end points. Other assessments include frequency and severity of adverse events, changes in laboratory parameters, health-related QoL, and biomarker analyses to better define predictiveness of response and drug resistance mechanisms. Final results are soon expected. LUME-Colon 2 is a phase II study assessing nintedanib alone or in combination with capecitabine in patients with refractory metastatic colorectal cancer after failure of at least two lines of standard treatment. Primary end point is PFS. Estimated enrollment is 100 patients. Results are expected in 2017 [http://ClinicalTrials.gov: NCT02780700]. A phase II study is testing first- or second-line docetaxel ± nintedanib in patients with HER2-negative metastatic or locally recurrent breast cancer. Docetaxel 75 mg/m^2 every 3 weeks could be increased to 100 mg/m^2 in the arm without nintedanib. Nintedanib is administered at 200 mg twice daily from day 2 of each cycle. Primary objective is PFS. Secondary end points are response rate, OS, QoL, and pharmacokinetic analyses. Estimated enrollment is 252 patients, and results are soon expected [http://ClinicalTrials.gov: NCT01658462].

Finally, an ongoing phase I trial is testing nintedanib and pembrolizumab in refractory solid tumors patients to define the toxicity profile of such combination [http://ClinicalTrials.gov: NCT02856425].

DISCUSSION AND CONCLUSION

Two randomized phase III clinical trials have evaluated to date the efficacy of nintedanib in patients with advanced NSCLC. The LUME-Lung 1 trial have showed, for the first time, an OS benefit in patients with advanced NSCLC from the addition of a targeted agent to chemotherapy in the second-line setting. In this trial, the addition of nintedanib to docetaxel significantly improved median OS in patients with adenocarcinoma histology (from 10.3 to 12.6 months), with a greater advantage in patients who progressed within 9 months after start of first-line treatment (from 7.9 to 10.9 months) and in patients who were most refractory to first-line chemotherapy (from 6.3 to 9.8 months). Moreover, nintedanib plus docetaxel improved PFS and disease control in the total study population. These results were partially confirmed by the LUME-Lung 2 trial that, despite early closure, showed that nintedanib plus pemetrexed resulted in a significant prolongation of PFS and disease control rate, while no difference in OS was seen between the arms, probably due to the final low power of the study. On these bases, the combination of docetaxel and nintedanib can be considered a new option for the second-line treatment for patients with advanced NSCLC with adenocarcinoma histology (37).

However, there are several issues that need to be addressed, including the following: (a) how to improve the tolerability profile of the combination of docetaxel and nintedanib; (b) the role of nintedanib in other settings, such as first line and neoadjuvant; (c) the feasibility of combining nintedanib with other drugs; (d) the activity of nintedanib in other tumors; and (e) the identification of predictive factors.

The most frequent adverse events of nintedanib as single agent were nausea, diarrhea, vomiting, increases in liver enzymes, and fatigue, generally of low to moderate intensity, while hypertension or thromboembolic events were rare. Combination of nintedanib with docetaxel revealed a similar toxicity profile as compared to nintedanib monotherapy, except for docetaxel-related toxicities. Chemotherapy with docetaxel 75 mg/mq administered once every 3 weeks has been proven to be a reasonable therapeutic choice for the second-line treatment of patients with advanced NSCLC, but myelosuppression is extremely frequent and severe: weekly scheduling of docetaxel has demonstrated to improve the toxicity profile of the drug in pretreated NSCLC patients without decreasing antitumor activity (38). Therefore, the addition of nintedanib to weekly docetaxel could be an attractive schedule to maintain the therapeutic efficacy of the combination with a better toxicity profile. An Italian multicenter, prospective, open-label study with two "cohorts" is evaluating the efficacy and safety profile of nintedanib plus docetaxel in patients with non-squamous NSCLC in stage IIIB/IV with two different combination schedules, including a weekly schedule of docetaxel (SENECA trial): the results of this trial should answer the question of the feasibility and activity of the combination of nintedanib with weekly docetaxel.

In other settings, a phase I study investigated nintedanib combined with paclitaxel (200 mg/mq) and carboplatin (AUC 6 mg/ml/min), in first-line setting in 26 patients with advanced NSCLC (21). This combination was well tolerated, without drug-to-drug interactions and demonstrated promising preliminary efficacy in patients with advanced NSCLC, supporting further investigation in patients with NSCLC. In neoadjuvant setting, a phase I study is evaluating the safety of nintedanib in combination with cisplatin and docetaxel before surgery in patients with stage I–III NSCLC.

A number of studies are evaluating the feasibility of the combination of nintedanib and other classes of drugs, including angiogenesis inhibitors such as bevacizumab, EGFR inhibitors such as afatinib, and immune checkpoint inhibitors such as pembrolizumab. The good safety profile of the drug allows to use nintedanib also in special populations, such as elderly patients, in combination with other chemotherapeutic agents: the VENUS-1 and VENUS-2 are dose-escalation trials to evaluate the feasibility of the combination of nintedanib with vinorelbine or with carboplatin and vinorelbine in elderly patients with advanced NSCLC.

Ongoing studies will clarify the activity of nintedanib in other tumors, including mesothelioma, colon, breast, ovarian, cervix, pancreatic cancer, and HCC.

Identifying molecular biomarkers that can predict a response to nintedanib remains an important goal to maximize the clinical

benefit of this agent. A phase II study is ongoing to examine the value of FGFR1 gene amplification as a predictor of nintedanib efficacy in patients with squamous cell NSCLC [http://ClinicalTrials.gov: NCT01948141]. Additional studies are planned that include translational approaches to identify more detailed mechanisms of action for nintedanib.

In conclusion, nintedanib is an effective second-line treatment in combination with docetaxel for patients with lung adenocarcinoma, also refractory to first-line chemotherapy. Future challenges are to indentify predictive factors to help the decision of using antiangiogenic agents in patients.

AUTHOR CONTRIBUTIONS

All the authors have made substantial contributions to the conception, drafting, and revision of the article and approved the final version.

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Focus on Alectinib and Competitor Compounds for Second-Line Therapy in *ALK*-Rearranged NSCLC

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The management of anaplastic lymphoma kinase rearranged (ALK+) non-small cell lung cancer (NSCLC) exemplifies the potential of a precision medicine approach to cancer care. The ALK inhibitor crizotinib has led to improved outcomes in the first- and second-line setting; however, toxicities, intracranial activity, and acquired resistance necessitated the advent of later generation ALK inhibitors. A large portion of acquired resistance to ALK inhibitors is caused by secondary mutations in the ALK kinase domain. Alectinib is a second-generation ALK inhibitor capable of overcoming multiple crizotinib-resistant ALK mutations and has demonstrated improved outcomes after crizotinib failure. Favorable toxicity profile and improved intracranial activity have spurred ongoing front-line trials and comparisons to other ALK inhibitors. However, important questions regarding comparability to competitor compounds, acquired alectinib resistance, and ALK inhibitor sequencing remain. Here, we review the key clinical data supporting alectinib in the second-line therapy of ALK+ NSCLC and provide context in comparison to other ALK inhibitors in development.

Keywords: alectinib, NSCLC, ALK, second line, crizotinib, resistance

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BACKGROUND

Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer and remains the leading cause cancer-related mortality in both men and women with a 5-year survival rate of less than 20% in US patients (1). Rapid advances in understanding the molecular pathogenesis of NSCLC have demonstrated that NSCLC is a heterogeneous group of diseases. Chromosomal rearrangements involving *ALK* and *ROS1* are present in 3–7% (2) and 2% (3) of patients with NSCLC, respectively. *ALK* translocations are found nearly exclusively in lung adenocarcinomas. Crizotinib, a first-generation ALK and ROS1 inhibitor, has resulted in improved progression-free survival (PFS) relative to chemotherapy in the first- and second-line settings for *ALK-rearranged (ALK+)* NSCLC. Compared to chemotherapy in treatment naïve *ALK*-rearranged patients, crizotinib led to higher objective response rate (ORR) (74 vs. 45%) and median PFS (10.9 vs. 7.0 months) but no difference in overall survival (hazard ratio for death with crizotinib, 0.82; 95% CI, 0.54–1.26; $P = 0.36$) (**Table 1**) (4). In *ALK*-rearranged patients with prior chemotherapy exposure, crizotinib also led to improved ORR (65 vs. 20%) and median PFS (7.7 vs. 3.3 months) (5). Like other oncogene driven

TABLE 1 | Comparison of second-line therapy trials in NSCLC.

Compound	Phase	*n*	Study population	Primary endpoint	PFS	ORR	Reference
ALK+ population							
Ceritinib	I	246	ALK+ naïve and crizotinib failure	RP2D 750 mg qd	ALK inh naïve: 18.4 months ALK inh expos: 6.9 months	ALK inh naïve: 72% ALK inh expos: 56%	(7)
Alectinib	II	138	ALK+, crizotinib failure	ORR 50%	8.9 months	ORR 50% CNS DCR 83% among 84 pts with CNS mets	(8)
Alectinib	II	87	ALK+, crizotinib failure	ORR 48%	8.1 months	ORR 48% CNS DCR 100% among 16 pts with CNS mets	(9)
Alectinib	I/II	47	ALK+, crizotinib failure	ORR 55%	NA	Overall ORR 55% CNS ORR 52%	(10)
Alectinib	I	46	ALK+ naïve	ORR 93.5%	NA	ORR 93.5%	(11)
Crizotinib vs. chemo	III	347	ALK+ prior chemo	PFS	7.7 vs. 3.0 months	65 vs. 20%	(5)
Crizotinib vs. chemo	III	343	ALK+ naïve	PFS	10.9 vs. 7 months 1 year survival rate 84 vs. 79%	74 vs. 45%	(4)
Unselected population							
Pembrolizumab vs. docetaxel	III	1,000	Unselected	OS: 12.7 vs. 8.5 months	4 vs. 4 months	18 vs. 9%	(12)
Nivolumab vs. docetaxel	III	272	SCC	OS: 9.2 vs. 6 months	1 year survival rate 42 vs. 24%	20 vs. 9%	(13)
Nivolumab vs. docetaxel	III	582	Non-SCC	OS: 12.2 vs. 9.4 months	1 year survival rate 51 vs. 39%	19 vs. 12%	(14)
Docetaxel + ramucirumab vs. docetaxel	III	1,253	Unselected pts after 1st line	OS: 10.5 vs. 9.1 months	4.5 vs. 3.0 months	23 vs. 14%	(15)
Erlotinib vs. docetaxel or pemetrexed	III	424	Unselected	OS: 5.3 vs. 5.5 months	1.4 vs. 2 months	NA	(16)
Pemetrexed vs. docetaxel	III	571	Unselected	OS: 9.3 vs. 8.0 months in non-squamous OS: 6.2 vs. 7.4 months in squamous	2.9 months each arm	9.1 vs. 8.8%	(17)
Docetaxel vs. placebo	III	104	Unselected	OS: 7.5 vs. 4.6 months	10.6 vs. 6.7 weeks	7.1 vs. 0%	(18)

Upper portion summarizes ALK+ trials and lower portion provides findings from key second-line chemotherapy and immunotherapy trials to provide context.

tumors, acquired resistance is nearly universal in *ALK*+ NSCLC, and most develop crizotinib resistance within 1 year of treatment with central nervous system (CNS) metastasis being a major site of progression (6).

While the propensity for intracranial failure on crizotinib is partly related to lower penetration of blood–brain barrier (19), systemic relapses are mediated by multiple mechanisms including secondary ALK mutations and compensatory bypass pathway activation. In nearly a third of patients, tumors have acquired secondary mutation in the ALK tyrosine kinase domain. The most common resistance mutation is the gatekeeper *L1196M* mutation, followed by the *G1269A* (20–22). Additional resistance mutations include *C1156Y*, *L1152R*, *G1202R*, *S1206Y*, *1151Tins*, *F1174C*, and *D1203N*, among many others (**Table 2**) (23–25). These mutations blunt the efficacy of crizotinib by either increasing the ALK kinase affinity for adenosine triphosphate (ATP) (*G1269A* and *1151Tins*), inducing conformational change causing steric hindrance (*G1202R* and *S1206Y*) or interfering with the downstream signaling pathway (*L1152R*) (23). Amplification of the *ALK* fusion gene was observed either alone or in combination with other resistance mechanisms in both *in vitro* studies (20) and resistant clinical specimens (26). Beyond the *ALK* dominant resistance mechanism, preclinical work and progression biopsies from patients on ALK inhibitors have revealed crizotinib resistance from amplification of epidermal growth factor receptor (EGFR) pathway, insulin-like growth factor pathway (*IGF-1R*), *cKIT* mutation, and *SRC* activity (26–28).

While crizotinib ushered in a new paradigm for *ALK*+ NSCLC, the emergence of acquired resistance and rates of intracranial progression suggested ongoing clinical needs in *ALK*+ disease. The management of crizotinib failure has largely been informed by data from later generation ALK inhibitors including alectinib; however, other recent second-line trials outside *ALK*+ disease are worth brief contextual mention (**Table 1**). The phase III REVEL trial demonstrated that the addition of ramucirumab (a vascular endothelial growth factor receptor 2 monoclonal antibody) to docetaxel in unselected advanced NSCLC patients yielded higher response rate (23 vs. 14%), median PFS (4.5 vs. 3 months), and median OS (10.5 vs. 9.1 months) than docetaxel monotherapy (15). Similarly, in the phase III CheckMate 017 trial nivolumab yielded superior ORR (20 vs. 9%), median PFS (3.5 vs. 2.8 months), and median OS (9.2 vs. 6.0 months) compared with docetaxel in heavily pretreated unselected advanced squamous NSCLC patients (13). The CheckMate 057 trial found higher ORR (19 vs. 12%) and median OS (12.2 vs. 9.4 months) in

TABLE 2 | Mutation coverage for ALK inhibitors in late stage clinical development.

Mutations	Crizotinib	Alectinib	Certinib	Brigatinib	Lorlatinib	Reference
EML4-ALK	S	S	S	S	S	(29, 30)
L1196M	R	S	S	S	S	(21, 22, 24, 29–32)
L1152P/R	R	S	R	S	S	(22, 30–32)
G1123S	R	S	R	NA	NA	(30, 33)
1151Tins	R	S	R	NA	S	(22, 24, 30, 31)
C1156Y	R	S	R	S	S	(21, 22, 29–31)
F1174V/C/L	R	S	R	S	S	(22, 29–31, 34)
I1171T/N/S	R	R	S	NA	NA	(30, 32, 35)
V1180L	R	R	S	NA	NA	(35)
G1202R	R	R	R	S	S	(22, 24, 30, 31)
G1269A/S	R	S	S	S	S	(22, 30–32)
F1245C	R	NA	S	NA	NA	(30, 36)
S1206C/Y/F	R	S	S	R	S	(22, 24, 30–32)
E1210K	R	S	S	S	S	(30)
L1198F	S	R	R	S	R	(30, 37)
D1203N	R	S	S	S	S	(30)
CMET amp	S	R	R	R	R	(38)

The letter S denotes mutations that are "sensitive" (clinical and/or preclinical data) to a given compound, and "R" denotes resistance. NA, data not available.

patients with non-squamous NSCLC compared with docetaxel (14). The efficacy of pembrolizumab was demonstrated in phase II/III KEYNOTE-010 trial which compared pembrolizumab vs. docetaxel in more than 1,000 patients (12). Pembrolizumab led to improved median OS in the overall population (12.7 vs. 8.5 months). Among 442 patients with at least 50% PD-L1 expression, the median OS for the pembrolizumab 2 mg/kg, 10 mg/kg, and docetaxel groups was 14.9, 17.3, and 8.2 months, respectively.

ALECTINIB OVERVIEW

The expanding appreciation of crizotinib-resistant ALK mutations spurred development of the second-generation ALK inhibitors. Alectinib is a potent and selective second-generation oral ALK inhibitor. Alectinib exhibits limited inhibitory activity against other protein kinases such as EGFR, fibroblast growth factor receptor 2 (FGFR2), human epidermal growth factor receptor 2 (HER2), hepatocyte growth factor receptor (MET), platelet-derived growth factor subunit B (PDGFB), and Janus kinase 1 (JAK1) (29). In cell free assays, the half maximal inhibitory concentration (IC_{50}) of alectinib for enzyme activity of ALK was 1.9 nM and the dissociation constant (KD) value for ALK in an ATP-competitive manner was 2.4 nM (29). *In vitro* experiments demonstrated that alectinib induces caspase-mediated apoptosis in *EML4-ALK* cell lines and results in dose-dependent tumor growth inhibition (ED50 = 0.46 mg/kg) and regression in animal models (29). More importantly, alectinib displayed significant efficacy against crizotinib-resistant *ALK L1196M* (IC_{50}, 2 nM) and G1269A (IC_{50}, 9 nM) mutations (22, 29). Alectinib was also active against ALK C1156Y, F1174L, 1151Tins, and L1152R but not ALK G1202R (IC_{50}, 70–80 nM) both *in vitro* and *in vivo* experiments (**Table 2**) (22).

ALECTININB FOR CRIZOTINIB FAILURE

Clinical trials evaluating the safety and efficacy of alectinib have been conducted in Japan and the US as both first-line untreated and *ALK*+ patient progressing on crizotinib. Support for alectinib activity in crizotinib failure comes from the AF-002JG study in which alectinib at 300–900 mg BID was well tolerated, with the most common adverse events (AEs) being fatigue (30%), myalgia (17%), and peripheral edema (15%) (10). The recommended phase II dose was 600 mg BID. Of the 44 evaluable patients with crizotinib resistance, 24 (55%) patients had response, 16 (36%) had stable disease (SD), and 4 (9%) had progressive disease. Alectinib also demonstrated activity against CNS metastases in 21 patients with an intracranial response rate of 52% [29% complete response (CR), 24% partial response (PR), and 38% SD] (10). Similar results were seen in a North American trial of 87 patients with advanced ALK-rearranged NSCLC who were refractory to crizotinib (9). The ORR for alectinib was 48% with a median PFS of 8.1 months (95% CI, 6.2–12.6). Fifty two patients had brain metastases at enrollment and 21 (40%) patients experienced CNS tumor regression, including 13 (25%) patients who achieved CR. Alectinib 600 mg BID was well tolerated with predominantly low grade constipation (36%), fatigue (33%), myalgia (24%), and peripheral edema (23%). Finally, the large phase II global study (NP2873) examined the ORR of alectinib for crizotinib-refractory ALK+ patients ($n = 138$) (8). This study is notable for a high rate of CNS metastases (61%) at baseline. The ORR determined by independent review committee was 50% (95% CI, 41–59%) and the median PFS was 8.9 months (95% CI, 5.6–11.3). Alectinib was highly effective for CNS metastases, with ORR of 57% and DCR of 83%. Of the 23 patients with baseline untreated CNS metastases, 10 (43%) had a complete CNS response. The authors note that the cumulative CNS progression rate (24.8%) was lower than the cumulative non-CNS progression rate (33.2%), which suggests that alectinib may delay or prevent the emergence of CNS metastases. Alectinib 600 mg BID was well tolerated with common side effects including low grade constipation (33%), fatigue (26%), and peripheral edema (25%). Overall the similar response rate to alectinib between the US and Japanese patients indicate

no ethnic difference in response. Additionally, there was no significant difference in alectinib exposure at 600 mg twice daily among a small subgroup of Caucasian and Asian patients who underwent pharmacokinetic analysis. Based on established activity, the Food and Drug Administration approved alectinib for the treatment of *ALK*+ NSCLC patients who progressed or were intolerant of crizotinib on December 11, 2015.

Based on promising second-line data and potential superiority over crizotinib, alectinib is being investigated in the first-line setting. In the phase I/II AF-001JP study conducted in Japan, patients with ALK inhibitor-naïve *ALK*+ NSCLC were treated with alectinib (11). Alectinib at 300 mg BID daily was well tolerated with few grade 3 toxicities or dose-limiting toxicities (DLTs) and ORR was observed in 43 out of 46 patients (93.5%) at this dose. On the other hand, the response rate for first-line crizotinib reported by Solomon et al. was 74% (4). Two phase III trials, ALEX (NCT02075840), and JapicCTI-132316, are currently comparing alectinib and crizotinib in ALK inhibitor-naive patients with *ALK*-rearranged NSCLC. Recently updated clinical data among 207 randomized patients in the J-ALEX trial were presented at the ASCO 2016 annual meeting (39). The primary endpoint was PFS and secondary endpoints included OS, ORR, CNS PFS, safety, and quality of life. In the alectinib arm, constipation (36%) was the only common event, while in the crizotinib arm nausea (74%), diarrhea (73%), vomiting (59%), visual disturbance (55%), dysgeusia (52%), constipation (46%), ALT elevation (32%), and AST elevation (31%) were seen in >30% patients. Alectinib was more tolerable than crizotinib with fewer grade 3/4 AEs (26.2 vs. 51.9%) which translated to a lower discontinuation rate (8.7 vs. 20.2%). The ORRs of the alectinib and crizotinib arms were 91.6 and 78.9%, respectively. The median PFS was not reached (CI, 20.3 to NR) but significantly higher than crizotinib 10.2 (CI, 8.2–12.0) with HR 0.34 (0.17–0.71). Complete data sets from first-line trials are eagerly awaited and may lead to additional indications for alectinib.

ADDITIONAL SECOND- AND THIRD-GENERATION ALK INHIBITORS

The second-generation ALK inhibitor ceritinib has *in vitro* activity against crizotinib-resistant mutations. Results from the open label multicenter ASCEND-1 trial showed that ceritinib yielded ORR of 72% (95% CI, 61–82) in 83 ALK inhibitor-naive patients and 56% (49–64) in 163 ALK inhibitor-resistant patients (7). Median PFS was 18.4 months in ALK inhibitor-naive patients and 6.9 months (5.6–8.7) in ALK inhibitor-pretreated patients. Among 94 patients with brain metastases, intracranial disease control was reported in 15 of 19 (79%) ALK inhibitor-naïve patients and in 49 of 75 (65%) ALK inhibitor-pretreated patients. In ALK inhibitor-resistant patients with CNS metastasis, the rates of intracranial CR, PR, and SD were 5, 13, and 47%, respectively. Common toxicities included diarrhea (80%), nausea (77%), vomiting (57%), fatigue (38%), abdominal pain (37%), decreased appetite (36%), constipation (30%), cough (29%), abdominal pain (23%), and dyspnea (21%). In April 2014, ceritinib 750 mg daily was approved by the US FDA for *ALK*+ previously treated with crizotinib.

Although both alectinib and ceritinib have shown promising systemic and CNS activity they are unlikely to be compared head to head in clinical trials. While ceritinib appears to have similar systemic response to alectinib, the intracranial response rate appears inferior to alectinib in crizotinib-resistant patients with CNS metastases. Accepting cross-trial comparison caveats the absolute median PFS is numerically shorter for ceritinib (6.9 months in the ASCEND-1 trial) than alectinib (8.9 months in the global NP2873 trial) in ALK inhibitor-resistant patients.

Other ALK inhibitors including brigatinib (*AP26113)* and lorlatinib (*PF-06463922*) have shown activity in crizotinib failure and highlight the non-overlapping resistance mutation coverage among current ALK inhibitors (**Table 2**). Briefly, brigatinib is a potent dual inhibitor of ALK and EGFR, including *ALK L1196M* and *EGFR T790M* mutants, shown in preclinical studies (40, 41). In the phase II ALTA study, 222 heavily pretreated *ALK*-rearranged patients were randomized to receive brigatinib 90 mg PO (arm A) vs. 180 mg PO qd (arm B) (42). The investigator-assessed ORRs of arm A and B patients were 46% (95% CI, 36–55%) and 54% (95% CI, 44–63%), respectively. Median PFS in arms A and B was 8.8 and 11.1 months, respectively. However, the median follow-up was only 8.3 months and longer follow-up is needed to confirm the higher PFS observed in arm B. Among patients with active brain metastases at baseline, intracranial ORRs, as assessed by independent review committee, in A and B were 37% (7/19) and 73% (11/15), respectively. Most common AEs in arms A/B included nausea (33/40%), diarrhea (19/38%), headache (28/27%), cough (18/34%), dyspnea (21/21%), fatigue (20/27%), constipation (19/15%), abdominal pain (17/8%), and vomiting (24/23%). Grade ≥ 3 treatment-emergent AEs (A/B) included: increased CPK (3/8%), hypertension (4/5%), pneumonia (3/5%), rash (1/4%), and pneumonitis (2/3%). Discontinuations and dose reductions due to AEs (A/B) were 3/6% and 7/18%, respectively. Due to the favorable efficacy and toxicity profile, brigatinib 180 mg PO daily was chosen as the optimal dose and is moving forward in the phase III ALTA-1L vs. crizotinib in the first-line setting.

Lorlatinib (PF-06463922) is a third-generation reversible, potent ATP-competitive small molecule, inhibitor of ALK and ROS1. Lorlatinib has demonstrated activity against the majority of known resistant ALK mutations, except for *L1198F* (**Table 2**) (31, 37). Early data from an ongoing phase I/II study of lorlatinib in mostly pretreated patients with *ALK*+ and *ROS1*+ NSCLC were presented at the ASCO 2016 annual meeting (43). Among the 54 evaluable patients who received dose escalation from 10 mg to 200 mg, the overall response rate was 50% and intracranial response rate was 44% for target and non-target lesions and 60% for target lesions. The most common treatment-related AEs were hypercholesterolemia (54%) and peripheral edema (37%). Hypercholesterolemia was the most common (9%) grade (G) ≥ 3 treatment-related AE and most frequent reason for dose delay/reduction. No patient was discontinued due to a treatment-related AEs. The phase II dose was identified as 100 mg once daily. Pharmacokinetic analysis of four patients revealed that the unbound CSF to plasma drug ratio ranged from 0.61 to 0.96, indicative of good CSF penetration. In contrast, the ratio of CNS to serum concentration of crizotinib has been in the range

of 0.0006–0.001 in previous reports (19, 44). Lorlatinib is effective against the *G1202R* mutation (**Table 2**).

CONCLUSION/FUTURE DIRECTIONS

Over the past decade, there has been a remarkable progress in the target therapy for the management of *ALK*-rearranged NSCLC. Second- and third-generation inhibitors demonstrate broader coverage against crizotinib-resistant *ALK* mutations and often more favorable side effect profiles. As discussed elsewhere in this issue, we are approaching a paradigm in which understanding the exact resistance mechanism will inform the optimal choice and perhaps sequencing of ALK inhibitors. The approval of alectinib for crizotinib failure highlights major areas of focus in *ALK*+ disease; toxicity profile, intracranial activity, and resistance mutation coverage. While alectinib compares favorably in these areas, ongoing results from first-line trials and direct comparison against current and emerging ALK inhibitors will be important to refine optimal alectinib usage. Here we have provided a review of the clinical data supporting the activity of alectinib in the management of *ALK*+ NSCLC with a focus on the second-line setting in advanced disease.

AUTHOR CONTRIBUTIONS

PT and SK are involved in the conception/design and drafting the manuscript. All the authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the manuscript.

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REVIEW
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Second-line Treatment of Non-Small Cell Lung Cancer: Focus on the Clinical Development of Dacomitinib

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Dacomitinib is a second-generation, irreversible, covalent pan-HER tyrosine-kinase inhibitor (TKI). It showed potent EGFR signaling inhibition in experimental models, including first-generation TKI-resistant non-small cell lung cancer (NSCLC) cell lines. This preclinical efficacy did not translate into clinically meaningful treatment benefits for advanced, pretreated, molecularly unselected NSCLC patients enrolled in two parallel phase III trials. Dacomitinib and erlotinib showed overlapping efficacy data in chemotherapy-pretreated *EGFR* wild-type (WT) patients in the ARCHER 1009 trial. Similarly, it failed to demonstrate any survival benefits as compared to placebo in *EGFR* WT subsets progressing on chemotherapy and at least one previous first-generation TKI (erlotinib or gefitinib) in the BR.26 trial. In the case of *EGFR*-mutant NSCLCs, a pooled analysis of the ARCHER 1009 and ARCHER 1028 trials comparing the efficacy of dacomitinib vs. erlotinib in chemotherapy-pretreated, EGFR TKI-naïve patients showed a trend to a longer progression-free survival (PFS) and overall survival in favor of dacomitinib that did not reach statistical significance, with a higher rate of treatment related adverse events (mainly skin rash, paronychia, and gastrointestinal toxicities). On the other hand, the clinical activity in patients with *EGFR*-mutant NSCLCs with acquired TKI resistance that were included in phase II/III trials was equally poor (response rate <10%; PFS 3–4 months). Therefore, with the results of the ARCHER 1050 trial (NCT01774721) still pending, the current clinical development of dacomitinib is largely focused on *EGFR*-mutant, TKI-naïve patients. Here, we review the most relevant clinical data of dacomitinib in advanced NSCLC. We discuss the potential role of dacomitinib in pretreated *EGFR* WT and *EGFR*-mutant (TKI-naïve and TKI-resistant) patients. Finally, we briefly comment the available clinical data of dacomitinib in HER2-mutant NSCLC patients.

Keywords: non-small cell lung cancer, second-line treatment, EGFR mutations, second-generation EGFR tyrosine-kinase inhibitors, dacomitinib, acquired resistance

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INTRODUCTION

Second-line treatment options for advanced non-small cell lung cancer (NSCLC) patients have substantially expanded in the past few years. Docetaxel- or pemetrexed-based chemotherapy and erlotinib were the only three drugs approved in our setting until year 2014, achieving an approximate 8–10% of response rates (RRs), median 4 months of progression-free survival (PFS) and 8–10 months of overall survival (OS) (1). Recently, antiangiogenics [ramucirumab (2), nintedanib (3), and bevacizumab (4)] and particularly PD-1/PD-L1 inhibitors [nivolumab (5, 6), pembrolizumab (7), and atezolizumab (8)]

have shown to prolong survival in pretreated patients, transforming the standardization of second-line NSCLC treatment.

In the absence of significant differences in terms of efficacy, the choice between pemetrexed- or docetaxel-based second-line chemotherapy is largely driven by three factors: histology, as pemetrexed is restricted to non-squamous tumors, type of platinum doublet used during first-line treatment, with pemetrexed being increasingly incorporated into the first-line or maintenance treatments, and differences in toxicity profiles. On the other hand, when deciding between chemotherapy and erlotinib, apart from clinical factors, EGFR mutation status is the main biomarker that determines treatment selection.

The IPASS trial definitely demonstrated that the clinical activity of EGFR tyrosine-kinase inhibitors (TKIs) in treatment-naïve patients was restricted to those with *EGFR*-mutant tumors (*EGFR*-sensitizing mutations). As the clinical activity of EGFR TKIs in TKI-naïve, *EGFR*-mutant tumors is comparable between treatment-naïve or platinum-pretreated patients (9), first- or second-generation EGFR TKIs are the preferred treatment options in patients with *EGFR*-mutant tumors. On the contrary, in patients with *EGFR* wild-type (WT) cancers, RRs and survival were significantly lower with gefitinib- compared to platinum-based chemotherapy in the IPASS study (10). However, whether this was also true in the second-line setting, a clinical context in which the efficacy of docetaxel- or pemetrexed-based chemotherapy hardly reaches 10% of RRs, has been a matter of extensive debate in the past few years. Some molecularly unselected randomized trials, initiated at a time where no definitive predictive biomarkers for the benefit or EGFR TKIs were discovered yet, initially suggested similar efficacy outcomes between erlotinib and second-line chemotherapy (11–13). More recent data, including molecularly selected or molecularly stratified randomized trials and large meta-analysis, have confirmed that second-line chemotherapy is superior to EGFR TKIs in patients with *EGFR* WT tumors, at least in terms of RRs and PFS. OS differences did not reach statistical significance (14–16).

In this therapeutic scenario, and considering that EGFR pathway activation might hypothetically contribute to cancer progression even in tumors with no *EGFR* activating mutations (17), to investigate if a more potent pan-HER inhibition with dacomitinib would add any clinical benefit seemed a rational approach, either from a biological or a clinical perspective. In addition, as the majority of patients with *EGFR*-mutant tumors treated with first-generation EGFR TKIs develop acquired resistance by ERBB-dependent mechanisms (18), and considering that dacomitinib showed activity in gefitinib-resistant preclinical lung cancer models (19), it was also rational to test its clinical activity in patients with *EGFR*-mutant, TKI-resistant cancers. Herein, we will succinctly discuss the potential role of second-line dacomitinib in *EGFR* WT and *EGFR*-mutant NSCLC.

DACOMITINIB: PRECLINICAL AND EARLY CLINICAL DATA IN NSCLC

Dacomitinib is a second-generation, irreversible, covalent-binding pan-HER TKI. As compared to first-generation EGFR TKIs, it has comparable inhibitory activity against the WT EGFR kinase *in vitro*. However, dacomitinib is more potent than gefitinib against cell lines harboring common *EGFR*-sensitizing mutations (del19, L858R). Moreover, it has inhibitory activity against gefitinib-resistant exon 20 insertions and acquired resistance exon 20 T790M mutations in preclinical lung cancer models. Unlike gefitinib or other first-generation TKIs, dacomitinib, as a pan-ERBB inhibitor, also inhibits the activity of both WT and mutant HER2 kinase (19, 20).

Three phase I trials, conducted both in Western and Asian patients, established that the maximum tolerated dose of dacomitinib was 45 mg daily, and this dose level was selected for further clinical evaluation. The most frequent dose-limiting drug-related adverse events were skin and gastrointestinal toxicities (21–23). The three trials consistently demonstrated that plasma concentrations and other pharmacokinetic parameters proportionally increased with increasing doses of oral dacomitinib (21–23), with no apparent food effect (21). Dacomitinib's half-life was estimated at 59–85 h in the phase I trial conducted in the United States (21). A modest preliminary clinical activity was observed in small cohorts of NSCLC patients previously treated with first-generation EGFR TKIs and/or chemotherapy. No objective responses were seen in EGFR TKI-resistant patients whose tumors harbored *EGFR* T790M mutations (21–23).

DACOMITINIB FOR PRETREATED NSCLC PATIENTS

Clinical Data in *EGFR* WT or NSCLCs Unselected by *EGFR* Status

The clinical activity of dacomitinib in pretreated NSCLC patients has been evaluated in four clinical trials (24–27). They are mostly molecularly unselected trials and, consequently, the vast majority of the patients included had *EGFR* WT tumors. An overview of the four clinical trials and the efficacy data in the overall study population are summarized in **Table 1**.

Two phase II trials initially suggested some degree of clinical activity in pretreated NSCLC patients. The ARCHER 1002 trial was a single-arm study that tested the activity of dacomitinib in patients that were refractory to one or two lines of chemotherapy and erlotinib. On the basis that *KRAS* mutant cell lines were primarily resistant to first- or second-generation EGFR TKIs, this study was enriched with patients with *KRAS* WT tumors. The trial failed to meet its primary end point, as dacomitinib yielded a disappointing 5.2 and 4.8% of RRs in the overall and adenocarcinoma subsets, respectively. Patients with *EGFR* WT/*KRAS* WT tumors included in this trial had comparable RRs (5%), PFS (8 weeks), and OS (26 weeks) to those of the overall study population (25) (**Table 1**). The second phase II trial (ARCHER 1028) compared the activity of dacomitinib and erlotinib in molecularly unselected patients progressing on one or two prior chemotherapy regimens. In this case, the trial met its primary endpoint, showing a statistically significant increase in PFS (2.86 vs. 1.91 months, HR 0.66, CI 95% 0.47–0.91) in favor of dacomitinib in the overall study population. Objective responses were also higher in dacomitinib treated patients (17 vs. 5.3%, $p = 0.01$). However, no differences in OS were noted (HR 0.80, CI 95% 0.56–1.10, $p = 0.20$) (**Table 1**). Comparable degree of PFS increment to the overall population was observed in *EGFR* WT NSCLCs (HR 0.70, CI 95% 0.47–1.05) and *EGFR* WT/*KRAS*

TABLE 1 | Clinical studies and efficacy data of dacomitinib in pretreated, advanced NSCLC patients.

Study	Phase	Clinical context	Molecular eligibility	*N* overall	*N* EGFR mutant	*N* EGFR WT	Response rates	PFS	OS
ARCHER 1002 (25)	Single arm phase II	Pretreated patients who failed at least one chemotherapy regimen, but no more than two, and erlotinib	KRAS WT[a]	66	26	23	5.2%	12 weeks	37 weeks
ARCHER 1028 (24)	Randomized phase II	Pretreated, TKI-naive patients who failed one or two chemotherapy regimens	Unselected	188	30	129	Dacomitinib: 17% Erlotinib: 5.3% $p = 0.01$	Dacomitinib: 2.86 months Erlotinib: 1.91 months HR 0.66 (CI 95% 0.47–0.91; $p = 0.01$)	Dacomitinib: 9.53 months Erlotinib: 7.44 months HR 0.80 (CI 95% 0.56–1.10; $p = 0.20$)
BR.26 (27)	Phase III	Pretreated patients who failed up to three chemotherapy regimen and erlotinib/gefitinib	Unselected	720	182	349	Dacomitinib: 7% Placebo: 1% $p = 0.001$	Dacomitinib: 2.66 months Placebo: 1.38 months HR 0.66 (CI 95% 0.55–0.79)	Dacomitinib: 6.83 months Placebo: 6.31 months HR 1.00 (CI 95% 0.83–1.21)
ARCHER 1009 (26)	Phase III	Pretreated, TKI-naive patients who failed one or two chemotherapy regimens	Unselected	878	91	662	Dacomitinib: 17% Erlotinib: 5.3% $p = 0.01$	Dacomitinib: 2.6 months Erlotinib: 2.6 months HR 0.94 (CI 95% 0.80–1.10; $p = 0.22$)	Dacomitinib: 7.9 months Erlotinib: 8.4 months HR 1.07 (CI 95% 0.91–1.27; $p = 0.81$)

[a]*Patients with EGFR-mutant tumors were assumed to be KRAS WT based on mutual exclusivity.*

PFS, progression-free survival; OS, overall survival; TKI, tyrosine-kinase inhibitor; NSCLC, non-small cell lung cancer; WT, wild-type.

WT NSCLCs (HR 0.61, CI 95% 0.37–0.99). Dacomitinib did not improve OS compared to erlotinib in patients with *EGFR* WT cancers (24).

This modest clinical activity served as the basis to launch two subsequent randomized phase III trials in similar therapeutic scenarios to their respective phase II trials. Unfortunately, both phase III studies were negative. First, in the BR.26 trial, whereas dacomitinib statistically significantly improved RRs (7 vs. 1%, $p = 0.001$) and PFS (2.66 vs. 1.38 months, HR 0.66 CI 95% 0.55–0.79) compared to placebo in patients progressing on chemotherapy and EGFR TKIs, it failed to demonstrate improved OS (primary end point; HR 1.00) (**Table 1**). Similarly, no trend for a clinically meaningful incremental efficacy was observed in patients with *EGFR* WT tumors or patients with both *EGFR* and *KRAS* WT NSCLCs compared to the overall patient population (27). And finally, Dacomitinib failed to improve the efficacy of erlotinib (control arm) in second- or third-line settings (ARCHER 1009), either in the overall population (**Table 1**) or in patients with *EGFR* WT tumors. In the latter subgroup, dacomitinib had overlapping objective RRs, PFS (1.9 vs. 1.9 months; HR 0.94, CI 95% 0.79–1.13), and OS (6.8 vs. 7.6 months; HR 1.07, CI 95% 0.90–1.29) compared to erlotinib. Results were almost identical for patients with either *KRAS* or *EGFR* WT NSCLCs (26).

Clinical Data in *EGFR*-Mutant, TKI-Naïve NSCLCs

In the particular case of pretreated, TKI-naïve subsets, a pooled analysis of the ARCHER 1009 and ARCHER 1028 trials comparing the efficacy of dacomitinib vs. erlotinib showed a comparable median PFS (14.6 vs. 9.6 months, respectively; HR 0.71, $p = 0.14$) and OS (26.6 vs. 23.2 months, respectively; HR 0.73, $p = 0.26$) outcomes that somehow favored dacomitinib (28) (**Table 2**). Both ARCHER 1028 and ARCHER 1009 trials showed that on target adverse events related to the inhibition of *EGFR* WT in normal tissues were significantly increased with dacomitinib compared to erlotinib, mainly skin rash, paronychia, and gastrointestinal toxicities (24, 26). These data are in line with the recently published LUX-Lung 7 trial, where afatinib significantly delayed PFS and the emergence of EGFR TKI resistance, albeit with a higher incidence of treatment related adverse events (29).

Clinical Data in *EGFR*-Mutant, TKI-Pretreated NSCLCs

In the context of EGFR TKI acquired resistance, the clinical efficacy of dacomitinib in patients with *EGFR*-mutant lung cancers progressing on first-generation EGFR TKIs that were included in these trials was disappointingly low, with an overall RR of about 8% (**Table 2**). No objective responses were reported among patients whose tumors harbored the secondary acquired resistance *EGFR* T790M mutation. In general, the PFS and OS data did not differ to those of the unselected patient population either (25, 27).

Clinical Data in *HER2*-Mutant, TKI-Naïve NSCLCs

In the largest prospective phase II study conducted to date in patients with *HER2*-mutant or *HER2*-amplified tumors ($n = 30$;

TABLE 2 | Clinical data of dacomitinib in EGFR-mutant NSCLCs.

Study	Phase	Clinical context	No. of patients with EGFR-mutant tumors (sensitizing mutations)	Response rates (%)	PFS	OS
A7471017 (30)	II	Treatment naive	45	76	18.2 months	–
Pooled analysis ARCHER 1009 and ARCHER 1028 (28)	II and III	Chemotherapy-pretreated, TKI naive	101	67.9	14.6 months	26.6 months
ARCHER 1002 (25)	II	TKI resistant	24	8	18 weeks	56 weeks
BR.26 (27)	III	TKI resistant	114	–	3.52 months	7.23 months

PFS, progression-free survival; OS, overall survival; TKI, tyrosine-kinase inhibitor; NSCLCs, non-small cell lung cancers.

83% had received at least one line of previous chemotherapy), dacomitinib showed only modest efficacy, with an objective RR of 12%, 3 months of median PFS, and 9 months of median OS. No responses were seen in patients with tumors harboring the most common *HER2* activating mutation (c. 2324_2325ins12) (31). Intriguingly, tumors with this genotype did respond to afatinib in other series (32). No responses were seen either in patients with *HER2*-amplified cancers ($n = 4$) (31). More studies are needed in order to determine which molecular contextures (i.e., possible coexistence with *HER2* amplification) and what specific *HER2* genotypes are true predictive targets for the benefit of dacomitinib.

CONCLUSION AND FUTURE PERSPECTIVES

Dacomitinib has failed to improve overall outcomes in pretreated NSCLC patients. An irreversible pan-HER inhibition is not superior to erlotinib in patients with no *EGFR*-sensitizing mutations and does not prolong OS compared to placebo in heavily pretreated patients either. Also, dacomitinib does not overcome *EGFR* T790M-mediated acquired resistance in *EGFR*-mutant NSCLCs at tolerable doses in humans. In non-T790M-mediated resistance, in which functional activation of HER pathway or acquired *HER2* activating mutations have been described in some cases (18, 33), no reliable clinical data are available, but a robust activity in this clinical setting seems unlikely. With these clinical data, together with recent regulatory approvals of third-generation, *EGFR*-mutant selective TKIs (e.g., osimertinib) with potent activity against the T790M mutation (34), current development of dacomitinib is focused to TKI treatment-naïve, molecularly selected patients with *EGFR*-mutant and *HER2*-mutant lung cancers. In a small phase II trial including a total of 45 treatment-naïve patients with tumors harboring common *EGFR*-sensitizing mutations, dacomitinib achieved an overall RR of 75.6% and a median PFS of 18.2 months (30).

In this regard, whether second-generation EGFR TKIs in TKI-naïve patients are superior to first-generation TKIs in EGFR-mutant NSCLCs is not fully answered to date. In the LUX-Lung 7 trial, afatinib significantly increased RRs (70 vs. 56%; $p = 0.0083$), median PFS (11 vs. 10.9 months; HR 0.73, CI 95% 0.57–0.95; $p = 0.0195$), and median time to treatment failure (13.7 vs. 11.5 months; HR 0.73, CI 95% 0.58–0.92; $p = 0.0073$) over gefinitib. However, there were no OS differences among treatment arms in this phase IIb trial ($n = 319$). Pre-specified subgroup analysis according to mutation type (exon 19 deletions vs. L858R mutations) did no show significant differences in OS either. Overall, treatment-related adverse events (mainly skin rash and diarrhea) and serious adverse events were more common with afatinib (33). Therefore, this trial suggests that the emergence of acquired resistance might be delayed with second-generation compared to first-generation TKIs, but whether these modest differences are clinically relevant for patients is arguable for many physicians. The ARCHER 1050 trial (NCT01774721) comparing first-line dacomitinb vs. gefitinib has recently completed accrual and will hopefully give a definitive answer in this regard, establishing the true role of front-line dacomitinib in *EGFR*-mutant NSCLCs.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work.

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REVIEW
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Next-Generation *EGFR* Tyrosine Kinase Inhibitors for Treating *EGFR*-Mutant Lung Cancer beyond First Line

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Tyrosine kinase inhibitors (TKIs) against the human epidermal growth factor receptor (*EGFR*) are now standard treatment in the clinic for patients with advanced *EGFR* mutant non-small-cell lung cancer (NSCLC). First-generation *EGFR* TKIs, binding competitively and reversibly to the ATP-binding site of the *EGFR* tyrosine kinase domain, have resulted in a significant improvement in outcome for NSCLC patients with activating *EGFR* mutations (L858R and Del19). However, after a median duration of response of ~12 months, all patients develop tumor resistance, and in over half of these patients this is due to the emergence of the *EGFR* T790M resistance mutation. The second-generation *EGFR/HER* TKIs were developed to treat resistant disease, targeting not only T790M but *EGFR*-activating mutations and wild-type *EGFR*. Although they exhibited promising anti-T790M activity in the laboratory, their clinical activity among T790M+ NSCLC was poor mainly because of dose-limiting toxicity due to simultaneous inhibition of wild-type *EGFR*. The third-generation *EGFR* TKIs selectively and irreversibly target *EGFR* T790M and activating *EGFR* mutations, showing promising efficacy in NSCLC resistant to the first- and second-generation *EGFR* TKIs. They also appear to have lower incidences of toxicity due to the limited inhibitory effect on wild-type *EGFR*. Currently, the first-generation gefitinib and erlotinib and second-generation afatinib have been approved for first-line treatment of metastatic NSCLC with activating *EGFR* mutations. Among the third-generation *EGFR* TKIs, osimertinib is today the only drug approved by the Food and Drug Administration and the European Medicines Agency to treat metastatic *EGFR* T790M NSCLC patients who have progressed on or after *EGFR* TKI therapy. In this review, we summarize the available post-progression therapies including third-generation *EGFR* inhibitors and combination treatment strategies for treating patients with NSCLC harboring *EGFR* mutations and address the known mechanisms of resistance.

Keywords: *EGFR*, T790M, NSCLC, osimertinib, third generation, brain metastasis

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INTRODUCTION

Over the past decade, scientific advances have progressively improved outcomes for patients diagnosed with lung cancers driven by target oncogene mutations. The first oncogenic driver in non-small-cell lung cancer (NSCLC) was discovered in 2004 with the identification of activating mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) among patients with dramatic

responses to *EGFR* tyrosine kinase inhibitors (TKIs) (1–3). *EGFR* mutations account for 10–17% of NSCLC cases in North America and Europe and 30–50% of NSCLCs in Asian countries and are most common among patients with adenocarcinoma NSCLC and a light or non-smoking history (4, 5). The first-generation TKIs gefitinib (Iressa®, AstraZeneca, London, UK) and erlotinib (Tarceva®, F. Hoffmann-La Roche, Basel, Switzerland), and the second-generation TKI afatinib (Giotrif®, Boehringer Ingelheim, Ingelheim, Germany) have shown higher response rates (RRs), improving progression-free survival (PFS) and quality of life compared to standard platinum-based chemotherapy in patients with good performance status (0–2) whose tumors harbor an activating (sensitizing) *EGFR* mutation (6–13).

These data established *EGFR* TKIs as the treatment of choice for patients with newly diagnosed *EGFR*-mutant advanced NSCLC. Of note, none of these studies demonstrated a benefit in terms of overall survival (OS) due to the high level of crossover. However, an unplanned pooled OS analysis of patients included in the LUX-Lung 3 or LUX-Lung 6 phase III trials demonstrated an OS benefit for afatinib compared to platinum-based chemotherapy in patients whose tumors harbor *EGFR* Del19 mutations vs. *EGFR* L858R mutations: 27.3 vs. 24.3 months, respectively [hazard ratio (HR) 0.81; 95% confidence interval (CI), 0.66–0.99; $p = 0.037$] (14). However, this benefit was not confirmed in the phase IIb LUX-Lung 7 designed to compare head-to-head afatinib with gefitinib in the first-line treatment of patients with *EGFR*-mutant NSCLC (15). Unfortunately, the majority of patients progress after a median of 12 months treatment with first-line TKIs, and multiple mechanisms of acquired resistance have been identified. Among them, the most common mechanism (~50% of cases) is the acquisition of a missense mutation within exon 20 of *EGFR*, the T790M mutation (p.Thr790Met) (16).

Until recently, standard chemotherapies were the main treatment option in a post-progression setting. For patients initiating chemotherapy, the role of *EGFR* TKI maintenance remains controversial. In a retrospective analysis, up to 23% of patients experience a disease flare after TKI discontinuation (17), which led many clinicians to continue *EGFR* TKIs when starting chemotherapy. It was hypothesized that some clones within a resistant cancer remained sensitive to *EGFR* inhibition and that withdrawal of the TKI could "let loose" these clones with resultant adverse outcomes. The randomized phase III IMPRESS trial provided the first prospective data to address this clinical question. Patients progressing on first-line gefitinib were randomized to receive cisplatin–pemetrexed with gefitinib or placebo. The trial did not confirm a benefit of maintaining the *EGFR* TKI, with comparable RRs and PFS in the two arms (18). The final OS analysis was presented recently; patients in the gefitinib arm had significantly lower OS compared to the placebo arm (13.4 vs. 19.5 months, HR = 1.44, $p = 0.016$), confirming the deleterious effect of maintaining the *EGFR* inhibition. Of note, this detrimental effect was predominantly observed among patients whose tumors harbored a T790M mutation detected *via* circulating tumor DNA (ctDNA; HR = 1.49; 95% CI, 1.02–2.21) (19).

To date, many third-generation *EGFR* TKIs have been developed to target both sensitizing *EGFR* mutations and *EGFR* T790M. In this review, we outline available post-progression therapies including osimertinib (previously known as AZD9291) as the only drug approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of patients with metastatic *EGFR* T790M+ NSCLC who have progressed on or after *EGFR* TKI therapy (20), and other next-generation irreversible *EGFR* TKIs in clinical development (**Table 1**).

IDENTIFYING THE ACQUIRED RESISTANCE MECHANISM TO FIRST/SECOND-GENERATION *EGFR* TKIs

For patients whose disease progresses on gefitinib, erlotinib, or afatinib, understanding the major mechanisms of resistance is essential to choosing the optimal post-progression treatment. To date, repeated biopsies are the standard of care; however, this approach comes with some limitations—not all patients are amenable to this procedure, and not all progressing lesions are accessible for biopsy. In addition, there is growing evidence that a single biopsy may not accurately represent the intrinsic heterogeneity of a resistant tumor. Liquid biopsy is a valid alternative to tissue rebiopsy. This approach, which has been validated (21), represents a surrogate DNA source and is a novel strategy for tumor genotyping, mainly applicable at the time of progression for *EGFR*-mutated patients (22–24). In cases when the T790M mutation is identified in peripheral blood, treatment with third-generation *EGFR* TKIs is justified (25).

In addition to T790M, other resistance mechanisms have also been identified. Globally, these can be categorized as target gene alterations (i.e., *EGFR* amplifications and mutations such as T790M), downstream bypass signaling pathway activation (i.e., *MET* and *HER2* amplifications or mutations in *BRAF*, *PIK3CA*), and phenotypic changes (including small-cell lung cancer transformation and epithelial to mesenchymal transition) (26, 27).

TARGETING *EGFR* T790M+ NSCLC

Second-Generation *EGFR* TKIs

Following the discovery that T790M is the main resistance mechanism against the first-generation *EGFR* TKIs gefitinib and erlotinib, many new drugs targeting T790M were developed. Although second-generation *EGFR* inhibitors such as neratinib, afatinib, and dacomitinib exhibited promising anti-T790M activity in the laboratory, their clinical activity in T790M+ NSCLC was poor, with RR less than 10% among patients resistant to gefitinib or erlotinib (28–30). In addition, increased toxicity, mainly skin and digestive (**Table 1**), was observed due to *EGFR* wild-type inhibition at lower concentrations than those required to inhibit T790M. Thus to date, none of the second-generation agents are considered as effective monotherapies in patients progressing on first-generation TKIs.

On the basis of preclinical observations that afatinib plus cetuximab (an anti-*EGFR* monoclonal antibody) overcame T790M-mediated resistance (31), this combination was evaluated in a phase Ib trial enrolling 126 heavily pretreated patients with advanced *EGFR*-mutant NSCLC who had developed resistance

TABLE 1 | *EGFR* TKI generations for metastatic *EGFR*-mutant NSCLC.

Generation TKI	Drug	Company	*EGFR* inhibition	Molecular targets	Most common adverse events	Status
First generation	Gefitinib	AstraZeneca	Competitive; reversible	*EGFR* L858R, Del19	Diarrhea, rash/acne, ALT/AST increased, decreased appetite	Phase III (approved)
	Erlotinib	F. Hoffmann-La Roche				
Second generation	Afatinib	Boehringer Ingelheim	Covalent; irreversible	wt-*EGFR*, *EGFR* L858R, L858R/T790M, L858R/T854A, wt-*HER2*, *HER2* amp., *HER4*	Skin rash, diarrhea	Phase III (approved)
	Dacomitinib	Pfizer		*EGFR* L858R, Del19, T790M, wt-*HER2*, mutant-*HER2*, *HER2* amp., *HER4*	Diarrhea, rash/acne	Phase III
	Neratinib	Puma Biotechnology		*EGFR* L858R, T790M, *HER2*, *HER4*	Diarrhea, dyspnea, nausea, vomiting	Phase III
Third generation	Osimertinib	AstraZeneca	Covalent; irreversible	*EGFR* L858R, Del19, T790M (limited activity against wt-*EGFR*)	Diarrhea, rash, nausea, decreased appetite	Phase III (approved)
	Rociletinib	Clovis			Hyperglycemia, long QT interval, nausea, fatigue, diarrhea	Phase II/III (stopped)
	Olmutinib	Hanmi/Boehringer Ingelheim			Diarrhea, rash, skin exfoliation, nausea, pruritus	Approved in South Korea[a]
	ASP8273	Astellas			Diarrhea, nausea, vomiting, platelet count decreased	Phase III
	Nazartinib	Novartis			Rash, diarrhea, pruritus	Phase I/II
	PF-06747775	Pfizer			No reported yet	Phase I/II
	Avitinib	Ace Bio			No reported yet	Phase I
	HS-10296	Jiangsu Hansoh			No reported yet	Phase I/II

[a]*Due to an unexpected increase of grade 3/4 skin toxicity, the ELUXA clinical trial program was temporally stopped.*

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Del, deletion; amp, amplification; TKIs, tyrosine kinase inhibitors; wt, wild-type; EGFR, epidermal growth factor receptor.

to erlotinib/gefitinib. The overall response rate (ORR) was 29% and was comparable in both T790M+ and T790M− tumors (32 vs. 25%), and median PFS was 4.7 months (95% CI, 4.3–6.4) (32). However, the dual *EGFR* inhibition resulted in increased toxicity with various grades 3–4 adverse events (AEs) (mainly rash, diarrhea, and fatigue) reported in up to 46% of patients (32). A randomized phase II/III trial (NCT02438722) of afatinib plus cetuximab vs. afatinib alone is currently open in treatment-naïve patients with advanced *EGFR*-mutant NSCLC.

Third-Generation *EGFR* TKIs

Many third-generation *EGFR* inhibitors are currently being consecutively developed to more effectively target the T790M mutation. Unlike second-generation TKIs, as these drugs exhibit increased specificity for T790M and thus mutant *EGFR* compared to wild-type *EGFR*, they are well tolerated resulting in few wild-type *EGFR* adverse effects. Among them, osimertinib (AZD9291) was the first to receive FDA and EMA approval in November 2015 and February 2016, respectively, for metastatic *EGFR* T790M+ NSCLC, which has progressed on or after *EGFR* TKI therapy. **Table 2** shows available efficacy data of new-generation *EGFR* TKIs.

Osimertinib (AZD9291; Tagrisso®)

Osimertinib is a mono-anilino-pyrimidine compound that acts as a covalent *EGFR* TKI. In *EGFR* recombinant enzyme assays, osimertinib showed potent activity against diverse *EGFR* mutations (L858R, L858R/T790M, exon 19 deletion, and exon 19 deletion/T790M) and exhibited nearly 200 times greater potency against L858R/T790M than wild-type *EGFR*. Osimertinib is metabolized to produce at least two circulating metabolites, AZ5104 and AZ7550. In biochemical assays, AZ7550 had a comparable potency and selectivity profile to osimertinib, although AZ5104 showed greater potency against exon 19 deletions, T790M mutants (both ~8-fold) and wild-type (~15-fold) *EGFR* (33). Its pharmacokinetic exposure did not significantly differ between Asian and non-Asian patients, showing a minimal food effect (34). Additionally, data from a clinical pharmacokinetic study (NCT02163733) showed that osimertinib exposure was not affected by concurrent administration of omeprazole (35). Thus, unlike first- and second-generation TKIs, gastric pH modifying agents can be concomitantly used with osimertinib without restrictions.

A phase I/II dose-escalation study of osimertinib (AURA, NCT01802632) was carried out in patients with locally advanced or metastatic *EGFR*-mutated NSCLC progressing on first- or second-generation *EGFR* TKIs. Patients were not preselected according to T790M status (36). The study included 253 patients who received osimertinib at five dose levels ranging from 20 to 240 mg daily and distributed between two cohorts, dose-escalation and dose-expansion cohorts. Among 31 patients enrolled in the dose-escalation cohort, no dose-limiting toxicity

TABLE 2 | Efficacy of third-generation tyrosine kinase inhibitors (TKIs) in activating epidermal growth factor receptor (*EGFR*) mutations and T790M+ NSCLC patients.

	Osimertinib				Rociletinib	Olmutinib	ASP8273	Nazartinib
Trial	AURA phase I	AURA phase I T790M+	AURA phase II ext.	AURA2 phase II	TIGER-X phase I/II[a]	HM-EMSI-101 phase I/II T790M+ (ongoing)	NCT02113813 phase I/II (ongoing)	NCT02108964 phase I/II (ongoing)
			T790M+					
			Pooled analysis					
Patients (*N*)	253 T790M+ = 138	63	201	210	69 T790M+ = 51	76	63 T790M+ = 58	152
Dose	20–240 mg qd	80 mg qd	80 mg qd		500, 625, or 750 mg bid	800 mg qd	300 mg qd	75–350 mg qd
ORR act*EGFR*m (%)	51 [95% CI, 45–58]	–	–		17 [95% CI, 4–41]	–	30	–
ORR T790M+ (%)	61 [95% CI, 52–70]	71 [95% CI, 57–82]	66 [95% CI, 61–71]		45 (95% CI, 31–60)	62	29 (central testing)	46.9
Overall mPFS (95% CI) mo	T790M+: 9.6 (8.3–NR) T790M−: 2.8 (2.1–4.3)	9.7 (8.3–13.6)	11.0 (9.6–12.4)		T790M+: 6.1 (4.2–9.6) T790M−: 1.8 (1.2–3.0)	6.9 (5.4–9.5)[b]	T790M+: 6.8 (5.5–NR)[c] T790M−: 6.0 (4.1–9.8)	9.7 (7.3–11.1)
Reference	Jänne et al. (36)	Yang et al. (37)			Sequist et al. (41)	Park et al. (44), Lee et al. (45)	Yu et al. (49)	Tan et al. (51)

[a]*Updated results from 69 reviewed cases included in the phase I TIGER-X trial.*

[b]*Updated results from 2016 ASCO Annual Meeting.*

[c]*mPFS from 28 NSCLC patients with central testing T790M+.*

actEGFRm, activating EGFR mutation; bid, twice daily; CI, confidence interval; ext., extension; mo, months; mPFS, median progression-free survival; NR, not reached; ORR, overall response rate; qd, once daily.

(DLT) occurred and the maximum tolerated dose (MTD) was not reached. An additional 222 patients were treated in five dose-expansion cohorts. The *EGFR*-T790M mutation was detected in tumors from 138 patients (62%) in the expansion cohorts. Of the 253 patients treated across all dose levels, 239 were evaluated for response. The ORR and disease control rate (DCR) in the whole population were 51% [95% CI, 45–58%] and 84% [95% CI, 79–88%], respectively. Among the 138 patients with a centrally confirmed *EGFR*-T790M mutation, 127 patients were evaluable for response. Outcomes were substantially better in the *EGFR* T790M+ population compared to T790M− tumor patients with an ORR of 61% [95% CI, 52–70%] vs. 21% [95% CI, 12–34%], a DCR of 95% [95% CI, 90–98%] vs. 61% [95% CI, 47–73%] and median PFS of 9.6 months [95% CI, 8.3–not reached] vs. 2.8 months [95% CI, 2.1 to 4.3], respectively (36). There were no DLTs at any dose level. The most common AE, mostly grade 1–2, were diarrhea (47%), skin toxicity (rash/acne, 40%), nausea (22%), and anorexia (21%). With increased incidence and severity of AEs (rash, dry skin, and diarrhea) in relation to the wild-type *EGFR* inhibition at higher dose levels (160 and 240 mg), 80 mg daily was selected as the recommended dose for further clinical trials (36).

The efficacy and safety data from the 80 mg expansion cohort in patients with centrally confirmed T790M NSCLC were recently updated (data cutoff: January 4, 2016). Among 63 patients, 61 patients were evaluable for response. The ORR and DCR were 71% [95% CI, 57–82%] and 93% [95% CI, 84–98%], respectively, with a median PFS of 9.7 months [95% CI, 8.3–13.6] (37).

The 80-mg daily dose evaluated in the phase II T790M+ extension cohort of the AURA trial (described above) was evaluated in an additional phase II "AURA2" study (NCT02094261) designed for patients with confirmed *EGFR*-mutant T790M+ locally advanced or metastatic NSCLC progressing on an approved *EGFR* TKI. A preplanned pooled analysis of both studies was performed. Among 411 patients (201 from the AURA extension and 210 from AURA2), 397 were evaluable. The ORR and DCR were 66% [95% CI, 61–71%] and 91% [95% CI, 88–94%], respectively. Median PFS was 11.0 [95% CI, 9.6–12.4] months with a median duration of response of 12.5 months [95% CI, 11.1 months to not calculable] (37).

Osimertinib has also demonstrated activity in the first-line setting. Data from two expansion cohorts in treatment-naïve *EGFR*-mutated advanced NSCLC patients were recently presented. Sixty patients received osimertinib 80 mg ($n = 30$) or 160 mg ($n = 30$) once daily and all were evaluable. The confirmed ORR was 77% [95% CI, 64–87%] with a DCR of 98% [95% CI, 89–100%]. Median PFS was 19.3 months [95% CI, 13.7 to not calculable] (38).

A number of phase III trials involving osimertinib in different settings are ongoing. The phase III FLAURA trial (First-Line-AURA; NCT02296125) in *EGFR*-mutated treatment-naïve NSCLC patients was designed to compare osimertinib 80 mg daily vs. the current standard of care gefitinib or erlotinib. The AURA3 trial (NCT02151981) is an open-label, randomized trial in the second-line setting, designed to compare osimertinib with platinum-based doublet chemotherapy in patients with *EGFR* T790M+ locally advanced or metastatic NSCLC. In a press

release dated July 18, 2016, AstraZeneca announced that the AURA3 trial, which included more than 400 patients, had met its primary endpoint demonstrating superior PFS compared to standard platinum-based chemotherapy.

In the adjuvant setting, the ongoing ADAURA trial (ADjuvant-AURA; NCT02511106) is a double-blind, randomized, placebo-controlled trial assessing the efficacy and safety of osimertinib vs. placebo in patients with *EGFR*-mutated stage IB–IIIA NSCLC following complete tumor resection. Results are not yet available.

Rociletinib (CO-1686)

Rociletinib is another oral, irreversible, mutant-selective inhibitor of commonly mutated forms of *EGFR*, including T790M, with minimal activity against wild-type *EGFR* in preclinical studies (39). A phase I/II trial (TIGER-X; NCT01526928) of rociletinib was performed in patients with *EGFR*-mutant NSCLC with acquired resistance to first- or second-generation *EGFR* TKIs (40). In the expansion (phase II) part of the study, patients with T790M+ NSCLC received rociletinib at doses of 500, 625, or 750 mg twice daily. At the time of report, 130 patients were enrolled. The MTD was not identified. The most common grade 3 AE was hyperglycemia, occurring in 20 of the 92 patients (22%) who received therapeutic doses. Among the 46 evaluable patients with T790M+, the ORR was 59% [95% CI, 45–73%]. For the 17 evaluable patients with T790M− disease, the ORR was 29% [95% CI, 8–51%] (40).

In November 2015, Clovis Oncology issued a press release that contained data from a pooled analysis of TIGER-X and TIGER-2 (NCT02147990), another phase II trial examining rociletinib in second line in patients with *EGFR* T790M+ NSCLC progressing on at least on *EGFR* inhibitor. Among 325 patients, the ORR (dose range, 500–750 mg twice daily) was 30.2% [95% CI, 25.2–35.5%]. The ORRs were 32% [95% CI, 25–40%] and 23% [95% CI, 14–34%] in patients receiving 625 mg ($n = 170$) and 500 mg ($n = 79$), respectively. The median duration of response for the two treatment doses was 8.8 and 9.1 months, respectively. Due to the different RR, an independent updated analysis was assessed in patients (intention-to-treat population) included in the TIGER-X trial confirming ORRs of 45% [95% CI, 31–60%] and 17% [95% CI, 4–41%] among patients with T790M+ and T790M− disease, respectively (41). Clovis thus decided to halt enrollment in all ongoing rociletinib studies, including the phase III TIGER-3 trial (NCT02322281), and has withdrawn its application for regulatory approval in the European Union.

Olmutinib (BI-1482694/HM61713; Olita™)

Olmutinib is an oral *EGFR* mutant-specific TKI active against mutant *EGFR* isoforms, including T790M, while sparing wild-type *EGFR* (42). A phase I/II trial HM-EMSI-101 (NCT01588145) was conducted to evaluate the safety, tolerability, pharmacokinetics, and preliminary activity of olmutinib in Korean patients with *EGFR* TKI-pretreated NSCLC (43). Patients received olmutinib at doses ranging from 75 to 1,200 mg/day. The ORR was 58.8% in the 34 patients who received olmutinib with a dose more than 650 mg. The most common DLTs involved gastrointestinal symptoms and increased aspartate aminotransferase, alanine aminotransferase, amylase, and lipase levels. The recommended phase II dose was 800 mg/day. In part II of the study, 76 patients with centrally confirmed T790M+ NSCLC were enrolled, 70 of whom were evaluable for response. The ORR was 61% and median PFS ($n = 76$) was 6.9 months [95% CI, 5.36–9.49]. The most common drug-related AEs (all grades) were diarrhea (59%), pruritus (42%), rash (41%), and nausea (39%) (44). These data validate previous preliminary trial results presented at the European Society for Medical Oncology Asia Congress in December 2015 (45). These results were the basis for Breakthrough Therapy Designation granted by the FDA in 2015 and the first approval for the treatment of patients with *EGFR* T790M+ NSCLC in South Korea in 2016. Following promising early clinical data, Boehringer Ingelheim launched the ELUXA clinical trial program to investigate olmutinib as a monotherapy in different settings as well as in combination with other anticancer treatments. Nevertheless, due to an unexpected increase in grade 3/4 skin toxicity (epidermolysis) in previous trials Boehringer decided to definitively stop the development of this drug.

ASP8273

ASP8273 is another oral, irreversible TKI that inhibits the kinase activity of *EGFR* mutations including T790M, with limited activity against *EGFR* wild-type (46). ASP8273 was further shown to suppress signaling *via* ERK and Akt. This agent showed activity in mutant *EGFR* cell lines that are resistant to other *EGFR* TKIs including osimertinib and rociletinib (47). ASP8273 was evaluated in an open-label phase I/II study (NCT02192697) for safety and efficacy (48). Thirty Japanese patients were enrolled in the phase I dose-escalation cohorts across seven dose levels (25–600 mg/day), and 15 patients were enrolled in the response expansion cohorts across four dose levels (100–400 mg/day). T790M status was 49% positive, 13% negative, and 38% unknown, respectively. Responses were observed in patients enrolled in ≥100 mg/day cohorts. Partial responses were achieved in 50% (18/36) of all evaluable patients and 80% (12/15) of patients with T790M+ NSCLC (including confirmed and unconfirmed). The most common AEs (all grades) were diarrhea (56%), nausea (31%), vomiting (31%), and thrombocytopenia (31%). Based on tolerability and preliminary antitumor activity, the recommended phase II dose selected was 300 mg once daily (48). The safety, tolerability, and antitumor activity for ASP8273 300 mg/day in patients with NSCLC *EGFR* mutation-positive and previously treated with an *EGFR* TKI were recently presented in a total of 63 patients, including seven treated in the dose-escalation part, 18 in the response expansion, 19 in recommended phase II dose part, and 19 from the food effect cohort (49). The majority of tumors (>90%) were positive for the T790M mutation based on local testing. All but one patient (98%) had been previously treated with an *EGFR* TKI, with erlotinib the most common inhibitor. Among the 63 patients treated with ASP8273 300 mg, the ORR was 30% [95% CI, 19.2–43.0%] and the median PFS was 6.0 [95% CI, 4.1–9.8] months. For the subgroups with T790M+ tumors the ORRs, assessed by local or central testing, were similar: 31% [95% CI, 19.5–44.5%] and 29% [95% CI, 13.2–48.7%], respectively. Median PFS for T790M+ patients (local testing) was 6.0 months [95% CI, 5.3–9.8] and 6.8 months [95% CI, 5.5 months to not evaluable] for T790M+ patients (central testing). The most

frequent drug-related AEs (all grades) were diarrhea (48%), nausea (27%), hyponatremia (19%), paresthesia (14%), and vomiting (13%). Six patients (10%) discontinued treatment due to treatment-related toxicity (49). Based on this study, the dose of ASP8273 300 mg daily was selected for a recently initiated, large ($n = 600$), international, randomized, phase III study (SOLAR) to compare the clinical efficacy and safety/tolerability of ASP8273 with erlotinib or gefitinib as initial treatment of advanced *EGFR*-mutant NSCLC (NCT02588261).

Nazartinib (EGF816)

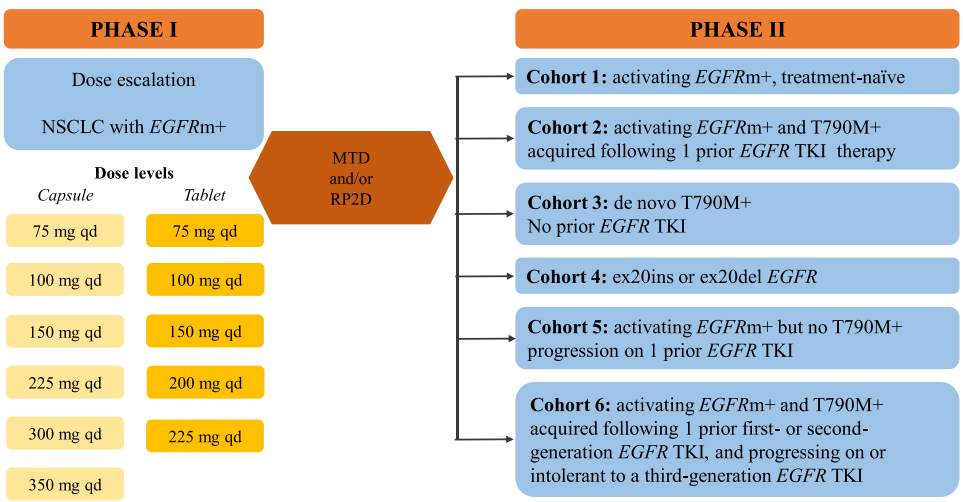
Nazartinib is a novel, irreversible mutant-selective *EGFR* inhibitor that specifically targets both *EGFR*-activating mutations (L858R, Del19) and the resistant T790M mutation, while sparing wild-type *EGFR* (50). NCT02108964 (EGF816X2101) is a phase I/II first-in-human study of nazartinib in patients with *EGFR*-mutated locally advanced or metastatic NSCLC. Updated results from the phase I dose-escalation part were recently presented. Patients were assigned to receive once-daily nazartinib with doses ranging from 75 to 350 mg. At the cutoff date of January 29, 2016, 152 patients had been treated across seven cohorts (51). Among them, 147 patients were evaluable for response. The confirmed ORR was 46.9% [95% CI, 38.7–55.3%] and the DCR was 87.1% [95% CI, 80.6–92.0%]. The estimated median PFS across all dose levels was 9.7 months [95% CI, 7.3–11.1]. Among 69 patients with confirmed responses at the cutoff date, the estimated median duration of response was 9.5 months [95% CI, 9.2–14.7]. The most common toxicities (all grades) were rash (54%), diarrhea (37%), and pruritus (34%). Interestingly, the rashes observed in the study tended to have a different pattern, location, and histology than those seen with other *EGFR* TKIs that target wild-type *EGFR*. Diarrhea was the most common grade 3/4 AE (16%), and of note, both incidence of diarrhea and rash tended to increase with increasing nazartinib doses (51). The phase II part, performed in six cohorts, is ongoing (**Figure 1**). In addition, the drug is being investigated in association with INC280, a specific *MET* inhibitor (based on the potential escape pathway for third-generation *EGFR* TKIs) in an ongoing phase Ib/II trial in patients with advanced *EGFR* mutant NSCLC (NCT02335944), and with nivolumab, an anti-PD-1 monoclonal antibody in a phase II trial in *EGFR* mutant/T790M+ NSCLC patients who have progressed on first-line *EGFR* TKI (NCT02323126).

Avitinib (AC0010)

Avitinib is another new-generation inhibitor of *EGFR* that, like the abovementioned agents, targets *EGFR*-activating mutations overcoming T790M-induced mutation with limited activity against wild-type *EGFR*. Clinical trials were initiated in China and the United States in parallel using avitinib as second-line therapy in NSCLC patients progressing on first-generation EGFR TKIs and who have acquired the gatekeeper T790M mutation. Two trials evaluating the safety, tolerability, pharmacokinetics, and antitumor activity of avitinib are ongoing; a phase I/II trial (NCT02274337) in advanced NSCLC patients progressing on prior therapy with an *EGFR* TKI agent and a phase I trial (NCT02330367) designed to determine the MTD and/or recommended phase 2 dose in previously treated mutant *EGFR* NSCLC patients with a T790M resistant mutation.

PF-06747775

PF-06747775 is another small molecule inhibitor of *EGFR* T790M with minimal activity against wild-type *EGFR*. It is being studied in a phase I/II clinical trial (NCT02349633) in advanced NSCLC patients with *EGFR* mutations (Del19 or L858R ± T790M). Results are not yet available.



*EGFR*m+, *EGFR* mutation-positive; MTD, maximum tolerated dose; qd, one daily; NSCLC, non-small cell lung cancer; RP2D, recommended phase 2 dose; TKI, tyrosine kinase inhibitor.

FIGURE 1 | Study design of nazartinib in *EGFR*m+ NSCLC patients (NCT0210896). *EGFR*m+, *EGFR* mutation-positive; MTD, maximum tolerated dose; qd, one daily; NSCLC, non-small-cell lung cancer; RP2D, recommended phase 2 dose.

HS-10296

HS-10296 is a small molecule inhibitor of *EGFR*-activating mutations and T790M-resistant mutation with limited activity against wild-type *EGFR*. An open-label, multicenter, phase I/II trial of HS-10296 with dose escalation, dose expansion, and extension cohorts in locally advanced or metastatic NSCLC patients who have progressed following prior therapy with an *EGFR* TKI agent is currently recruiting participants (NCT02981108).

THIRD-GENERATION *EGFR* TKIs IN CENTRAL NERVOUS SYSTEM (CNS) METASTASES

Incidence data of brain and leptomeningeal metastasis in *EGFR*-mutated NSCLC patients come from retrospective cohorts, reporting 24 and 9%, respectively. Gefitinib, erlotinib, and afatinib have impressive intracranial activity, with RR of 60–80% (52, 53). However, for patients with CNS progression on these first- and second-generation agents, further effective therapies are limited. Among the third-generation *EGFR* TKIs, osimertinib was the only inhibitor demonstrating sustained tumor regression in both preclinical and clinical models (54). Osimertinib has greater penetration of the mouse blood–brain barrier than gefitinib, rociletinib, or afatinib, and induced sustained tumor regression in an *EGFR* mutant PC9 mouse brain metastasis model at clinically relevant doses, while rociletinib did not achieve tumor regression (55). CNS activity was confirmed in the AURA study phase II extension cohort (NCT01802632) and the AURA2 phase II study (NCT02094261) (56). The phase I BLOOM trial (NCT02228369) was designed to assess for the first time the safety, tolerability, pharmacokinetics, and preliminary antitumor activity of two third-generation *EGFR* TKIs, orsimetinib and AZD3759. AZD3759 was the first *EGFR* TKI primarily designed to effectively across the blood–brain barrier to tackle CNS metastases in patients with *EGFR* mutant NSCLC (57, 58). The osimertinib cohort of the trial included 21 Asian patients with advanced or metastatic NSCLC harboring the L858R mutation ($n = 13$) or an exon 19 deletion ($n = 9$), and a confirmed diagnosis of leptomeningeal metastasis by positive cerebrospinal fluid cytology. At study entry, the T790M mutation was detected in cerebrospinal fluid in two patients and in plasma in six. Patients received osimertinib at 160 mg/day. All patients were evaluable for efficacy; seven (33%) had a confirmed radiologic response, nine (43%) had stable disease, and neurological function improvement was seen in five (24%) patients (59). Preliminary results from the AZD3759 cohort were recently presented (60). Twenty-nine patients with advanced *EGFR* mutant NSCLC and brain metastases, including leptomeningeal metastasis were treated in escalating dose cohorts of 50–500 mg, twice daily (BID). The pharmacokinetic analysis demonstrated excellent CNS penetration, with a 1:1 ratio with plasma. The tolerability profile of AZD3759 was consistent with *EGFR* TKI class effects and included grade 3/4 rash (7%), pruritus (7%), diarrhea (3%), and acne (3%). The MTD was 300 mg BID but study investigators recommended 200 mg BID for phase II dosing. AZD3759 demonstrated encouraging intracranial antitumor activity. Among 21 patients with measurable brain metastases, 11 demonstrated tumor shrinkage in the target brain lesion at AZD3759 doses of ≥50 mg BID. In this group, there were six partial responses (three confirmed, three unconfirmed). Among 22 patients with measurable extracranial lesions, eight experienced tumor shrinkage, with one unconfirmed partial response (60). Based on these promising findings, the BLOOM trial is continuing to enroll patients in the AZD3759 brain and leptomeningeal metastasis expansion cohorts.

MECHANISMS OF RESISTANCE TO THIRD-GENERATION *EGFR* TKIs

As is the case with first and second-generation *EGFR* TKIs, mutations mediating resistance to third-generation *EGFR* TKIs are emerging (61–65). Among them, while the C797S mutation in exon 20 of *EGFR* was the most common mechanism responsible for resistance to osimertinib (62), it occurs in less than 3% of patients treated with rociletinib (66). The C797S mutation was also reported in one case that led to resistance to olmutinib (65). Very recently, two novel tertiary *EGFR* mutations were described. The acquired L798I mutation was observed in *cis* with T790M in one patient following rociletinib therapy (66). Subsequently, another mutation in the same codon (L798Q) was reported in one patient at the time of progression under osimertinib (67).

The acquired resistance associated with the *EGFR* T790M mutation can occur by selection of preexisting *EGFR* T790M+ clones or *via* genetic evolution of initially *EGFR* T790M− drug-tolerant cells, suggesting that cancer cells that survive third-generation TKIs may serve as a key reservoir from which acquired resistance can emerge during treatment (68).

Additional *EGFR*-independent mechanisms of resistance have been reported. *NRAS* mutations, including a novel E63K mutation, and amplifications of wild-type *NRAS* or *KRAS* have been described as mechanisms of acquired resistance to osimertinib but also to gefitinib and afatinib (69). Amplifications in *HER2* and *MET* genes were also described as potential mechanisms of acquired resistance to osimertinib and rociletinib in *EGFR* T790M+ NSCLC patients (66, 70). Additionally, loss of T790M at the time of progression may be mediated by overgrowth of cells harboring *HER2* amplification, or *BRAF* V600E or *PIK3CA* mutations, as was recently detected in plasma of patients included in the phase I AURA trial (71).

Finally, small-cell lung cancer transformation was seen in two cases of rociletinib resistance and one osimertinib-resistant patient; the T790M was lost while the original *EGFR* mutation was maintained in the small cell transformed cancer in each case (72, 73).

OVERCOMING RESISTANCE TO THIRD-GENERATION *EGFR* TKIs

The favorable toxicity profiles of the third-generation *EGFR* TKIs make them particularly attractive candidates for combination therapy, and many trials are currently planned or ongoing (**Table 3**).

TABLE 3 | Ongoing and forthcoming third-generation *EGFR* TKIs-based combination trials.

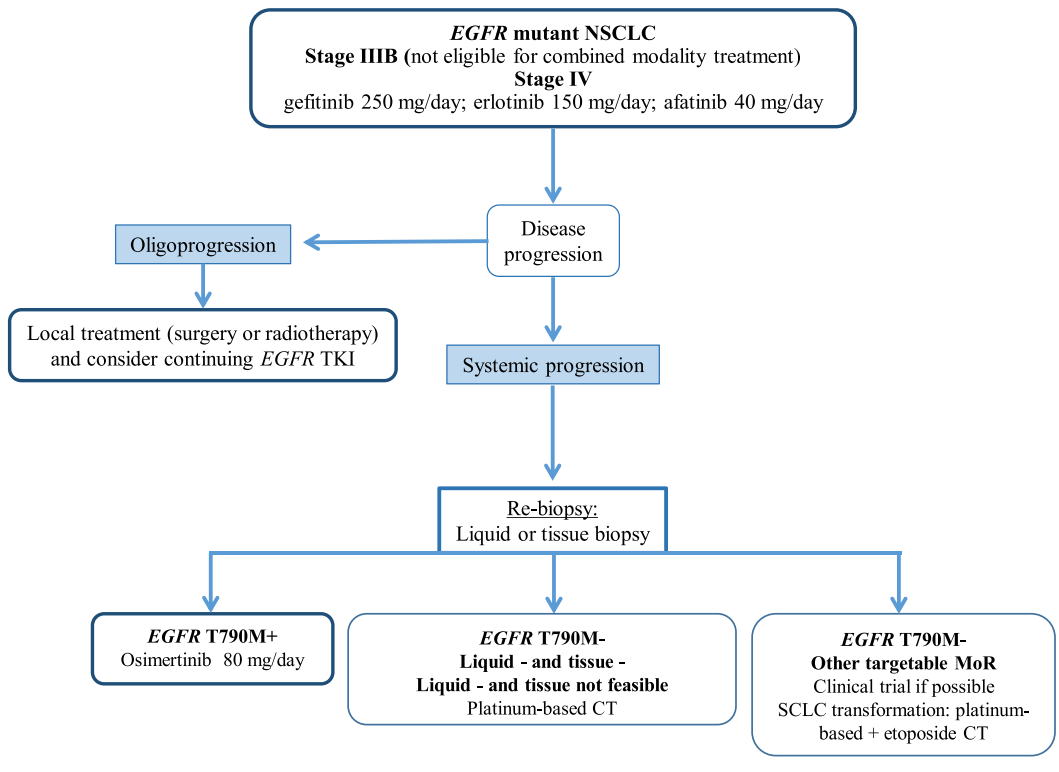
Third-generation *EGFR* TKI	Trial, NCT number	Drug combination	Mechanism of action	Population and setting	Primary endpoint	Status
Osimertinib	NCT02143466; TATTON Phase Ib	Durvalumab Savolitinib Selumetinib	Anti-PD-L1 antibody *MET* inhibitor *MEK* inhibitor	Advanced *EGFR*-mutant NSCLC progressing under *EGFR* TKI	Part A: safety and tolerability Part B: safety, tolerability and efficacy	On hold Recruiting Recruiting
Osimertinib	NCT02454933; CAURAL Phase III	Osimertinib monotherapy		*EGFR* mutant/T790M+ NSCLC progressing under *EGFR* TKI	PFS	On hold
		Durvalumab	Anti-PD-L1 antibody			
Osimertinib	NCT02496663; phase I	Necitumumab	Anti-*EGFR* antibody	Advanced *EGFR*-mutant NSCLC progressing under *EGFR* TKI	Safety and tolerability	Recruiting
Osimertinib	NCT02803203; phase I/II	Bevacizumab	Anti-*VEGF* antibody	Advanced *EGFR*-mutant NSCLC in the first-line setting	Phase I: MTD Phase II: PFS	Recruiting
Osimertinib	NCT02789345; phase I	Necitumumab Ramucirumab	Anti-*EGFR* antibody Anti-vascular endothelial growth factor receptor 2 antibody	*EGFR* mutant/T790M+ NSCLC progressing under first-line *EGFR* TKI	ORR	Forthcoming
		Necitumumab + ramucirumab				
Osimertinib	NCT02520778; phase Ib	Navitoclax	Bcl-2 family inhibitor	Advanced *EGFR*-mutant NSCLC progressing under *EGFR* TKI	Safety and tolerability	Recruiting
Osimertinib	NCT02503722; phase I/II	Sapanisertib	TOR1/2 inhibitor	Advanced *EGFR*-mutant NSCLC progressing under *EGFR* TKI	Safety and recommended phase II dose Safety and efficacy in T790M– population	Recruiting
Nazartinib	NCT02335944; phase Ib/II	INC280	*MET* inhibitor	*Ph. Ib/Ph. II Group 1*: advanced *EGFR*-mutant NSCLC progressing under G/E/A *Ph. II Group 2*: advanced NSCLC not been previously treated with any *EGFR* TKI and harbor *de novo* T790M mutation	Phase Ib: MTD or RP2D of nazartinib Phase II: ORR	Recruiting
Nazartinib	NCT02323126; phase II	Nivolumab	Anti-PD-1 antibody	*EGFR* mutant/T790M+ NSCLC progressing under first-line *EGFR* TKI	PFS	On hold

G/E/A, gefitinib/erlotinib/afatinib; MTD, maximum tolerated dose; NCT number, http://clinicaltrials.gov identification number; NSCLC, non-small-cell lung cancer; ORR, objective response rate; PFS, progression-free survival; RP2D, recommended phase 2 dose.

Preclinical *EGFR* L858R/T790M/C797S mutation cell models exhibited *in vitro* sensitivity to cetuximab, an antibody that blocks *EGFR* dimerization (74, 75), but this was not confirmed in *in vivo* analyses. However, the allosteric inhibitor EAI045 in combination with cetuximab exhibited mechanistic synergy and was effective in mouse models of lung cancer driven by *EGFR* L858R/T790M and by *EGFR* L858R/T790M/C797S (76). Interestingly, the allelic context in which C797S was acquired may predict responsiveness to subsequent TKI treatments. For example, if the C797S and T790M mutations are in *trans*, cells will be resistant to third-generation *EGFR* TKIs but are sensitive to a combination of first and third-generation TKIs, and when C797S develops in T790 wild-type cells, this results in resistance to third-generation TKIs, while sensitivity to first-generation TKIs is retained (61). These data are of great clinical value in sequencing for this mutation in patients with acquired resistance to osimertinib.

Navitoclax (ABT-263), a BCL-2 family inhibitor, enhances the apoptotic response of late-resistant *EGFR* T790M cells with decreased sensitivity to *EGFR* inhibition. The combination of navitoclax with the third-generation *EGFR* TKI WZ4002 (in preclinical development) induced more apoptosis compared to WZ4002 alone in both *in vivo* and *in vitro* analyses. This approach could be an effective strategy for treating *EGFR* T790M-positive cancers that have a decreased apoptotic response to *EGFR* inhibition (68). Additionally, the combination of WZ4002 with trametinib, another *MEK* inhibitor, prevents the development of acquired resistance in *EGFR*-mutant lung cancer models (77). A phase Ib trial is ongoing to evaluate the safety and tolerability of the osimertinib/navitoclax combination in patients with *EGFR*-mutant NSCLC following resistance to prior *EGFR* TKIs (NCT02520778).

In vitro, a combination of osimertinib with the *MEK* 1/2 inhibitor selumetinib prevented emergence of resistance in PC9 (Ex19del) cells and delayed resistance in NCI-H1975 (L858R/T790M) cells. *In vivo*, concomitant osimertinib with selumetinib caused regression of osimertinib-resistant tumors in an *EGFR*-mutant/T790M transgenic model (69). This association, among others, is being evaluated in the phase Ib TATTON trial (NCT02143466) designed to evaluate the safety, tolerability, and preliminary antitumor activity of osimertinib in combination with durvalumab (an anti-PD-L1 monoclonal antibody), savolitinib (*MET* inhibitor) or selumetinib in patients with advanced *EGFR*-mutant NSCLC who have progressed on an *EGFR* TKI. Preliminary results from the osimertinib/durvalumab arm were recently presented (78). The investigator-assessed ORR was 67% in nine patients with T790M+ tumors, compared to 21% in 14 T790M− NSCLC. Interstitial lung disease was reported in 38% (13/34) of patients, which is higher than would be expected with either drug alone, including five grade 3/4 events (78). Thus, recruitment in the osimertinib + durvalumab arm was stopped



CT, chemotherapy; MoR, mechanism of resistance; SCLC, small-cell lung cancer; TKI, tyrosine kinase inhibitor

FIGURE 2 | Potential treatment algorithm for advanced *EGFR*-mutated NSCLC patients. CT, chemotheraphy; *EGFR*, epidermal growth factor receptor; MoR, mechanism of resistance; NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; TKI, tyrosine kinase inhibitor.

but expansion cohorts of the *MET* and *MEK* inhibitor combinations are ongoing. In addition, the phase III CAURAL trial (NCT02454933) is being conducted in second-line metastatic *EGFR*-mutant T790M+ NSCLC patients testing osimertinib plus durvalumab vs. osimertinib monotherapy. This study was also stopped prematurely due to the pulmonary toxicity observed in the TATTON trial.

Dual *EGFR* blockage is being evaluated in a phase I trial (NCT02496663) combining osimertinib with the anti-*EGFR* monoclonal antibody necitumumab to assess safety and determine the optimal dose in patients with *EGFR*-mutant advanced NSCLC who have progressed on a previous *EGFR* TKI.

As was reported, the dual vascular endothelial growth factor receptor (VEGFR) and *EGFR* blockade inhibit tumor growth in *EGFR* TKI resistance xenograft models (79). This hypothesis was confirmed in two phase II clinical trials in *EGFR*-mutant NSCLC treatment-naïve patients, the randomized Japanese (JO25567) trial comparing erlotinib plus bevacizumab vs. erlotinib alone, and the single-arm Caucasian (BELIEF) trial. Median PFS was similar and encouraging in both trials supporting the combination in the first-line setting (80, 81). Following this strategy, a phase I trial was designed to evaluate the safety of two osimertinib-based combination strategies, with necitumumab or ramucirumab (an anti-*VEGFR2* monoclonal antibody) in patients with advanced *EGFR* T790M+ NSCLC after progression on first-line *EGFR* TKI therapy (NCT02789345). Finally, the osimertinib/bevacizumab combination will be evaluated in another phase I/II 3 + 3 dose-escalation study (NCT02803203) to test the safety of combining these drugs.

For patients whose tumors undergo small-cell lung cancer transformation, platinum-based plus etoposide chemotherapy is recommended.

CONCLUSION

Over the last decade, we have seen considerable advances in the treatment of patients with *EGFR* mutant NSCLC. Three *EGFR* TKIs are currently FDA and EMA approved for first-line treatment of patients with sensitizing *EGFR* mutations in metastatic NSCLC. Despite this progress, the development of acquired resistance is an unfortunate reality and remains an important challenge in the clinical setting. No second-generation TKIs have been successfully developed, and to date, osimertinib is the only third-generation *EGFR* mutant/T790M+ TKI approved by the FDA and EMA for patients with advanced T790M NSCLC who progress on a first-line *EGFR* TKI. Osimertinib has demonstrated strong efficacy and safety data in phase I and II studies, mainly in a second- or post-second-line setting but also as first-line treatment, placing it as a very attractive drug in this scenario. The clinical development of osimertinib represents one of the fastest cancer drug development programs, taking just 2 years, 8 months, and 1 week from the first patient dosed to the first approved indication. Until recently, patients with advanced NSCLC with *EGFR*-activating mutations who progress on a first-line *EGFR* TKI have traditionally been treated with a platinum-doublet chemotherapy. These combinations show ORRs of approximately 30%, marginally higher than those observed in the T790M− populations, but significantly lower than those reported in T790M+ cohorts across osimertinib phase I–III trial development. In addition, given the encouraging CNS efficacy, osimertinib is also attractive as frontline treatment for patients with brain and/or leptomeningeal metastases. The phase III FLAURA (NCT02296125) trial will hopefully soon answer the issue of where osimertinib should be positioned. Among the other new-generation *EGFR* TKIs and considering that the development of rociletinib and olmutinib as monotherapies has been stopped, ASP8273 is now the most advanced agent in the clinic.

Figure 2 illustrates potential post-progression treatment algorithms for *EGFR*-mutated advanced NSCLC patients. The heterogeneity of resistant cancers seems to play an important role in both efficacy and resistance to these novel T790M-specific agents, and combination strategies could be effective in delaying and/or preventing resistance. Finally, in an era of personalized medicine, the analysis of both tumor tissue and ctDNA should be a priority to improve our knowledge to the benefit of our patients.

AUTHOR CONTRIBUTIONS

IS prepared the manuscript. DP supervised and accepted the final version.

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MINI REVIEW
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Liquid Biopsy in Non-Small Cell Lung Cancer

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Liquid biopsy analyses are already incorporated in the routine clinical practice in many hospitals and oncology departments worldwide, improving the selection of treatments and monitoring of lung cancer patients. Although they have not yet reached its full potential, liquid biopsy-based tests will soon be as widespread as "standard" biopsies and imaging techniques, offering invaluable diagnostic, prognostic, and predictive information. This review summarizes the techniques available for the isolation and analysis of circulating free DNA and RNA, exosomes, tumor-educated platelets, and circulating tumor cells from the blood of cancer patients, presents the methodological challenges associated with each of these materials, and discusses the clinical applications of liquid biopsy testing in lung cancer.

Keywords: ctDNA, ctRNA, CTCs, exosomes, tumor-educated platelets, mutations, gene fusions, lung cancer

INTRODUCTION

The so-called "liquid biopsy" is quickly moving from research into clinical practice in lung cancer, as well as in other human malignancies. Although its full potential has not yet been reached, the "liquid biopsy" is no longer a promise but a reality that is allowing a better treatment selection and monitoring of lung cancer patients in hospitals and oncology departments worldwide. We can already foresee a day when "liquid biopsy"-based tests will be as widespread and useful as "standard" biopsies and imaging techniques, offering invaluable diagnostic, prognostic, predictive, and monitoring information. In this mini review, we will summarize the state of the art in this exciting area, placing a particular emphasis on the clinical utility of the "liquid biopsy" and the variety of applications, methodologies, and results that can be derived from it.

"Liquid biopsies" are usually defined as tests done in blood samples or other body fluids. In the case of cancer patients, the objective of those tests is to detect materials originated in the tumor. Although the term "liquid biopsy" is universally used, many pathologists argue that it is incorrect. The so-called "liquid biopsies," they claim, are not true biopsies. A "true" biopsy is usually performed by a surgeon or a pneumologist and involves the extraction of sample cells or tissues that are subsequently examined by a pathologist under a microscope, commonly after some kind of fixation and staining. Paraffin embedding is also widespread. In contrast, "liquid biopsies" are not obtained by surgeons; involve the extraction of blood or other fluids and not of solid tissues, pathologists only

occasionally intervene and fixation, embedding, or staining are equally infrequent. In addition to the "biopsy" half, the "liquid" half in the term "liquid biopsy" can also be misleading. The materials originated in the tumor that are to be detected in such "biopsies" are never liquid. Some of them are cells or fragments of cells, such as circulating tumor cells (CTCs), exosomes, or tumor-educated platelets (TEPs); others are nucleic acids dissolved in the blood, such as circulating tumor DNA or RNA (ctDNA, ctRNA). Each of these materials offers unique opportunities to test different biomarkers and analyze particular characteristics of the tumors (**Table 1**).

The differences between a "real" and a "liquid" biopsy—or "liquid sample," as the pathologists would probably prefer to call them—explain the advantages of the latter. "Liquid" biopsies will never replace real biopsies, which are irreplaceable sources of information that cannot be obtained by any other means, such as tumor type and histology. However, they offer all sorts of additional data that cannot be obtained in any other way. In patients who cannot be biopsied, or where biopsies do not have enough tissue, "liquid biopsy" is the only alternative to perform genetic testing for targeted therapy. Also, in patients with advanced disease, it is not feasible to obtain biopsies of every metastasic site. But blood reaches both the primary tumor and the metastases, and materials coming from all can be found in a "liquid biopsy." Finally, unlike "real" biopsies, blood can be repeatedly obtained without the risk of comorbidities and used to monitor the course of the disease, including early detection of response and relapse or emergence of resistance to a particular therapy.

CIRCULATING TUMOR DNA

Circulating free DNA (cfDNA) can be found dissolved in plasma and serum, at variable amounts. In the case of cancer patients, a fraction of the cfDNA is tumor derived, and ctDNA represents from less than 0.1% to more than 10% of the total cfDNA. This percentage has been shown to depend on stage, tumor burden, vascularization of the tumor, biological features like apoptotic rate and metastatic potential of the cancer cells, and factors affecting the blood volume of the patient (1, 2). In addition, variations on the relative abundance of ctDNA correlate with response to therapy (3–5). ctDNA is released by passive mechanisms, such as lysis of apoptotic and necrotic cells or digestion of tumor cells by macrophages, and also by active mechanisms. In this respect, cfDNA shows and enrichment in 150–180 bp fragments typical of the nucleosomal pattern of DNA fragmentation during apoptosis (6–9). The ctDNA carries the same somatic alterations as the tumor itself and can be used to detect clinically relevant mutations such as those in the epidermal growth factor (*EGFR*) or *KRAS* genes. This is particularly useful when no biopsy is available for genetic analyses and, in this setting, the European Medicine Agency recommends *EGFR* testing in liquid biopsies to select patients for tyrosine kinase inhibitor (TKI) therapy (10). However, many standard techniques for mutation detection are not useful for ctDNA analyses due to an insufficient sensitivity. Since ctDNA often represents a small percentage of the total cfDNA, somatic mutations coming from the tumor can be present at allele fractions as low as 0.01%. Highly sensitive methodologies, or variations of preexisting methodologies, have been developed in order to detect low abundance mutations in cfDNA (6, 11).

TABLE 1 | Biological materials that can be isolated from liquid biopsies and their applications in lung cancer.

Material	Applications
Circulating tumor DNA (ctDNA)	Somatic mutations[a] DNA methylation changes Copy number alterations
ctRNA	Gene fusion Splicing variants
Tumor-educated platelets	Gene fusions Splicing variants Cancer diagnosis RNA profiling
Exosomes	Gene fusions Splicing variants miRNA analyses RNA and protein-based molecular profiling
Circulating-tumor cells (CTCs)	Monitoring (total CTC counts)[b] Culture of CTCs DNA, RNA, and protein-based molecular profiling Somatic mutations Gene fusions

Applications used in routine clinical practice in ([a]) NSCLC or ([b]) metastatic breast, prostate, and colon cancer patients. Unmarked, research use.

Modified real-time PCR techniques have been widely used to identify genetic alterations in the cfDNA of cancer patients. They include amplification-refractory mutation system [ARMS (12),], Scorpion-ARMS (13), and peptide nucleic acid (PNA) or locked nucleic acid (LNA) mutant-enriched PCR (14–17). The diagnostic sensitivity of these techniques, when compared to tumor tissue, ranges from 43 to more than 90%, while the specificity is usually close to 100%; and the two commercially available methods to determine *EGFR* mutations in the cfDNA of cancer patients (Therascreen Plasma from Qiagen and COBAS Blood from Roche Diagnostics) are based on them. In our group, we have developed a quantitative PCR technique in the presence of PNA to detect *EGFR*, *KRAS*, and BRAF mutations in the cfDNA of advanced lung, colon, and cancer patients that achieves 75–80% sensitivity with 100% specificity (18, 19). Digital PCR, droplet digital PCR, and beads, emulsion, amplification, and magnetics (BEAMing) system constitute further refinements of the PCR-based techniques and have also been used to determine mutations in cfDNA (14, 20–26) (**Table 2**).

Most modified PCR techniques are easy, comparatively unexpensive, and have a quick turnaround time (19), but have the disadvantage that can only detect mutations in a limited number of loci, usually within a single gene. Next-generation sequencing methodologies can overcome these limitations but, while tissue-based NGS genotyping is already well established, the application of NGS technologies to liquid biopsies is challenging and an ultra-deep sequencing approach is commonly used in order to improve sensitivity. In this approach, the gene panels are limited so that each read is sequenced thousands of times (39, 50, 51). However, this requirement of a high sensitivity may easily lead to false-positive results and requires a careful

TABLE 2 | Summary of reports on detection of genetic alterations in liquid biopsy materials from advanced NSCLC patients.

Technique	*n*	Type of sample	Alteration detected	Sensitivity (%)	Reference
ARMS	86	Circulating free DNA (cfDNA) (plasma)	Epidermal growth factor (EGFR)-sensitizing mutations	68	(27)
SARMS	42	cfDNA (serum)	EGFR-sensitizing mutations	75	(13)
SARMS	11	cfDNA (serum)	EGFR-sensitizing mutations	50	(13)
SARMS	21	cfDNA (plasma)	EGFR-sensitizing mutations	39	(28)
SARMS-based DxS EGFR mutation test kit	86	cfDNA (serum)	EGFR-sensitizing mutations	43	(15)
SARMS-based EGFR mutation detection kit	652	cfDNA (plasma)	EGFR-sensitizing mutations	66	(12)
Mass spectrometry-based genotyping	31	cfDNA (plasma)	EGFR-sensitizing mutations	39	(29)
Mutant-enriched PCR			EGFR-sensitizing mutations	33	
Mutant-enriched PCR	18	cfDNA (plasma)	EGFR-sensitizing mutations	100	(30)
Mutant-enriched PCR	111	cfDNA (plasma)	EGFR-sensitizing mutations	56	(31)
EGFR array, PNA-PCR	37	cfDNA (plasma)	EGFR-sensitizing mutations	100	(32)
Digital PCR	35	cfDNA (plasma)	EGFR-sensitizing mutations	92	(22)
Droplet digital PCR	46	cfDNA (plasma)	EGFR-sensitizing mutations	67	(33)
Droplet digital PCR	50	cfDNA (plasma)	EGFR mutations	76	(34)
Droplet digital PCR	25	cfDNA (plasma)	EGFR mutations	81	(35)
Cobas® EGFR blood test	199	cfDNA (plasma)	EGFR-sensitizing mutations	61	(20)
Cobas® EGFR blood test	38	cfDNA (plasma)	p.T790M (EGFR)	73	(36)
Cobas® EGFR blood test	238	cfDNA (plasma)	EGFR mutations	76	(14)
DHPLC	230	cfDNA (plasma)	EGFR-sensitizing mutations	82	(37)
DHPLC	822	cfDNA (plasma)	EGFR-sensitizing mutations	77	(36)
BEAMing	44	cfDNA (plasma)	EGFR-sensitizing mutations	73	(24)
BEAMing[b]	915	cfDNA (plasma)	EGFR, KRAS, BRAF, PIK3CA mutations	83–99[c]	(23)
BEAMing	153	cfDNA (plasma)	EGFR-sensitizing mutations p.T790M	82 73	(26)
Cobas® EGFR blood test			EGFR-sensitizing mutations p.T790M	73 64	
PNA-Q-PCR	97	cfDNA (serum/plasma)	EGFR sensitizing mutations	78	(18)
PNA/LNA-Q-PCR	35	cfDNA (serum)	EGFR, KRAS mutations	73	(17)
NGS (CAPP-Seq)	142	cfDNA (plasma)	EGFR mutations	81	(38)
NGS (Ion Torrent)[a]	107	cfDNA (plasma)	EGFR, HER2, KRAS, BRAF, PIK3CA mutations	58	(39)
NGS (deep sequencing)	288	cfDNA (plasma)	EGFR mutations	73	(40)
Melting curve PCR	8	Circulating tumor cells (CTCs)	EGFR mutations	100	(41)
NGS	37	CTCs	EGFR mutations	84	(42)
Mutant-enriched PCR	21	CTCs	p.T790M (EGFR)	57[c]	(43)
	25	cfDNA (plasma)		60[c]	
ISET + fluorescence *in situ* hybridization (FISH)	5	CTCs	ALK fusions	100	(44)
ISET + filter-adapted FISH	32	CTCs	ALK fusions	100	(45)
ISET + filter-adapted FISH	4	CTCs	ROS1 fusions	100	(46)
Antibody-independent CTC isolation + FISH	31	CTCs	ALK fusions	≥90[c]	(47)
NanoVelcro System + FISH	41	CTCs	ALK fusions	100	(48)
Retrotranscription PCR	77	cfRNA (plasma)	ALK fusions	22	(49)
		Platelets	ALK fusions	65	

[a]*Samples in the study include stages I–IIIA.*

[b]*Samples in the study include tumors other than NSCLC.*

[c]*Concordance value.*

validation of the whole testing process. Examples of NGS protocols specifically developed for ctDNA analysis include TAm-Seq (tagged-amplicon deep sequencing), which combines site-specific primers with universal tails (52, 53); Safe-SeqS (Safe-Sequencing System) (54), and CAPP-seq (capture based sequencing), which relies on hybridization-based capture of target regions followed by amplification (38, 55) (**Table 2**).

The detection of mutations in cfDNA by modified PCR or NGS techniques is not only useful in lung cancer patients at presentation. It has also been successfully employed for patient monitoring, including early evaluation of response and relapse, which are associated with changes in the *EGFR* or *KRAS* mutational burden in cfDNA; and for early detection of acquired resistance to EGFR TKIs, associated in many patients with the emergence of the p.T790M mutation in blood (26, 56). In this respect, p.T790M testing in cfDNA has been recently recommended in patients eligible for osimertinib treatment, in order to avoid unnecessary rebiopsies (33, 36, 56).

CIRCULATING TUMOR RNA

Similar to ctDNA, RNA derived from tumor cells (ctRNA) is present in the plasma of cancer patients and can be used for detection of the clinically relevant *ALK*, *ROS1*, and *RET* fusion genes and METΔ14 splicing variant. However, genetic analyses in cfRNA present specific challenges and have not been widely used. Unlike cfDNA, cfRNA degrades very quickly and needs to be purified rapidly after blood extraction. The alternative is adding a preservative such as Trizol and freezing the sample at −80°C, but this procedure is not easily accessible to many clinical sites. Despite these limitations, our group has a 5-year experience in detection of *EML4-ALK* fusion transcripts in plasma cfRNA by retrotranscription PCR (RT-PCR) (49) and, using improved processing and purification methods, we have demonstrated that the sensitivity of the technique can be significantly improved.

TUMOR-EDUCATED PLATELETS

Platelets have been recently demonstrated to sequester tumor RNA by a microvesicle dependent mechanism, and the so-called TEPs (57, 58) can be used as a source of tumor RNA for genetic analysis. Platelets can be isolated from blood by simple centrifugation steps, and its RNA content easily purified and used for the detection of gene fusions and splicing variants. Using a RT-PCR approach, our group has detected *EML4-ALK* fusion transcripts in TEP RNA from advanced lung cancer patients with 65% sensitivity and 100% specificity (49). In addition, we have demonstrated that the disappearance of fusion transcripts in platelets correlates with response to crizotinib treatment. Regarding splicing variants, the clinical relevance of METΔ14 in lung cancer was only described in 2015 (59–61), and there are no reports in the literature about detection of METΔ14 transcripts in liquid biopsy. However, we have recently detected the alteration in the TEP RNA of a NSCLC patient positive in tumor tissue, who attained a partial response to crizotinib (unpublished data).

Platelet RNA can also be analyzed by multiplexing techniques, and a recent report has demonstrated the diagnostic potential of this approach. Using mRNA sequencing and surrogate TEP RNA profiles of 283 samples, 228 cancer patients of six different origins were discriminated from 55 healthy individuals with 96% accuracy. Tumors with specific genetic alterations, such as *KRAS* or *EGFR* mutations, were also distinguished and the location of the primary tumor identified with 71% accuracy (58).

EXOSOMES

Exosomes are small vesicles present in blood and other body fluids (62–64). With a 30–100 nm diameter, they are constitutively released through exocytosis by many cells, including tumor cells, in physiological and pathological conditions. Exosomes contain lipids, proteins, mRNA, several types of non-coding RNAs, and double-stranded DNA; and their composition partly reflects that of the parental cells (65). In addition, being generated by the cell secretion pathway, all exosomes carry some common proteins independent of their origin, such as ALIX, CD63, or TSG-101 (66). Exosomes are generally isolated by sucrose gradient ultracentrifugation or immune-bead isolation techniques (such as magnetic activated cell sorting), and there are commercial kits available. Once isolated, exosomes are characterized by transmission electron microscopy, Western blot, FACS, or other methodologies (67).

Although being more difficult to purify than other materials, the lipid bilayer of exosomes makes their cargo particularly stable, theoretically allowing the identification of the tumor of origin, genetic alterations or resistances to treatments. In this respect, EML4-*ALK* fusion transcripts have been recently identified in the exosomal RNA of NSCLC patients (68). In addition, some studies indicate that micro RNA (miRNA) analysis of exosomes might be useful for the diagnosis of lung adenocarcinoma (69–71) and that particular miRNAs can offer prognostic information in advanced NSCLC. For example, downregulation of miRNA-373 and miRNA-512 has been associated with a poor prognosis (72), miR-208a and miR-1246 with resistance to radiotherapy (73, 74), and miR-221-3p and 222-3p with good response to osimertinib in *EGFR* mutated patients (75).

CIRCULATING TUMOR CELLS

Together with ctDNA, CTCs are the most widely investigated material in liquid biopsies of cancer patients. First observed in 1869 (76), they are cancer cells detached from the solid tumor mass that circulate in the blood and lymphatic system (77) as single cells or as aggregates, the so-called circulating tumor microemboli (78–80). In advanced NSCLC patients, CTCs are relatively rare, 1–10 per mL against a background of 10^6–10^7 peripheral blood mononuclear cells. This low abundance poses formidable challenges for the development of robust and sensitive enrichment protocols (81).

Some CTC capture methods are label dependent, based on specific epithelial cell surface markers, such as epithelial cell adhesion molecule (EpCAM) for positive selection or CD45 for negative depletion. One of such techniques, the CellSearch® system (Veridex), has been approved by the FDA for monitoring some type of tumors (82–84), but not lung cancer. In advanced NSCLC, CellSearch® has shown a limited detection efficiency, with CTCs detectable in only 20–40% of patients (85–87). Label-dependent methods do not select CTCs that have undergone epithelial to mesenchymal transition (88) or those with stem cell characteristics that have not started epithelial differentiation. Label-independent techniques, which are based on physical characteristics such as size, can overcome this limitation. Isolation by Size of Epithelial Tumor cells (ISET®, Rarecells), based on filtration and cytological characterization, has shown an increased sensitivity in NSCLC (89–92) with an 80% detection rate of CTCs in blood from 40 stage IIIA–IV patients compared with 23% using CellSearch® (85). Another technology based on size, ScreenCell®, allows not only the detection but also the isolation of CTCs, which can be subjected to further morphological studies and used for genetic testing. Isolated CTCs can be cultured or injected into mice to generate xenografts (93–96) and CTC-derived tumor cells can thus be obtained in enough numbers for molecular and pharmacological profiling.

CTC counts have been extensively researched as a prognostic factor in NSCLC (97). In early-stage patients, the decrease or disappearance of CTCs after surgery has been reported to correlate with better clinical outcomes (98, 99), while its persistence was associated with shorter progression-free survival (PFS) (100). Regarding advanced NSCLC, some studies have reported that a higher CTC count at presentation correlates with advanced stage and shorter PFS and overall survival (85, 101). Also, the decrease or disappearance of CTCs after chemotherapy or targeted therapy has been consistently associated with better outcomes (102–104).

Finally, CTCs have also been investigated as a tool to identify clinically relevant genetic alterations in NSCLC (**Table 2**). Using NGS and modified PCR techniques, *EGFR*-sensitizing mutations and the p.T790M resistance mutation have been detected in the CTCs of *EGFR*-positive patients at presentation and after progression to TKI treatments, respectively (28, 41, 42, 105). The sensitivities reported range from 47 to 100%. However, unlike cfDNA, CTCs are not used for *EGFR* testing in the routine clinical practice. EML*4*-*ALK* fusions have been identified by fluorescence *in situ* hybridization (FISH) and immunochemistry in CTCs isolated using ISET (44) or other enrichment methodologies (47, 48). In some cases, filter-adapted FISH was employed, a methodology that has also been demonstrated to successfully identify ROS1 rearrangements in CTCs isolated by ISET (46).

AUTHOR CONTRIBUTIONS

All the authors contributed to the writing and critical revision of the review.

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REVIEW
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Focus on Nivolumab in NSCLC

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Immunotherapy is changing the treatment of non-small cell lung cancer (NSCLC). The PD-1 inhibitor nivolumab has demonstrated meaningful results in terms of efficacy with a good safety profile. The novel approach to treating NSCLC using immunotherapy still has unsolved questions and challenging issues. The main doubts regarding the optimal selection of the patient are the role of this drug in first line of treatment, the individualization of the correct methodology of radiologic assessment and efficacy analysis, the best management of immune-mediated adverse events, and how to overcome the immunoresistance. The aim of this review is to analyze literature data on nivolumab in lung cancer with a focus on critical aspects related to the drug in terms of safety, the use in clinical practice, and possible placement in the treatment algorithm.

Keywords: nivolumab, immunotherapy, NSCLC, PD-1, PDL1, checkpoint inhibitors

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RATIONALE FOR IMMUNE CHECKPOINT INHIBITORS

Several clinical observations foresaw the promising results arising from the employment of immunotherapy in lung cancer. Indeed, the lungs are involved in many autoimmune disorders. In addition to hyperplasia of fibroblasts, diminished collagen breakdown and production of autoantibodies, the pathophysiology of pulmonary disease includes activation of T cells, B cells, and alveolar macrophages. Activated T cells produce cytokines, such as interleukin-4 and interleukin-10, which enhance fibroblasts proliferation. Furthermore, activated T cells produce an altered form of interferon gamma (IFNγ) with a reduced skill to inhibit fibroblasts proliferation (1). Moreover, spontaneous tumor regressions, not only in cutaneous melanoma (2) but also in lung cancer (3), have been described and confirm the involvement of immune system in cancer control.

At the beginning of the twentieth century, Paul Ehrlich first proposed the idea that transformed cells can elicit immune system to repress them (4). The discovery of rejection of transplanted tumors in mice and the existence of tumor-associated antigens (5) led then Burnet to propose the hypothesis of cancer immune surveillance (6) with the assumption that "tumour cells provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence" (6). However, immune surveillance is not sufficient to explain the occurrence and growth of cancer in immunocompetent individuals. Indeed, tumors acquire ability to resist to host's immune system. The term "cancer immunoediting" has been proposed to explain this complex interaction between cancer and host and includes three phases: elimination, equilibrium, and escape. During elimination phase, tumor growth induces the release of inflammatory signals that activate cells of the innate immune system. These are natural killer (NK), NK T cells, γδ T cells, macrophages, and dendritic cells (DCs). They produce IFNγ, which has antiproliferative and apoptotic effect, and induce chemokines such as CXCL10, CXCL9, and CXCL11. These chemokines block angiogenesis and recruit more NK and macrophages that promote the maturation of DCs. DCs capture necrotic tumor cells, migrate to lymph nodes, and present tumor antigens (TAs) to naïve $CD4^+$ T cells leading to their differentiation in effector $CD4^+$ T cells, development of TA-specific $CD8^+$ T cells, and their expansion. Finally,

TA-specific T cells can home to tumor site and eliminate tumor cells. Some tumor cells that withstand the elimination phase enter the equilibrium process. During this phase, activated T cells and IFNγ manage to limit tumor growth without removing it. Nevertheless, tumor cells with reduced immunogenicity for low levels of TAs survive and become resistant to immune system. They enter the escape phase and expand in an uncontrolled way (7). To become effector T cells, naïve T cells must recognize their specific TAs and interact with DCs through major histocompatibility complex. This interaction involves both costimulatory and coinhibitory signals. In normal tissues, there is a balance between these signals. By contrast, inhibitory receptors and ligands are overexpressed on tumor cells and in tumor microenvironment. For example, high proportions of $CD4^+CD25^+$ T cells are present in the tumor-infiltrating lymphocytes (TILs) of patients with non-small cell lung cancer (NSCLC) (8). These T cells show high expression of CTLA-4 on their surface and inhibit the activation of T cells (9).

Immune checkpoints, such as CTLA4 and PD-1, are crucial to maintain the balance between costimulatory and inhibitory signals limiting excessive immune response against self-antigens. Thus, they are potential targets for cancer therapies.

CTLA4 is expressed on $CD8^+$ T cells, $CD4^+$ T cells, and on regulatory T cells (T_{reg}) and is involved in early stages of T cell activation. Its ligands are CD80 (B7.1) and CD86 (B7.2) expressed on antigen-presenting cells (APCs) like DCs (10). CD28 is a costimulatory receptor also expressed on T cells, which binds to CD80 and CD86 with consequent activation of T cells. CTLA4 interacts with CD80 and CD86 with higher affinity than CD28 does and inhibits $CD4^+$ T cell activation (11).

Even though CTLA4 is expressed by activated $CD8^+$ effector T cells, the major physiological role of CTLA4 seems to be through distinct effects on the two major subsets of $CD4^+$ T cells: downmodulation of helper T cell activity and enhancement of T_{reg} activity. The latter is crucial for the maintenance of self-tolerance (12).

PD-1, as CTLA4, is expressed on T cells, but contrary to CTLA4, it is involved in the late phases of immune reactions and mostly within the tumor microenvironment. Its ligands are PD-L1 (B7-H1) and PD-L2 (B7-DC) that are expressed on APCs and tumor cells. The interaction of PD-1 with its ligands results in reduced effector T cell proliferation, exhaustion of T cell activity, and enhancement of T_{reg} proliferation (13). Tumors are able to escape immune control because of upregulation of PD-1 on their surface. Indeed, PD-L1 is expressed in about 50% of NSCLC, mostly in squamous subtypes at advanced stage, and seems to correlate with poor prognosis (14, 15).

Two mechanisms of PD-1 ligands upregulation are present, known as innate immune resistance and adaptive immune resistance. The first refers to the constitutive expression of PD-L1 through involvement of oncogenic signaling pathways, such as AKT and STAT3, as in ALK-positive lung cancer (16, 17). In adaptive immune resistance, PD-1 ligands are overexpressed on tumor cells in response to cytokines, in particular IFNγ (18). The adaptive immune resistance is probably involved in most NSCLC without an oncogenic driver. Indeed, higher neoantigen burden seems associated with clinical benefit of PD-1 blockade (19).

Due to strong rationale and promising preclinical data, monoclonal antibodies anti-CTLA4 and anti-PD-1/PD-L1 have been extensively studied in advanced NSCLC. The therapeutic interference of immune synapse was a strategy adopted in preclinical model from 2010, and nivolumab was the "first in class" MoAb to be employed in clinical trials in advanced NSCLC immediately the unripe experience of Ab anti-CTLA4.

NIVOLUMAB DEVELOPMENT IN CLINICAL PRACTICE: STATE OF THE ART

Nivolumab was evaluated in the Phase Ib dose-escalation trial Checkmate 003 (20) (**Table 1**) in 129 heavily pretreated NSCLC patients. It was administered at 1, 3, and 10 mg/kg i.v. every 2 weeks for up to 96 weeks. Median OS for 3 mg/kg cohort was longer than mOS for 1 and 10 mg/kg (14.9 vs. 9.2 months). Median progression-free survival (mPFS) was 2.3 months, median duration of response was 17.0 months, and the overall response rate (ORR) was 17%, similar for squamous and non-squamous NSCLC. Eighteen patients discontinued the study without progression and 50% of these continued to respond 9 months after the last dose. The dose of 3 mg/kg every 2 weeks of nivolumab was determined as the dose to be employed in further trials.

CheckMate 063 (21) (**Table 1**), a Phase II, single-arm trial, evaluated nivolumab activity in 117 pretreated advanced squamous NSCLC patients. ORR was the primary endpoint. About 14.6% (17/117) of patients obtained a response, 26% (30/117) had stable disease (SD). Response was achieved in a median time of 3.3 months, and the majority of responses were ongoing at the time of the report. Patients with SD had a duration of response of 6 months. Nivolumab demonstrated activity irrespective of PD-L1 expression, using a cutoff of 5%. PD-L1 was assessed in 76 patients, 33% (25/76) had PD-L1 expression and among them 6 patients had a partial response, whereas 7 patients of 51 with PD-L1-negative obtained a response.

After these promising results, nivolumab was compared with chemotherapy in two randomized Phase III trials in second line in advanced squamous and non-squamous NSCLC.

CheckMate 017 (22) (**Table 1**), a randomized open-label Phase III trial, employed nivolumab or docetaxel in advanced squamous (SCC) NSCLC after progression to first-line chemotherapy. OS was the primary endpoint, and it was significantly longer in the nivolumab arm compared to docetaxel (9.3 vs. 6.0 months). Nivolumab decreased the risk of death of 41% (hazard ratio 0.59; 95% CI, 0.44–0.79; $P < 0.001$). In the experimental arm, ORR (20 vs. 9%) and PFS (3.5 vs. 2.8 months; hazard ratio for death or disease progression, 0.62; 95% CI 0.47–0.81; $P < 0.001$) were also increased.

There was no correlation between PD-L1 expression and nivolumab activity (PD-L1 analysis was performed retrospectively).

Nivolumab was also compared to docetaxel in the CheckMate 057 (23) (**Table 1**), a randomized Phase III trial in non-squamous advanced NSCLC after platinum-based doublet chemotherapy (PT-DC). OS was the primary endpoint, and as previously seen in SCC, it was improved for nivolumab-treated

TABLE 1 | Major clinical trials of nivolumab in lung cancer.

Trial	No. patients	Phase	Histology	Setting	Treatment	Outcome	Safety	Notes
CheckMate 003 (20)	129	Phase I	Non-small cell lung cancer (NSCLC)	Pretreated	Nivolumab dose escalation	OS 3 mg/kg 14.9 months vs. mOS 1 and 10 mg/kg 9.2 months	3 treatment-related deaths (associated with pneumonitis)	
CheckMate 063 (21)	117	Phase II	Squamous NSCLC	Pretreated	Nivolumab 3 mg/kg	OS 8.2 months 1-year OS 41%	17% of the pts reported Grade 3 or 4 treatment-related AEs. Two treatment-associated deaths (pneumonia and ischemic stroke)	PD-L1 cutoff of 5%; nivolumab demonstrated activity irrespective of PD-L1 expression
CheckMate 017 (22)	272	Phase III	Squamous NSCLC	Pretreated	Nivolumab vs. docetaxel	OS 9.3 vs. 6.0 months	Grade 3 or 4 treatment related were reported in 7% of the pts in the nivolumab arm vs. 55% in the docetaxel arm	Nivolumab demonstrated activity irrespective of PD-L1 expression
CheckMate 057 (23)	582	Phase III	Non-squamous NSCLC	Pretreated	Nivolumab vs. docetaxel	OS 12.2 vs. 9.4 months	Grade 3 or 4 treatment-related AEs were reported in 10% of the pts in the nivolumab arm vs. 54% in the docetaxel arm	PD-L1 cutoff ≥1, ≥5, and ≥10%; relevant predictive association between OS, median progression-free survival, overall response rate (ORR), and PD-L1 expression
CheckMate 012 (24)	52	Phase I	NSCLC	I line	Nivolumab 3 mg/kg	OS 19.4 months 12-month OS 73%	19% of pts reported Grades 3–4 treatment-related AEs; 12% discontinued because of a treatment-related AE	PD-L1 cutoff ≥1 and <1%, ≥5 and <5%; clinical activity regardless of PD-L1 expression, but higher ORR for greater PD-L1 expression. Not clear correlation between PFS, OS, and PD-L1 expression
CheckMate 012 (25)	56	Phase I	NSCLC	I line	Nivolumab + platinum-based doublet chemotherapy (PT-DC)	OS PT-DC + Nivo 10 mg/kg from 11.6 to 19.2 months; plus Nivo 5 mg/kg not reached	45% of pts reported Grade 3 or 4 treatment-related AEs. 21% of pts discontinued because of a treatment-related AEs	Nivolumab demonstrated activity irrespective of PD-L1 expression
CheckMate 032 (26)	216	Phase I/II	Small cell lung cancer	Pretreated	Nivolumab or sequentially cohorts nivolumab + ipilimumab	OS Nivo 4.4 months; OS Nivo + IPI 6–7.7 months; 1-year OS 33 and 35–43%	Grade 3 or 4 treatment-related AEs events occurred in 13% of pts in the nivolumab 3 mg/kg cohort, 30% in the nivolumab 1 mg/kg + ipilimumab 3 mg/kg, and 19% in the nivolumab 3 mg/kg + ipilimumab 1 mg/kg. Two pts who received nivolumab 1 mg/kg + ipilimumab 3 mg/kg died from treatment-related AEs (myasthenia gravis and renal failure); 1 who received nivolumab 3 mg/kg + ipilimumab 1 mg/kg died from treatment-related pneumonitis	No correlation between PD-L1 expression and response

patients (12.2 vs. 9.4 months, hazard ratio for death, 0.73; 96% CI, 0.59–0.89; $P = 0.002$). OS rate at 1 year and 18 months was longer for the experimental arm (51 and 39% vs. 39 and 23%) in addition, there was an advantage also for ORR (19 vs. 12%) with a longer duration of response and a median time to response of 2.1 vs. 2.6 months. Immunotherapy was not superior to chemotherapy in terms of mPFS (2.3 and 4.2 months). PD-L1 expression was assessed retrospectively on archival or recent tumor tissue. PD-L1 cutoff was ≥1, ≥5, and ≥10%. It was observed a relevant predictive association among OS, mPFS, ORR, and PD-L1 expression. Subgroup analysis revealed that patients who received third line of chemotherapy, the presence of central nervous system metastases, EGFR mutation, and patients who lived in South America, Asia, and Australia obtained more benefits from chemotherapy. Kaplan–Meyer curves of OS and PFS revealed a chemotherapy early advantage, however, later curves crossed showing a nivolumab advantage. This unexpected finding may be explained by an initial benefit from chemotherapy in patients who do not expressed PD-L1 but presented EGFR mutations. In fact, in this setting, the experimental drug provided less advantage respect to chemotherapy By contrast, in CheckMate 017 trial, Kaplan–Meyer curves had an early separation, particularly for OS. It can be related to nivolumab benefit in overall squamous NSCLC population.

CheckMate 012 trial (24, 25) (**Table 1**) was conducted in I line in advanced NSCLC. It is a Phase I multicohort study that evaluated the safety and efficacy of nivolumab monotherapy or combined to PT-DC. Pretreatment tissue was used only for biomarker evaluation and not for patients' selection. In monotherapy, nivolumab was administered to 52 patients. ORR was 23%, 27% of patients had SD with a disease control rate of 50%. mOS in overall population was of 19.4 months (16.8 months in squamous histology and NR in non-squamous), 12-month OS rate in overall population was 73% (76% in squamous histology and 72% in non-squamous), and 18-month OS rate in overall population was 57% (42% in squamous histology and 63% in non-squamous). In overall population, mPFS was 3.6 months and 24-week PFS was 41%.

Clinical activity was observed regardless of PD-L1 expression, and higher ORR was related to greater PD-L1 expression. The correlation between PFS, OS, and PD-L1 expression is not clear. Smoking history seems to be associated with higher activity of nivolumab. In the combination arm, nivolumab was administered to 56 patients for four cycles every 3 weeks at 10 mg/kg + cisplatin–gemcitabine in squamous histology, plus cisplatin–pemetrexed in non-squamous histology or at dose of 5 or 10 mg/kg + carboplatin–paclitaxel in all histologies. After the planned chemotherapy cycles, patients received nivolumab alone. Nivolumab dose of 5 mg/kg was emended when trial was ongoing. mPFS ranged from 4.8 to 7.1 months, 24-week PFS rate from 38 to 71%. Range of mOS of PT-DC + nivolumab at 10 mg/mg was from 11.6 to 19.2 months, but it was not reached for nivolumab at 5 mg/kg + carboplatin–paclitaxel. ORR was 48% for patients with PD-L1 expression >1 and 43% if PD-L1 was <1%. Nivolumab activity also occurred if PD-L1 was absent or low expressed, whereas smoking history was related to higher clinical activity.

Small cell lung cancer (SCLC) is strongly related to tobacco use, and as a result, it is characterized by high mutational burden. Response to second-line chemotherapy is around 9–23% depending on platinum sensitivity.

CheckMate 032 (26) (**Table 1**) is a muticentre, Phase I/II open-label trial. Patients affected by limited or extended SCLC, after at least platinum-based chemotherapy, received: nivolumab 3 mg/kg every 2 weeks, nivolumab + ipilimumab every 3 weeks for four cycles (1 + 1, 1 + 3, and 3 + 1 mg/kg), then nivolumab 3 mg/kg every 2 weeks. Patients were enrolled sequentially in the four cohorts. The cohort nivolumab 1 mg/kg + ipilimumab 1 mg/kg is the smaller with only 3 patients of 216 overall patients. At interim analysis, ORR was 10% for nivolumab, 23% for nivolumab 1 mg/kg + ipilimumab 3 mg/kg, and 19% for nivolumab 3 mg/kg + ipilimumab 1 mg/kg. mOS was 4.4 months for nivolumab, 7.7 months for nivolumab 1 mg/kg + ipilimumab 3 mg/kg, and 6.0 months for nivolumab 3 mg/kg + ipilimumab 1 mg/kg. One-year overall survival was 33, 43, and 35%. mPFS was 1.4, 2.6, and 1.4 months. Most frequent Grade 3 or 4 AEs were diarrhea and increase of lipase occurring in 4, 30, and 15%. PD-L1 was evaluated retrospectively on archival or fresh tissue collected. PD-L1 expression in SCLC was lower compared to NSCLC, and there was no correlation found between PD-L1 and response.

This trial evidenced similar responses between platinum-resistance and platinum-sensitive patients. The reason is probably due to the mechanism of action of immune checkpoint that is completely different from chemotherapy (i.e., topotecan), and it works better in presence of high mutational burden. No differences were found between patients pretreated with one or more line of chemotherapy. Unfortunately, the absence of randomization does not allow to a comparison between the different arms. Nivolumab achieves rapid and durable responses. The majority of nivolumab studies are limited by the evaluation of PD-L1 expression that can change over time, so tissue collection deriving from archival or recent biopsy does not offer a PD-L1 real status even if this point remains a major concern to debate.

Trials of immune checkpoint inhibitors used different test to establish PD-L1 expression, so there is no unique test for PD-L1 evaluation and a comparison among PD-1/PD-L1 inhibitors is not possible. For this reason, the Blueprint development group has proposed a way to compare different diagnostic assays for future clinical practice that requires validation.

Interesting future development of nivolumab (**Table 2**) in lung cancer are as adjuvant therapy (NCT02595944), after chemo-radiotherapy (NCT02768558), in association with RT in case of intracranial metastasis (NCT 02696993), as maintenance treatment (NCT02538666; NCT02713867), and in combination with ipilimumab/chemotherapy/TKIs (NCT02477826, NCT02785952, NCT02659059, NCT02154490, NCT02041533, NCT02613507, NCT02481830, and NCT02864251).

NIVOLUMAB – SAFETY PROFILE

As mentioned earlier, nivolumab demonstrated an improvement over current available therapies with a risk profile acceptable relative to the clinical benefit offered.

TABLE 2 | Selected future development of nivolumab in lung cancer.

Trial	Phase	Histology	Setting	Treatment	Status	Association
CheckMate 227 NCT02477826	Phase III	Non-small cell lung cancer (NSCLC)	I line	Nivo, NIvo + IPI, Nivo + platinum-based doublet chemotherapy (PT-DC), PT-DC	Recruiting	CT and Immunotherapy
ANVIL NCT02595944	Phase III	NSCLC	IB–IIIA adjuvant	Nivo	Recruiting	Immunotherapy
Lung-MAP NCT02785952	Phase III	Squamous NSCLC	II line	Nivo, Nivo + IPI	Recruiting	Immunotherapy
CheckMate 451 NCT02538666	Phase III	ED-small cell lung cancer (SCLC)	Maintenance after I line CT	Nivo + Placebo, Nivo + Ipilimumab	Recruiting	Immunotherapy
CheckMate-026 NCT02041533	Phase III	NSCLC PD-L1$^+$	I line	Nivo, investigator's choice CT	Active, not recruiting	CT and Immunotherapy
Cisplatin and etoposide + RT followed by Nivo/placebo for locally advanced NSCLC NCT02768558	Phase III	NSCLC	Unresectable, medically inoperable disease, or patients who refuse resection stage IIIA or stage IIIB disease	Thoracic RT, cisplatin, etoposide ± Nivo	Not yet recruiting	RT, CT, and Immunotherapy
CheckMate 078 NCT02613507	Phase III	NSCLC	II line, after platinum-based CT	Nivo, docetaxel	Recruiting	CT and Immunotherapy
Phase I/II trial of nivolumab with radiation or nivolumab and ipilimumab with radiation for the treatment of intracranial metastases from NSCLC NCT02696993	Phase I/II	NSCLC	Stage IV metastatic disease with intracranial disease	Nivo + IPI + WBRT, Nivo + IPI + SRS	Not yet recruiting	RT and Immunotherapy
CheckMate 331 NCT02481830	Phase III	SCLC	II line, after platinum-based CT	Nivolumab, topotecan, amrubicin	Not yet recruiting	CT and Immunotherapy
CheckMate 384 NCT02713867	Phase III	NSCLC	Nivo 240 mg every 2 W vs. Nivo 480 mg every 4 W after up to 12 months of Nivo at 3 mg/kg or 240 mg every 2 W	Nivo 240 mg every 2 W vs. nivolumab 480 mg	Recruiting	Immunotherapy
CheckMate 568 NCT02659059	Phase II	NSCLC	I line	Nivo + IPI	Recruiting	Immunotherapy
Lung-MAP NCT02154490	Phase II/III	Squamous NSCLC	II line	Docetaxel, durvalumab, erlotinib, hydrochloride, FGFR, AZD4547, IPI, laboratory biomarker analysis, Nivo, palbociclib, rilotumumab, taselisib	Recruiting	Immunotherapy, CT, and target therapy
CheckMate 722 NCT02864251	Phase III	NSCLC EGFR mut, T790M	After 1 line EGFR TKI therapy	Nivo + IPO vs. Nivo + PEM + CDDP/CBDCA	Not yet recruiting	Immunotherapy, CT, and target therapy

CT, chemotherapy; Nivo, nivolumab; IPI, ipilimumab; PEM, pemetrexed; W, week.

Phase I

In Phase I study of nivolumab, treatment-related select adverse events of any grade were observed in 41% of 129 patients with NSCLC, and the most common included skin, gastrointestinal, and pulmonary events (16, 12, and 7%, respectively). Grades 3–4 treatment-related adverse events occurred in 14% of cases, with fatigue (3.1%) and pneumonitis (2.3%) being the most common. There were three treatment-related deaths associated with pneumonitis. No clear relationships between the occurrence of pneumonitis and dose level or treatment duration were noted (20).

Phase II

In the non-comparative Phase II trial (ONO-4536-06) conducted in Japanese population (currently not published), any grade drug-related adverse events were reported in 68% of patients. Decrease appetite, malaise, pyrexia, and rash were the most frequent toxicities. Grade 3/4 toxicities were experienced in

5.7%. Regarding the immune-related adverse events, the most common was skin rash (reported in 28% of patients), followed by endocrine (11.4%), pulmonary, gastrointestinal, infusion reactions (each occurring at 5.7%), and renal (2.9) toxicity. No Grade 3/4 toxicities occurred (27).

CheckMate 063: SCC

In the Phase II, single-arm study CA209063 (CM063), any grade treatment-related adverse events were reported in 74% of patients and included fatigue (33%), decreased appetite (19%), nausea (15%), asthenia (12%), rash (11%), and diarrhea (10%). Grades 3–4 treatment-related adverse events were observed in 17% of subjects, with fatigue (4%), pneumonitis (3%), and diarrhea (3%) being the most frequent. Treatment-related adverse events led to discontinuation of the drug in 12% of patients. Immune-mediated adverse reactions, defined as cases requiring use of systemic corticosteroids with no clear alternative cause were immune-mediated pneumonitis (6.0%), hypothyroidism (4.3%), hyperthyroidism (1.7%), motor dysfunction (1.7%), rash (1.7%), adrenal insufficiency (0.9%), vasculitis (0.9%), colitis (0.9%), and renal dysfunction (0.9%). These immunological side effects were treated with administration of high-dose corticosteroids followed by a taper and interruption of nivolumab therapy. Of note, no patients were rechallenged with nivolumab following corticosteroid taper. Finally, two treatment-associated deaths (one due to pneumonia and one due to stroke) occurred (21).

CheckMate 017

In the Phase III open-label randomized trial CheckMate 017 comparing nivolumab vs. docetaxel in SCC NSCLC, the incidence of adverse events was 58% in the nivolumab group vs. 86% in the docetaxel arm. The most frequent adverse events in patients treated with nivolumab were fatigue (16%), reduced appetite (11%), and asthenia (10%), whereas in patients treated with docetaxel, neutropenia (33%), fatigue (33%), alopecia (22%), and nausea (23%) were commonly observed. In the overall study population, treatment-related Grade 3/4 adverse events were more common with docetaxel (55%) with a high number of hematologic toxic events and infections. On the contrary, only 6.9% of patients in the nivolumab arm reported Grade 3/4 treatment-related adverse events, and they were commonly represented by fatigue, decreased appetite, and leukopenia. Overall, 3.1% of patients in the nivolumab arm discontinued treatment due to an AE compared with 10.1% for docetaxel. The most frequently reported (≥3% of patients) selected treatment-related AEs of any grade were hypothyroidism (4 vs. 0%), diarrhea (8 vs. 20%), and pneumonitis (5 vs. 0%) for nivolumab and docetaxel, respectively. Discontinuation due to toxicity issues occurred in 10% of patients on docetaxel, mostly due to peripheral neuropathy, while only 3% interrupted nivolumab mainly for pneumonitis. Finally, no treatment-related deaths were reported for patients treated with nivolumab, whereas three deaths occurred (one death each from interstitial lung disease, pulmonary hemorrhage, and sepsis) in docetaxel arm (22).

Data regarding longer follow-up of the study showed no unpredicted adverse events with nivolumab and a good safety profile compared to docetaxel (28).

CheckMate 057: nsq NSCLC

In the Phase III CheckMate 057 having similar characteristics in terms of design, endpoints, drugs, and schedules of treatment of CheckMate 017, but with a larger samples (in the CheckMate 057:292 and 290 patients in the nivolumab and docetaxel arm, respectively, in the CheckMate 017:135 patients in the nivolumab arm and 137 in the docetaxel arm), the safety profile was in line with the previous reports. More in details, safety analysis demonstrated that AEs of any grade occurred in 69% of patients receiving nivolumab and 88% of patients receiving docetaxel. Among them, the most frequent were fatigue, nausea, decreased appetite, and asthenia in the nivolumab group, whereas neutropenia, fatigue, nausea, alopecia, diarrhea, and anemia were the most common in the docetaxel group. Treatment-related Grade 3/4 adverse events were reported by 10% of the patients treated with nivolumab, with fatigue, nausea, and diarrhea being the most common and each reported in 1% of subjects. In comparison, 54% of patients in the docetaxel group experienced mainly neutropenia (27% of cases), febrile neutropenia (10%), leukopenia (8%), fatigue (5%), and anemia (3%). Treatment-related select adverse events of any grade reported in ≥2.5% of patients were rash (9% of patients vs. 3%, respectively, in the nivolumab and docetaxel arm), pruritus (8 vs. 1%), erythema (1 vs. 4%), diarrhea (8 vs. 23%), hypothyroidism (7 vs. 0%), increased alanine aminotransferase levels (3 vs. 1%), increased aspartate aminotransferase (AST) levels (3 vs. 1%), infusion-related reactions (3 vs. 3%), and pneumonitis (3 vs. 0.4%). Grades 3–4 treatment-related select adverse events experienced in patients receiving nivolumab were pneumonitis (1.0% of patients), diarrhea and increased γ-glutamyl transferase levels (each reported in 0.7% of cases) and rash, dermatitis, colitis, increased AST levels, transaminases increased and interstitial lung disease (each reported in 0.3% of patients). Treatment discontinuation due to adverse events occurred in 5% of patients receiving nivolumab (mainly because of pneumonitis) and in 15% of subjects treated with docetaxel (mostly because of fatigue) (23).

CheckMate 012: I Line

Recently, the results of the first-line monotherapy with nivolumab for advanced NSCLC in the Phase I, multicohort, CheckMate 012 trial were published. Also in this setting, nivolumab was well tolerated, with 19% of patients reporting Grades 3–4 treatment-related AEs and no treatment-related deaths. According to prior nivolumab data (20–23, 27), treatment-related select AEs affected the skin (any grade, 25%; Grades 3–4, 4%), endocrine (any grade, 14%; Grades 3–4, 0%), gastrointestinal (any grade, 12%; Grades 3–4, 2%), and pulmonary organ (any grade, 6%; Grades 3–4, 2%) (24). These toxicities were easily manageable using established guidelines.

Recently, the results of the cohort of the CheckMate 012 study investigating nivolumab + PT-DC in first-line advanced NSCLC were published. A total of 56 patients were enrolled and treated with the following regimens: nivolumab 10 mg/kg + gemcitabine–cisplatin (squamous) or pemetrexed–cisplatin (non-squamous), or nivolumab 5 or 10 mg/kg + paclitaxel–carboplatin (all histologies). No dose-limiting toxicities occurred during the first 6 weeks of treatment. In patients treated with nivolumab

full dose + PT-DC, treatment-related AEs of any grade occurred in 93% of patients, whereas Grade 3/4 AEs occurred in 50% of patients. In the overall population, 95 and 45% of patients experienced any Grade and Grade 3 or 4 treatment-related AEs, respectively. The most frequent (≥30% of patients) treatment-related AEs of any grade were fatigue, nausea, decreased appetite, and alopecia. Regarding treatment-related Grade 3 or 4 AEs, they were mainly (≥5% of patients) pneumonitis, fatigue, and acute renal failure. The majority of patients experienced a treatment-related select AE during the combination period than during nivolumab monotherapy. Treatment-related AEs led to discontinuation of all study therapy in 21% of patients and Grade 3 or 4 treatment-related AEs led to discontinuation in 14% of patients. However, no treatment-related deaths were reported. Because of the high percentage of discontinuation due to AEs, the potential regimen for future indication could be the nivolumab 5 mg/kg + paclitaxel–carboplatin (25).

Recently, the results from CheckMate 026 were presented. The study was one of the first trial in chemotherapy-naïve patients with stage IV or recurrent NSCLC to compare nivolumab with a platinum-based regimen. A total of 541 patients received nivolumab 3 mg/kg every 2 weeks or investigator's choice of PT-DC every 3 weeks for up to six cycles. Despite an enriched population with PD-L1-positive tumors (threshold defined as ≥1%; $n = 423$), nivolumab did not show superior mPFS compared with chemotherapy (4.2 vs. 5.9 months; HR 1.15, $P = 0.25$) (29).

In this context, the CheckMate 227 Phase III open-label study evaluating platinum-based chemotherapy alone or in combination with nivolumab + ipilimumab or nivolumab in previously untreated advanced NSCLC (NCT02477826) is largely awaited.

A Toxicity Profile Never Seen Before

As mentioned, the introduction of immunotherapy in clinical trials showed a specific toxicity profile that is peculiar from the known side effects of cytotoxic chemotherapy or targeted therapies (30). As a result, some patients experienced a novel type of AE considered to be linked to an immune-mediated response directed to different tissues: an immume-related AE (irAE). The percentage of the incidence is around 9%, and the most common irAEs are skin rash, hypothyroidism, diarrhea and colitis, pneumonitis, and increased hepatic function test. These side effects are generally manageable but can be fatal in some cases (31–34). Moreover, their appearance may be subclinical and early diagnosis and management could be extremely challenging. For these reasons, it is important to underline the need to act a careful monitoring of patients receiving nivolumab in order to offer a prompt and optimal management of irAEs. For this reason, physicians should be aware about the use of the established safety guidelines (20, 23, 35, 36). In addition, education of patients and caregivers on recognition of irAEs has a relevant role. Finally, input from other specialties may be valuable for difficult cases (**Table 3**).

TABLE 3 | Management of selected immune-related adverse events.

Organ (disorder)	Grade 1–Grade 2	Grade 3–Grade 4
Gastrointestinal (diarrhea colitis)	Supportive care measures Loperamide If no improvement in 5 days, or if worsening of symptoms, commence steroids at a dose of 0.5–1 mg/kg/day of prednisolone (or IV equivalent)	Withheld the drug Steroids at 1–2 mg/kg prednisolone or IV equivalent If no improvement consider infliximab 5 mg/kg Grade 4: permanent discontinuation of drug
Dermatologic (diffuse, maculopapular rash)	Manage symptomatically If persistent Grade 2, the drug should be withheld for one dose	Grade 3: the drug should be withheld for one dose Grade 4: permanent discontinuation of drug
Hepatic (elevation in liver function tests)	High-dose IV glucocorticosteroids for 24–48 h, followed by an oral steroid taper (dexamethasone or prednisone)	Grade 3/4: permanent discontinuation of the drug
Lung (pneumonitis)	Observation Delay drug administration Consider steroids (e.g., prednisone 1 mg/kg/day PO or methylprednisolone 1 mg/kg/day IV)	Discontinue drug administration High-dose steroids with methylprednisolone (e.g., 1 g/day IV) Add prophylactic antibiotics If not improving after 48 h or worsening, administer additional immunosuppressive therapy (e.g., infliximab, mycophenolate, and immunoglobulins). If improving, taper steroids Discontinue treatment permanently
Endocrine (hypophysitis)	Asymptomatic, no intervention needed: monitor only	Withhold the treatment Use methylprednisolone 1–2 mg/kg intravenously (IV). This should be followed by prednisone 1–2 mg/kg orally (PO) once daily with gradual tapering over 4 weeks and replacement hormones during the tapering. The drug can be restarted with Grade 2, but Grade 3/4 endocrinopathy requires permanent drug discontinuation
Renal injury	Monitor renal function, promote hydration and cessation of nephrotoxic drugs	Prednisolone 1–2 mg/kg or IV equivalent. Discontinue the drug
Nephritis	Consider prednisolone 0.5–1 mg/kg	

Adapted from Ref. (35, 36).

Peculiar Aspects

Combination

The combination of nivolumab with different drugs in NSCLC is under investigation. Of note, combinations of the anti-CTLA4 antibody ipilimumab + nivolumab have showed promising results (37), and several trials are ongoing (NCT02477826, NCT02659059, NCT02864251, NCT01454102, and NCT02869789). Toxicity management is a challenging issue, and new dosages and schedules are under evaluation.

Onset

The onset of immune adverse events occurs on average 6–12 weeks after starting of therapy. It should be considered that these events can happen within days of the first dose, after several months of treatment, and even after discontinuation of therapy.

Open Questions

Currently, many questions are still unsolved. First, the toxicity profile in "real-world," since patients included in clinical trials do not represent the total population in clinical practice. In this setting, there is a lack of data as well as people with pre-existing autoimmune conditions. In such cases, physicians have to consider if benefit exceeds the risk.

A number of case reports about rare irAEs are publishing in literature demonstrating the need to improve the recognition of clinical abnormalities and their association with nivolumab treatment. The awareness of nivolumab safety will grow as experience of physician will increase as well.

Second, immunotherapy has improved survival and as a consequence, a new set of survivorship issues may arise for management. For instance, there may also be sequelae due to an interplay between late effects of radiotherapy in addition to immunotherapy and association among immunotherapeutics MoAbs or targeted therapies must be deeply explored in order to unveil newer and unexpected safety concerns.

NIVOLUMAB ON REAL-WORLD POPULATION: THE STRENGTHS AND WEAKNESSES

After the unprecedented clinical results regarding the activity and the long-term response duration even in heavily pretreated NSCLC squamous and non-squamous subtypes, nivolumab quickly became an undebatable gold standard in second-line setting. These results are noteworthy also because adverse events are generally manageable and or reversible.

The strength of nivolumab arose from clinical trials, especially those well-designed Phase III (22, 23). In order to maximize these astonishing results in real-world population, it is necessary to understand in which patients this drug must be employed and in which nivolumab does not work at all. In addition, it is important to highlight the challenging "gray zones" coming from nivolumab experience in the past 2 years of clinical practice.

In squamous and in non-squamous patients, nivolumab shows nearly 20% of RR and approximately two-third of response are durable and persisting with a plateau after more than 24 months of follow-up in overall survival. As a consequence, it has been demonstrated that nivolumab can provide a real control of the disease leading to the concept of disease chronicization. Unfortunately, 80% of patients have a temporary control of the disease, and in the era of precision medicine, it is essential to understand the main reasons. Looking at the cross-over shape of the CheckMate 057 (23) overall survival curves between docetaxel and nivolumab, the main reason for this particular aspect can be due to the activity of the immune checkpoint inhibitor in one undefined subpopulation. This point led investigators to analyze one or more predictive biomarkers, and as a result, PD-L1 tumoral staining has became an important putative biomarker to select the patient who would benefit more with of this class of drugs (38).

Nivolumab has been studied in all-comers patients, regardless of PD-L1 expression; however, a *post hoc* analysis analyzing the percentage of positivity of tumoral PD-L1 was carried out and different cutoff (>1, >5, and >10%) were reported.

In non-squamous histotype, the PD-L1 tumor expression is predictive of nivolumab activity in term of ORR, DOR, mPFS, and mOS. In particular, higher ORRs were observed when PD-L1 was expressed ranging from 31 to 37% respect to 18% in overall population and 9% in PD-L1-negative patients. Median DOR was longer with nivolumab than with docetaxel across different PD-L1 expression levels (16 vs. 5.6 months). Among PD-L1-negative patients responsive to nivolumab, the mDOR was higher respect to docetaxel (18.3 vs. 5.6 months). This result highlights how PD-L1 alone is a defective predictive biomarker.

A further sub-analysis in strong PD-L1-positive tumors (i.e., >50%) has confirmed the axiom "more PD-L1 expression on tumor and more nivolumab clinical activity." There are many reasons to consider PD-L1 expression as a weak predictive biomarker. First of all, the confounding role between predictivity and prognosis. Many studies associated PD-L1 overexpression with poor prognosis (39); however, prognosis depends on the characteristic of PD-L1 expression and on lymphocyte population forming tumor-infiltrating cells. In fact, CD8 T cells infiltrations strongly correlates with good prognosis in NSCLC, while high B cells and CD4 T cells seem to not impact on prognosis (40–42). It is possible to assume that the subtypes of TILs and the frequency of $CD8^+$ T cells infiltrating tumor and PDL1 tumoral expression are all important to predict the activity of nivolumab more than PD-L1 expression alone. In fact, like chronic infection, in cancer antigen, persistency leads to T cell exhaustion with a high number of T reg and other immunosuppressive myeloid cells constituting TILs. In this situation, tumor PD-L1 expression is not enough to predict the activity of nivolumab on the contrary in TILs rich in T cells $CD8^+$ even with PD-L1 low expression the immune checkpoint inhibitor could stimulate the awakening of competent immune system.

Some elegant models seem to corroborate this hypothesis: the frequency of $CD8^+$ T cells may be associated with better clinical response to immune checkpoint blockade (43, 44), while an immunosuppressive protumoral microenvironment defines intrinsic resistance to anti-PD1 therapy (45). Moreover, myeloid-derived suppressor cells (MDSCs) are recently emerged since

they produce many factors stimulating angiogenesis and immunosuppression with a reduction of viability and number of $CD8^+$ T cells in TILs (46).

Furthermore, MDSCs accumulate in tumor and blood of NSCLC patients, and they are associated with poor prognosis (47, 48). Their quantity reflects a higher number of neutrophil count and a simple and easy calculation of neutrophil to lymphocyte ratio could be a predictive marker of response to immunotherapy (49, 50).

Regarding clinical features associated with a major probability of response, data from a subgroup analysis showed that smoking habit has an important role, especially in non-squamous histology. Ever smoker has a great possibility to have a clinical benefit from nivolumab as demonstrated from CheckMate 057 study (23). This aspect is related to a higher rate of non-synonimous load mutation due to genetic instability of tumors occurring more in smokers than in never-smokers patients. These neoantigens may elicit an immune response in particular when their expression is represented in most tumor cells generating the theory that a clonal mutation has a better possibility to generate a neoantigen recognized by immune system rather than a subclonal expression (51).

Tumors with low mutational burden seem to benefit less from nivolumab according to a subgroup analysis from CheckMate 057. Moreover, it was shown that EGFR-mutated tumors and never-smokers patients had a similar benefit if treated with docetaxel or nivolumab.

The expression of PD-L1 in tumors harboring EGFR mutations or ALK translocations is generally high; however, no reliable data and final conclusions can be drawn from literature data (52, 53).

Recently, in a larger cohort of EGFR/ALK-positive patients, the lack of expression of PD-L1 and the absence of $CD8^+$ T cells in TILs surrounding these tumors were seen. This aspect could classify oncogenic driven tumors as non-inflamed tumors, suggesting a scarce probability to induce an immune awakening and a low activity from immune checkpoint inhibitor agents (54).

The mutational load combined with PD-L1 expression and the analysis of lymphocyte subpopulation of TILs may represent a sort of signature of prediction of response to nivolumab. However, no standard cutoff are available, and there are still many methodological issues regarding the definition of "high" vs. "low" mutational rate tumors.

Nivolumab demonstrates higher efficacy than docetaxel in second line irrespective to PD-L1 expression and in non-squamous patients this benefit increases with the expression of PD-L1. However, the mDOR of nivolumab and its better safety profile renders this drug a reasonable choice even in PDL1-negative patients. This finding led the FDA and EMA approval of nivolumab for all-comers patients and several guidelines do not recommend PDL1 testing.

The issue of a specific predictive biomarker is an important challenge since nivolumab is not a treatment that fits for all patients for several reasons.

First of all the safety: in a *post hoc* analysis from CheckMate 057, a higher risk of death emerged in the first 3 months of treatment with nivolumab respect to docetaxel in particular in poor prognosis patients, especially those with worse ECOG PS and heavy disease burden (55). This aspect is partially explained by a delayed pattern of response of nivolumab, but other characteristics may contribute to contraindicate the use of nivolumab instead of chemotherapy. Second, the sustainability of nivolumab therapy for all patients, in particular in non-squamous histology, across countries.

Some authority regulation agencies like UK National Institute for Health and Care Excellence and Canadian Agency for Drugs and Technologies in Health rejected the use of nivolumab merely due to costs defining this drug as non-cost-effective (56, 57).

Recently, the Swiss Health System conducted a study in order to investigate the cost-efficacy of nivolumab compared with docetaxel. A way to consider this drug effective and sustainable is to select patients with non-squamous histology and testing PD-L1 (cutoff >10%). However, an acceptable ICER threshold of CHF 100,000/QALY is reached only reducing the price of the drug or the dosage or the duration of treatment (58).

It is probable that the absence of a predictive marker of activity will not allow nivolumab to confirm its usefulness largely demonstrated in many trials in a real-world population due to accessibility disparity across countries.

OVERCOME THE RESISTANCE: FUTURE STRATEGIES

There are two main causes of resistance to immune checkpoint inhibitors: the first one is an intrinsic resistance and the second one is an acquired resistance. The former, excluding the mechanism of pseudo-progression, is due to an immunologic ignorance or an adaptive immune resistance. The combination of PD-L1 expression and TIL presence surrounding and within a tumor may classify carefully this situation (59).

The immune-ignorant phenotype lacks a precise strategy; however, the combination of chemotherapy and nivolumab could switch this situation toward and "immune-awakening" due to the delivery of neoantigens as killing effect to chemotherapy use. In the Phase I multicohort study, CheckMate 012 nivolumab was combined with PT-DC (25). In this non-pretreated cohort, the combination showed a good safety profile and encouraging activity in particular when nivolumab at 5 mg/kg was combined with the paclitaxel–carboplatin regimen leading to a 62% of 2-year OS rate. Data are still immature to definitely suggest the application of this strategy only to immune-adaptive resistance or ignorance. Nevertheless, it is intriguing to think about a different strategy in cases where the use of nivolumab alone predicts a worse clinical benefit.

Another approach is to combine nivolumab with the anti-CTLA4 agent ipilimumab in order to enhance T-cell antitumor activity through distinct and complementary mechanisms.

Based on the sole PD-L1 expression, it could be presumed that in PD-L1-positive tumor nivolumab alone should be enough and in PD-L1-negative tumors the combination with ipilimumab could restore the sensitivity to nivolumab.

Several cohorts of CheckMate 012 explored the combination of different doses of nivolumab and ipilimumab. Recently, the combination of nivolumab 3 mg/kg every 2 weeks + ipilimumab

1 mg/kg every 6 or 12 weeks demonstrated a good tolerability profile and promising efficacy with an ORR of 39–47% with mDOR not reached in first-line treatment (37). Patients with higher levels of PD-L1 expression had especially robust responses to the nivolumab/ipilimumab combination. Among patients with tumor PD-L1 expression levels of ≥50% treated with nivolumab every 2 weeks and ipilimumab Q12w, the ORR was 100% and the median PFS was 13.6 months. However, the nivolumab/ipilimumab combination demonstrated efficacy across all tumor PD-L1 expression levels, even among patients with <1% tumor PD-L1 expression.

The combination of ipilimumab 1 mg/kg q6w + nivolumab 3 mg/kg q2w in PD-L1 unselected population is ongoing in a Phase III trial in first-line treatment (CheckMate 227).

In order to circumvent the intrinsic or acquired resistance, other strategies are under investigation. Early phase trials suggest an activity in particular with the combination with other inhibitors or agonists of immune synapse like Abs targeting CSF1R, LAG3, TIM3, IDO, GITR, and OX40. Finally, the combination of nivolumab and radiotherapy (60) or CAR-engineered T cell ACT and vaccines (61) may represent a fascinating strategy to enhance the activity of nivolumab alone.

In EGFR-positive tumors where there is a lack of response of nivolumab in patients previously treated with TKIs, the research is currently focused on naïve EGFR TKI population. This approach is based on the link between the high probability to generate a response with EGFR TKIs in naïve population and the induction of upregulation of PDL1 and TILs. Nivolumab was studied in pretreated and in EGFR TKIs naïve population with promising results observed in the naïve group (62). With the same rationale nivolumab is currently being studied with crizotinib (NCT01998126) and results are largely awaited.

Another strategy to explore is the combination between immune-checkpoint inhibitors and antiangiogenic agents due to cross talk between this two systems and the possibility to influence the angiogenic power and immune-tolerance against tumor. However, even if the rational is strong, the huge number of factors regulating these two axes renders difficult to forecast the results.

In conclusion, nivolumab currently represents the gold standard for the therapy of advanced, pretreated SCC NSCLC and may represent, with some criticism about the role of PDL tumor expression, a valid option in pretreated nsq NSCLC.

The sustainability and disparity across countries lead the affordability of this drug a main concern for the future. Even if for the first time, we have observed a long and durable response in lung cancers using nivolumab in second line, many questions remain to be answered. In particular, the understanding of the right selection of the patient who would benefit more from the drug and the next step of moving toward a first-line treatment with nivolumab in all-comers to control cancer growth from the beginning.

Finally, it is crucial to understand and overcome the immunoresistance mechanisms in order to develop future studies not only trying a combination based on "*in vitro*" rationale but orienting the discoveries of older trials in biologically based Phase I studies.

Nivolumab is not a "one-size fits all" treatment and the main risk is to deny one of the most powerful drug ever employed in clinical practice.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to this paper and agreed to be accountable for the content of the work.

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