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Clinical and molecular features of Epidermal Growth Factor Receptor (EGFR) mutation positive non-small-cell lung cancer patients treated with tyrosine kinase inhibitors: predictive and prognostic role of co-mutations

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Introduction

Lung cancer

Lung cancer is the first cause of cancer-related death worldwide, accounting for more than 1,790,000 deaths each year [1], and it ranks second in incidence, just behind breast cancer [2]. The main risk factor for lung cancer is cigarette smoking, as demonstrated by the seminal works by Doll and Hill [3] and Wynder and Graham [4]. Indeed, smoking causes 90% and 75-80% of male and female lung cancer deaths in the United States, respectively [5]. Despite this evidence, approximately 23% of adult world population still smokes tobacco products, including 1 billion males and 250 million females [6]. Beside tobacco consumption, lung cancer risk factors include family history and rare hereditary syndromes (e.g. Li-Fraumeni syndrome), specific genetic polymorphisms, high meat and alcohol intake, chronic inflammation, pulmonary tuberculosis, exposure to ionising radiation, occupational exposures (e.g. asbestos, chemicals and some metals), air pollution [7].

Lung cancer encompasses multiple histological subtypes, as defined by the WHO/IARC classification (table 1), the most common being adenocarcinoma, squamous cell carcinoma, and small-cell carcinoma.

Adenocarcinoma is a malignant epithelial tumour with glandular differentiation, mucin production, or pneumocyte marker expression. The WHO classification recognizes five major subtypes of lung adenocarcinoma: lepidic, acinar, papillary, micropapillary, and solid. The most important risk factor for developing lung adenocarcinoma is tobacco smoking, although it could be diagnosed even in never-smokers, among whom it is the most frequent histological type. The genetic profile of lung adenocarcinoma comprises several driver alterations including epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene analogue (*KRAS*), *BRAF*, human epidermal growth factor receptor 2 (*ERBB2/HER2*), anaplastic lymphoma kinase (*ALK*), *ROS1*, Rearranged during Transfection (*RET*), Neurotrophic Receptor Tyrosine Kinase-1 (*NTRK1*), and Neuregulin 1 (*NRG1*) [8].

Squamous cell carcinoma is another malignant epithelial tumour that shows keratinization and/or intercellular bridges, or expresses immunohistochemical markers of squamous cell differentiation when

morphologically undifferentiated. Most patients affected by squamous cell lung carcinoma are smokers. These tumours are characterized by a high rate of mutations. Common mutated genes include *TP53*, *CDKN2A*, *PTEN*, *PIK3CA*, *KEAP1*, *MLL2*, *HLA-A*, *NFE2L2*, *NOTCH1*, and *RB1* [8].

Small cell carcinoma is a tumour consisting of small cells with scant cytoplasm, poorly defined cell borders, finely dispersed nuclear chromatin, and absent or inconspicuous nucleoli. Mitotic index is typically high and necrosis is extensive. Small cell carcinomas usually express neuroendocrine markers. They account for 13% of all lung tumours worldwide, and they are mostly diagnosed in heavy smokers. *TP53* is frequently mutated along with *RB1*, while some subsets of tumours harbour *PTEN*, *SLIT2*, *EPHA7* mutations, *FGFR1* or *SOX2* amplifications, *RLF-MYCL* fusions [8].

Adenocarcinoma	
	Lepidic adenocarcinoma
	Acinar adenocarcinoma
	Papillary adenocarcinoma
	Micropapillary adenocarcinoma
	Solid adenocarcinoma
	Invasive mucinous adenocarcinoma Mixed invasive mucinous and non-mucinous adenocarcinoma
	Colloid adenocarcinoma
	Fetal adenocarcinoma
	Enteric adenocarcinoma
	Minimally invasive adenocarcinoma Non-mucinous Mucinous
	Preinvasive lesions Atypical adenomatous hyperplasia Adenocarcinoma in situ Non-mucinous Mucinous
Squamous cell carcinoma	
	Keratinizing squamous cell carcinoma
	Non-keratinizing squamous cell carcinoma
	Basaloid squamous cell carcinoma
	Preinvasive lesion Squamous cell carcinoma in situ
Neuroendocrine tumours	
	Small cell carcinoma Combined small cell carcinoma

	Large cell neuroendocrine carcinoma Combined large cell neuroendocrine carcinoma
	Carcinoid tumours Typical carcinoid Atypical carcinoid
	Preinvasive lesion Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
Large cell carcinoma	
Adenosquamous carcinoma	
Pleomorphic carcinoma	
Spindle cell carcinoma	
Giant cell carcinoma	
Carcinosarcoma	
Pulmonary blastoma	
Other and unclassified carcinomas	
	Lymphoepithelial-like carcinoma
	NUT carcinoma
Salivary gland-type tumours	
	Mucoepidermoid carcinoma
	Adenoid cystic carcinoma
Papillomas	
	Squamous cell papilloma Exophytic Inverted
	Glandular papilloma
	Mixed squamous cell and glandular papilloma
Adenomas	
	Sclerosing pneumocytoma
	Alveolar adenoma
	Papillary adenoma
	Mucinous cystadenoma
	Mucous gland adenoma

Table 1. Lung epithelial tumours classification according to 2015 WHO Classification [see reference 8]

Molecular diagnostic in non-small-cell lung cancer (NSCLC)

Until the beginning of this century, histology was the only factor guiding the treatment choice in advanced non-small-cell lung cancer (NSCLC) patients. Indeed, the low activity of pemetrexed observed in squamous cell lung cancer as well as the high risk of fatal bleeding with the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab in the same group of patients, limited the use of these agents to

non-squamous tumours [9,10]. In 2004 the discovery that specific activating mutations of Epidermal Growth Factor Receptor (EGFR) predict sensitivity to tyrosine kinase inhibitors, paved the way to a paradigm shift in NSCLC treatment [11,12]. Indeed, previous treatment with this class of drugs was not based on accurate patient selection, leading to impressive responses and long-term disease control in a subset of patients, mainly adenocarcinoma in never smokers, females and patients of Asian ethnicity. Since this important milestone, other pharmacological targets have been discovered leading to the introduction of specific targeted treatments in *ALK*, *ROS1*, *RET*, *NTRK* rearranged tumours as well as for patients harbouring *BRAF* and *MET* exon 14 skipping mutations as well [13]. Moreover, drugs developed to target specific *KRAS* mutations as well as *HER2* alterations are in advanced clinical development [14,15]. Due to this expanding drug arsenal, the molecular characterization of patients with advanced non-squamous NSCLC has become of paramount importance? and the promise of a wide precision medicine approach in thoracic oncology appears closer than ever.

EGFR mutation-positive NSCLC

EGFR activating mutations are found in 10-15% of Caucasian NSCLC patients and in up to 50% of Asian ones, especially in those with adenocarcinoma, female sex and never- or former smokers [16]. The most common mutations are exon 19 deletions and exon 21 L858R point mutation (usually defined as “common mutations”), accounting for approximately 90% of cases, while mutations occurring in exon 18 and 20 as well as other exon 21 mutations are rare [17]. Indeed, mutation type could predict sensitivity to specific tyrosine kinase inhibitors [18,19].

Such predictive power was confirmed in multiple phase 3 randomized trials comparing first (reversible)- or second (irreversible)-generation TKIs with platinum-based first-line chemotherapy in EGFR mutation positive advanced NSCLC patients. All these studies demonstrated the superior activity and efficacy of targeted therapy over chemotherapy in terms of overall response rate (ORR) and progression-free survival (PFS), and many studies showed superiority in terms of quality of life. Notably, the superiority in ORR and PFS was consistent across subgroups. Interestingly, TKIs did not show to increase overall survival (OS) as compared to chemotherapy, probably due to the high cross-over rate, the well-known high sensitivity to EGFR TKI of patients already treated with chemotherapy, and the inadequate size of the studies (designed

with PFS as primary end-point) [20-22]. A phase 2B clinical trial comparing the second-generation irreversible EGFR TKI afatinib with the first-generation TKI gefitinib, failed to demonstrate an OS advantage of afatinib in the front-line setting [23]. Following these results, first- and second- generation EGFR TKIs have become the standard treatment for EGFR-mutation positive advanced NSCLC in the first-line setting.

Recently, novel TKIs as well as combination treatments have been shown to potentially improve EGFR-mutation positive patients'outcomes. The phase 3 ARCHER 1050 trial compared dacomitinib (a second-generation TKI) with gefitinib in treatment-naïve stage IV EGFR-mutated lung cancer patients without central nervous system (CNS) metastases. The study showed a statistically significant improvement in terms of PFS with the experimental treatment (mPFS 14.7 versus 9.2 months; HR 0.59, 95% CI 0.47–0.74, P<0.0001). The median OS was 34.1 months with dacomitinib versus 26.8 months with gefitinib (HR 0.76, 95% CI 0.58–0.993, P<0.04) [24,25]. As alternative approach, two different phase 3 trials compared single-agent TKI versus the combination of chemotherapy and TKI. The Japanese NEJ009 study compared gefitinib alone or in association with carboplatin and pemetrexed in the front-line setting. The experimental treatment significantly improved both PFS (mPFS: 20.9 versus 11.2 months, HR 0.49, 95% CI 0.39–0.62, p<0.001) and OS (mOS: 50.9 versus 38.8 months, HR 0.72, 95% CI 0.52–0.92, p=0.021) as compared to the standard of care [26]. Similar results were reported by Noronha et al from a single-institution Indian phase 3 trial as the combination regimen led to significantly longer PFS (mPFS: 16 versus 8 months, HR 0.51, 95% CI 0.39-066, p<0.001) and OS (mOS: not reached vs 17 months, HR 0.45, 95% CI 0.31-0.65, p<0.001) [27]. Moreover, two other phase 3 randomized trials assessed the efficacy of anti-angiogenics drugs in combination with the first-generation TKI erlotinib. The NEJ206 study showed that adding bevacizumab significantly increase PFS as compared to placebo (median PFS 16.9 versus 13.3 months, HR 0.60, 95% CI 0.41–0.87), although no differences in terms of OS were observed [28,29]. The RELAY study compared the combination of erlotinib and ramucirumab, a monoclonal antibody directed against VEGF-receptor 2, versus erlotinib plus placebo showing the superiority of the investigational regimen in terms of PFS (mPFS 19.4 versus 12.4 months; HR 0·59; IC 95% ·46–0·76; p<0·0001) [30].

Finally, the third-generation highly specific EGFR TKI osimertinib was compared with first-generation TKIs as first-line treatment in advanced EGFR-mutation positive NSCLC patients in the phase 3 FLAURA study.

Osimertinib significantly improved the PFS (mPFS 18.9 versus 10.2 months; HR 0.46, 95% CI 0.37–0.57, P<0.0001), and led to superior OS (mOS 38.6 vs 31.8 months, HR 0.80; 955% CI, 0.64 to 1.00; p=0.046) [31,32]. However, osimertinib has shown great efficacy even in patients who experience disease relapse during first- or second-generation EGFR TKI treatment. Indeed, this agent was initially developed to tackle the T790M mutation on EGFR exon 20, which is responsible of approximately 50% of acquired resistances to old-generation TKIs. The phase 3 AURA 3 trial demonstrated osimertinib superiority as compared to platinum-pemetrexed chemotherapy in patients harboring this resistance mutation upon progression to old-generation EGFR TKIs. Osimertinib significantly increase both overall response rate (ORR, 71% vs 31%; HR 5.39, 95% CI 3.46–8.48, P<0.001) as well as PFS (mPFS 10.2 versus 4.4 months; HR 0.30, 95% CI 0.23–0.41, P<0.0001). Moreover, osimertinib showed higher central nervous system (CNS) activity as compared to chemotherapy [33]. Based on these results, according to National and International guidelines, the presence of T790M mutation is mandatory to use osimertinib in patients progressing to first- or second-generation EGFR TKIs. This mutation could be detected either on circulating tumor DNA (ctDNA) or by analyzing a novel tissue biopsy, the latter being the recommended approach in case on negative results from the ctDNA, due to the risk of false negative results [34,35]. Taken all together these data fueled the scientific debate about the best treatment approach to this special NSCLC population, as some clinicians prefer to use the best treatment upfront while other advocate for a sequence. However, beside EGFR mutation type, we still miss other strong predictive and prognostic factors that could be clinically used to stratify patients and, consequently, to personalize the therapeutic approach.

Once the targeted therapeutic options in these patients are exhausted, chemotherapy remains the standard treatment. Indeed, differently from other subgroups, immune checkpoint inhibitors directed against programmed death -1 (PD-1) and its ligand (PD-L1) are rarely active [36]. Recently, a subgroup analysis of a phase 3 study suggested a role of the combination of chemotherapy bevacizumab and the anti PD-L1 monoclonal antibody atezolizumab in EGFR-mutation positive patients, although the low number of patients analyzed do not allow any strong conclusion [37]. A phase 3, randomized, placebo-control trial investigating the combination of anti-PD-1

pembrolizumab with chemotherapy in this group of patients would add further insights about the efficacy of chemo-immunotherapy combinations after TKI failure (KEYNOTE-789, NCT03515837).

Heterogeneity among oncogene-addicted NSCLC patients

During the last years, mounting evidence suggests a substantial heterogeneity among oncogene addicted defined subgroups of NSCLC both at the molecular and clinical level (the so called "intra-driver heterogeneity"). Such intra-driver diversity could at least partially explain different clinical behaviour and response to targeted agents in patients with similar baseline characteristics. Indeed, response rates in clinical trials with EGFR TKIs range between 50% and 83% (with few complete responses and some patients showing primary resistance to targeted agents), and survival is also variable [20]. One of the main factors that could influence the results is the type of EGFR mutation. Indeed, exon 19 deletions confer a better prognosis as compared to exon 21 L858R mutation, as reported by different studies [38,39]. To date, the biological basis of such differences is still unknown. As far as possible, due to these differences, tumours with EGFR exon 19 deletions and exon 21 L858R mutations should be regarded as different disease even if the therapeutic approach is the same.

Co-mutations in EGFR mutation-positive NSCLC

The introduction of novel technologies evaluating multiple genomic alterations with a single test, enabled oncologists to better characterize tumours genomic landscape. Along with increasing the detection of rare oncogenic alterations, these tests could detect concomitant genetic mutations in specific populations. However, to what extent such concomitant alterations could affect the outcome of patients with an oncogenic driver is still unclear. Co-mutations in advanced EGFR mutation positive NSCLC occur more frequently in genes such as *TP53*, *RB1*, *CTNNB1* and *PIK3CA*, but also amplifications involving *EGFR* itself, *NKX2-1*, *CDK4*, *CDK6*, and *CCNE1* could be present [40]. The type and prevalence of co-alterations is similar in patients with exon 19 deletions, exon 21 L858R mutations and exon 20 insertions, and prior treatments seem to increase their number [41]. The most common co-alterations in advanced EGFR-mutated NSCLC patients are somatic *TP53* mutations. *TP53* encodes a transcription factor, p53, involved in cell cycle progression, DNA repair and

apoptosis. p53 is activated by p14, therefore both *TP53* inactivating mutations and p14 inactivation result in p53 loss and cell cycle progression. Typically, somatic *TP53* mutations occur on exons 5-8 [42]. By using the MSK-IMPACT Clinical Sequencing Cohort of the TCGA, Jiao and colleagues evaluated the prevalence and prognostic value of *TP53* and *EGFR* mutations in 1441 advanced NSCLC patients [43]. *TP53* mutation rate was 53% and was associated with worse prognosis, especially when occurring on exons 4, 6 or on multiple exons. Moreover, patients with both *TP53* and *EGFR* mutations showed a significantly worse prognosis than patients with *EGFR* activating mutations only.

A plethora of retrospective studies which analyze patients with *EGFR*-mutation positive NSCLC tried to evaluate the role, if any, of co-mutations in predicting the benefit of EGFR TKIs (table 2).

An Italian multicenter study evaluated 18 consecutive patients with EGFR-mutated advanced NSCLC treated with gefitinib [44]. By using a 22-genes panel (Lung Cancer Panel v2, on Ion Torrent Platform), the Authors found a total of 13 co-occurring mutations in 10 patients. The most common co-mutated genes were TP53 (33.3%) and KRAS (11.1%). Co-mutations was confirmed by Sanger sequencing. The Authors observed that co-mutations significantly affected both PFS (median 3.0 vs 12.3 months, p=0.03) and OS (median 3.6 vs not reached, p=0.03).

A retrospective Australian study using MassArray technology assessing 19 oncogenes found a low prevalence (12.9%) of co-mutations in 62 EGFR-mutated advanced NSCLC treated with first- and second-generation TKIs [45]. Such alteration occurred on *EGFR* and *PIK3CA* genes and were associated with significantly shorter PFS and lower ORR. The single-centre retrospective Chinese study by Hu and colleagues evaluated co-alterations in 320 stage IV and IIIB EGFR mutation positive NSCLC patients treated with old generation TKIs [46]. Real time polymerase chain reaction for *HER2*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* mutations and for *ALK*, *ROS1* and *RET* rearrangements was performed. The most frequent concurrent alterations were found on *PIK3CA* (2.8%) *KRAS* (0.9%), *ALK* (1.9%), *RET* (0.8%) and *ROS1* (0.8%). Concomitant alterations were associated with significantly shorter PFS, while no OS differences were found. Another retrospective single-centre study explored the role of *TP53* mutational status in 60 EGFR-positive NSCLC patients treated with first-generation TKIs [47]. Mutations were detected by Sanger sequencing or NGS. 56% of patients showed *TP53* mutations,

although only 17% had missense mutations. Only the latter were associated with significantly shorted mPFS (HR 1.91, 95% CI 1.01-3.60, p=0.04). A comprehensive genomic profiling with either SNaPshot-NGS or JAX-Cancer Treatment Profile platform was performed on 20 EGFR-positive NSCLC specimens in a single-centre retrospective study [48]. Sixteen patients were treated with first- or second-generation TKIs. The most frequent co-occurring alterations were *TP53* (50%), PIK3CA (10%), *PTEN* (5%) mutations, and no impact on survival was observed comparing patients with and without co-alterations. A similar study by Hong et al evaluated specimens from 58 EGFR-positive Chinese NSCLC patients treated with first-generation TKI [49]. The 49 cancer-related genes with Ion Pi Sequencing 200 kit v2 (Thermo Fisher Scientific) showed that *TP53* (41.4%), *EGFR* T790M (13.8%), *KRAS* (6.9%), and *PIK3CA* (5.2%) mutations were the most prevalent alterations. Co-occurring mutations were associated with significantly lower ORR and shorter PFS and OS. Jakobsen and colleagues evaluated specimens from 23 EGFR positive NSCLC using a 22 lung/colon-cancer associated gene panel along with *MET* and *ALK* immunohistochemistry and *MET* fluorescence *in situ* hybridization [50]. The prevalence of *TP53* mutations was 67%, while 13% of specimens harbored *CTNNB1* mutations, and 9 had *MET* dysregulation. However, no differences in terms of survival were observed when comparing patients with and without co-alterations.

NGS using MSK-Impact panels was used by Yu et al on 200 EGFR-mutated NSCLC patients treated with first- or second-generation TKI in a U.S. single-institution cohort [51]. The most frequent co-occurring alterations were *TP53* (60%), *PIK3CA* (12%), *CTNNB1* (9%), *RB1* (10%) mutations, and *EGFR* (22%), *TTF1*(15%), *MDM2 (12%), CDK4* (10%) and *FOXA1* (10%) amplifications. Only ERBB2 and MET amplifications along with TP53 mutations were associated with shorter time to progression (HR 2.42, p=0.018; HR 3.65, p=0.029; HR 1.68, p=0.006, respectively). A Korean retrospective cohort of 75 patients showed a similar prevalence of co-occuring alterations [52]. In this study the most common co-mutations found by NGS (CancerSCAN panel v1 and v2) were on *TP53* (57.3%), *CTNNB1* (9.3%), *PIK3CA* (8%), and *RB1*(6.7%). TP53 mutations were independently associated with worse PFS (HR 2.02, 95% CI 1.04-3.93; p=0.038). The study by Chang and colleagues showed that only FGFR3 mutations and CDKN2 CNV loss are associated with shorter PFS, while concomitant mutations predict

worse OS [53]. This retrospective cohort included 33 specimens from EGFR-positive NSCLC treated with first- or second-generation TKIs. Specimens were analysed with ACTonco®+ panel. *TP53* mutations occurred in 32% of cases, followed by *CDK4* (26%) and *CDKN2A* (23%) alterations (mainly CNV gain or loss). Chen and colleagues compared the genomic landscape of EGFR-positive patients selected according to PFS (≤6 months or ≥24 months) on first-generation EGFR TKIs [54]. 416 cancer-relevant genes were analysed on 71 specimens using Illumina Hiseq 4000 NGS platform. The most common alterations were *TP53* (51%), *MAP2K2* (15%), *NKX2-1* (15%), *CTNNB1* (15%), *RB1* (12%) mutations, and *EGFR* amplification (18%). *TP53* missense and *PIK3CA* missense mutations were more frequent in the short PFS group as well as co-occurring driver mutations (*ALK* rearrangement, *MET* amplification, *BRAF V600E* mutation). However, no difference in *TP53* mutation prevalence was observed between short and long PFS group. A retrospective Italian multicentre cohort of 133 patients treated with old-generation TKIs showed that concomitant mutations, excluding *TP53*, are associated with significantly shorter PFS [55]. Specimens were analysed by 22 cancer-related genes NGS panel. Interestingly, the prevalence of *TP53* mutations were lower as compared to other cohorts (17.3%), while *KRAS* mutations occurred in 14% of cases. Recently, Chen et al reported a retrospective cohort including 160 patients with EGFR mutations and treated with EGFR TKIs (18 with 3rd generation TKIs) with or without other agents (chemotherapy, anti-angiogenics monoclonal antibodies) as first-line treatment [56]. Specimens were analysed with a 520 or 168 genes NGS panel. The most frequent co-mutations occurred on *TP53* (57%), PIK3CA (6%), *PMS2* (6%), *DMT3A* (6%), *APC* (6%), *MYC* (6%) mutations while 6.9 % of cases showed other driver alterations (*ERBB2* and *MET* amplifications, *ERBB2*, *BRAF* and *KRAS* mutations). *TP53* mutations, and *ERBB2* and *FGF19* amplifications were associated with significantly worse OS upon treatment with 1st generation but not 2nd generation TKIs. CNV was associated with significantly shorter PFS on 3rd generation TKIs. Christopoulos and colleagues evaluated a multicentre cohort of EGFR positive advanced NSCLC [57]. The Authors analysed 261 specimens with a custom NGS panel covering 38-42 genes. They found that only *TP53* mutations independently predict OS and PFS among 219 patients treated with old-generation TKIs.

All these studies are extremely heterogenous in terms of selection criteria, sequencing technologies and genes assessed. Moreover, only one of them explored the role of genomic co-alterations in the era of first-line 3rd generation TKIs.

Author, year	Type of study, Country	Number of advanced patients analysed	Type of first-line treatment (number treated)	Type of test	Most frequent concurrent alterations	Outcomes
Bria, 2016[44]	Multicentre, retrospective	18	1st generation (n:18)	22 genes (Lung Cancer Panel v2 on Ion Torrent platform) plus Sanger sequencing validation	*TP53* (33%), *KRAS* (11.1%), *PIK3CA* (5.5%), *MET* (5.5%),*SMAD4* (5.5%)	Co-mutations significantly affected both mPFS (3.0 vs 12.3 months, p=0.03) and mOS (3.6 momths vs not reached, p=0.03)
Barnet, 2017[45]	Single-centre, retrospective, Australia	62	1st and 2nd generation TKIs (n: 62)	19 oncogenes, MassArray (OncoCarta v1.0 panel)	*EGFR* (8%) and *PIK3CA* (3.2%) mutations	Significantly shorter mPFS in patients with co-mutations (5.7 vs 12.3 months; p=0.02) and lower ORR (38% vs 89%, p<0.001)
Hu, 2017[46]	Single-centre, retrospective, China	320 (stage IIIB and IV)	1st or 2nd generation TKIs (n: 320)	PCR for *HER2*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* mutations and for *ALK*, *ROS1* and *RET* rearrangements	*PIK3CA* (2.8%) and *KRAS* (0.9%) mutations, *ALK* (1.9%), *RET* (0.8%) and *ROS1* (0.8%) rearrangements	Concomitant mutations are associated with significantly shorter PFS; no difference in OS were observed
Labbé, 2017[47]	Single-centre, retrospective, Canada, focused on *TP53* only	60	1st generation TKIs (n: 60)	*TP53* Sanger sequencing or NGS	*TP53* 56% (missense: 17%)	Significantly shorter mPFS in patients with *TP53* missense mutations (HR 1.91, 95% CI 1.01-3.60, p=0.04)
VanderLaan, 2017[48]	Single-centre, retrospective, United States	20	1st or 2nd generation EGFR TKIs (n: 16)	SNaPshot-NGS or JAX-Cancer Treatment Profile	*TP53* (50%), *PIK3CA* (10%), *PTEN* (5%) mutations	No significant differences between patients with and without co-mutations

Hong, 2018[49]	Single-centre, retrospective, China	58	1st generation TKIs (n: 58)	49 cancer-related genes with Ion Pi Sequencing 200 kit v2 (Thermo Fisher Scientific)	*TP53* (41.4%), *EGFR* T790M (13.8%), *KRAS* (6.9%), *PIK3CA* (5.2%) mutations	Concomitant mutations were associated with lower ORR, and shorter PFS and OS
Jakobsen, 2018[50]	Single-centre, retrospective, Denmark	18 (+ 5 with stage IIIA/B)	1st generation TKIs (n: 23)	22 lung/colon-cancer associated genes (Ion AmpliSeq Colon—Lung Cancer Research Panel v2 on Ion Torrent, Thermo Fisher Scientific); *MET* IHC and FISH, and *ALK* IHC were also performed	*TP53* (67%), *CTNNB1* (13%). 5 and 4 samples had *MET* overexpression and amplification, respectively.	No differences in terms of PFS or OS between patients with or without co-alterations
Yu, 2018[51]	Single-centre, retrospective, United States	200	1st or 2nd generation EGFR TKIs (n: 200)	NGS: MSK Impact, version 1, 2 or 3	*TP53* (60%), *PIK3CA* (12%), *CTNNB1* (9%), *RB1* (10%) mutations, and *EGFR* (22%), *TTF1*(15%), *MDM2 (12%), CDK4* (10%) and *FOXA1* (10%) amplifications	Shorter time to progression on TKI associated with *ERBB2* (HR 2.42, p=0.018) and *MET* (HR 3.65, p=0.029) amplifications, and *TP53* mutations (HR 1.68, p=0.006)
Kim, 2019[52]	Single-centre, retrospective, Korea	75	1st or 2nd generation EGFR TKIs (n: 75)	NGS: CancerSCAN panel, version 1 or 2	*TP53* (57.3%), *CTNNB1* (9.3%), *PIK3CA* (8%), *RB1*(6.7%) mutations	*TP53* mutations independently associated with worse PFS (HR 2.02, 95% CI 1.04-3.93; p=0.038)
Chang, 2019[53]	Single-centre, retrospective, Taiwan	33	1st and 2nd generation TKIs (n: 33)	ACTonco®+ panel using Ion Proton sequencer with Ion PI chip (Life Technologies)	*TP53* (32%) mutations, *CDK4* (26%) and *CDKN2A* (23%) alterations (mainly CNV gain or loss)	FGFR3 mutations and CDKN2A CNV loss associated with shorter PFS; patients with any concomitant mutations

						have worse OS (24.1 vs 40.8 momths; p=0.029)
Chen, 2019[54]	Singe-centre, retrospective, China	71 (selected according to PFS ≤6 months or ≥24 months)	1st generation TKIs (n: 71)	416 cancer-relevant genes (Illumina Hiseq 4000 NGS platforms)	*TP53* (51%), *MAP2K2* (15%), *NKX2-1* (15%), *CTNNB1* (15%), *RB1* (12%) mutations, *EGFR* amplification (18%).	*TP53* missense and *PIK3CA* missense mutations more frequent in the short PFS group as well as co-occurring driver mutations (*ALK* rearrangement, *MET* amplification, *BRAF V600E* mutation). No difference in *TP53* mutation rate between short and long PFS group.
Rachiglio, 2019[55]	Multicentre, retrospective, Italy	133	1st and 2nd generation TKIs (n: 133)	22 cancer-related genes panel (Ion AmpliSeq Colon—Lung Cancer Panel on Ion Torrent, Thermo Fisher Scientific)	*TP53* (17.3%), *KRAS* (14%), *PIK3CA* (9%), *EGFR* T790M (6.8%) mutations	Concomitant mutations (but not *TP53*) are associated with significantly shorter PFS on EGFR TKIS
Cheng, 2020[56]	Single-centre, retrospective, China	175 (stage IIIA or higher) 160 with EGFR mutations	1st, 2nd, 3rd generation TKIs +/- chemotherapy or anti-angiogenics (n: 110, 1st; n: 35, 2nd; n: 15, 3rd)	520 or 168 cancer-related gene panel based on NextSeq 500 (Illumina technology)	*TP53* (57%), *PIK3CA* (6%), *PMS2* (6%), *DMT3A* (6%), *APC* (6%), *MYC* (6%) mutations. 6.9% other driver alterations (*ERBB2* and *MET* amplifications, *ERBB2*, *BRAF* and *KRAS* mutations)	*TP53* mutation, *ERBB2* and *FGF19* amplifications associated with significantly worse OS upon treatment with 1st but not 2nd generation TKIs; CNV associated with significantly shorter PFS on 3rd generation TKIs

Christopoulos, 2020[57]	Multi-centre, retrospective, Germany	261	1st and 2nd generation TKIs (n: 219)	38-42 genes custom panel (NGS, ThermoFisher Scientific)	*TP53* (44%), *CTNNB1* (4.6%)	*TP53* mutations independently associated with PFS and OS

Table 2. Studies evaluating co-mutations in advanced NSCLC patients with activating EGFR mutations. EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitors; NGS: next-generation sequencing; HR: hazard ratio; CI: confidence interval; CNV: copy number variation; PFS: progression-free survival; OS: overall survival; ORR: objective response rate; IHC: immunohistochemistry; FISH: fluorescence *in situ* hybridization.

Study design

This retrospective study evaluated all patients with EGFR activating mutations and treated with first-line TKIs (both first, second and third generation) at the Thoracic Oncology Unit of S. Luigi Gonzaga University Hospital along with patients treated with first-line osimertinib at the Oncology Unit at Mauriziano Umberto I University Hospital. The study aimed at the characterization of clinical characteristics and outcomes of patients by the presence or absence of co-mutations. Patients alive were enrolled under the ProMole protocol, a prospective observational study on NSCLC molecular data. The study was approved by the local Ethic Committee.

Materials and methods

Patient demographics and outcome measurements

Patients with advanced NSCLC with activating EGFR mutation treated with TKIs at the Thoracic Oncology Unit of the AOU San Luigi Gonzaga (Orbassano) and, in case of first-line osimertinib, also at the Oncological Unit of the Mauriziano Umberto I Hospital (Turin) were included.

EGFR mutations (exons 18-21) were detected by RT-PCR (Reverse transcriptase-polymerase chain reaction) or NGS (next generation-sequencing). PFS was defined as the time interval from the start of EGFR TKIs treatment and disease progression or death. OS was defined as the time from the start of cancer treatment and patient's death. Performance status (PS) was assessed according to ECOG (Eastern Cooperative Oncology Group) score. The definition of treatment response and disease progression was determined by the investigators using the response evaluation criteria in solid tumors (RECIST) version 1.1. The date of the last follow-up corresponds to January 30th 2021.

Demographic data, data on smoking history, PS and clinical outcomes were collected from medical records. The database used for the study included the following variables:

• Patient characteristics: age, sex, smoking habit

• Disease characteristics: date of first diagnosis, method and site of diagnosis, histology, staging (TNM VIII edition), date of diagnosis of metastatic disease, number and sites of metastases.

• Molecular characteristics: EGFR mutation type (exon 19 deletion, exon 21 L858R mutation, non-T790M exon 20 mutations, exon 20 T790M mutation, others), diagnostic technique, PD-L1 expression level, presence/absence of co-mutations.

• Clinical course: starting date of first-line treatment, PS, best response, suspension of therapy and motivation, progression of the disease with relative sites, local treatments (radiotherapy, surgery or other), site and result of biopsy at the time of progression (liquid and /or tissue). Similar data were collected for second and third lines of therapy.

• Survival outcome: date of the last follow-up, status of patients (dead or alive).

Patients treated with treatment other than single-agent first-line EGFR TKI as well as those with resistant mutations such as EGFR exon 20 insertions were excluded.

NGS sequencing

NGS sequencing has been performed using the Ion Torrent platform (ThermoFisher Scientific) with Oncomine solid tumor DNA and RNA kit assays allowing both the analysis of coding sequence variants of 22 genes (including *EGFR, ALK, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, NTRK, MAP2K1, STK11, NOTCH1, CTNNB1, SMAD4, FBXW7, TP53*), and the identification of 4 gene rearrangements (i.e. *ALK, RET, ROS1, NTRK1*).

After sample adequacy assessment, tumor DNA or RNA have been extracted by automated purification kits. The amplicon libraries have been prepared with Ion AmpliSeq Library Kit (Thermo Fisher, MA, USA). After PCR amplification and barcodes ligation, the amplicon libraries have been equalized and pooled in equal molar ratio. Emulsion PCR and template preparation has been performed using Ion OneTouch Template Kit and Ion OneTouch system (Thermo Fisher) and sequenced. Data analysis have been conducted automatically by Ion Reporter Software. Post-sequencing bioinformatics analysis matched the complementary strands of each barcoded DNA fragment to remove false-positive results. The variant allele fraction (VAF) was computed as the number of mutated DNA molecules divided by the total number (mutated plus wild type) of DNA fragments at that allele; variants have been called if the variant frequency

was ≥5%. Only pathogenic or likely pathogenic variants were included in analyses, based on the current literature.

Immunohistochemical scoring for PD-L1

Immunohistochemical (IHC) detection of tumor PD-L1 expression was performed using the PD-L1 Clone 22C3 kit (pharmDx) and the Ventana platform. The percentage of tumor cells with PD-L1 expression (positive membrane staining) was obtained by counting at least 100 viable cells, and this was the so-called TPS. The evaluation of PD-L1 expression followed the specific requests of the treating clinician in terms of selection of the tested population and timing (diagnostic biopsy or rebiopsy).

Definitions

Based on recent literature, patients were defined as having co-mutations if they harbored mutations of pathogenic or unknown significance according to the COSMIC database and the FATHMM-MKL algorithm. Therefore, patients with co-occurring benign mutations were considered without co-mutations. An exploratory analysis considering all patients with co-alterations (pathogenic or not) was also performed.

Statistical analysis

Descriptive analyzes such as medians, intervals, frequencies, percentages were used to describe the baseline characteristics of the patients.

The χ^2 test was used to analyze the differences between clinical and genetic parameters of patients. The survival curves were estimated using the Kaplan-Meyer method and the log rank test was used to determine the differences in the survival curves between the groups. A $p < 0.05$, with 2-sided testing, was defined as statistically significant. Cox proportional hazard regression models were used to evaluate the association between co-mutations (present/absent) and PFS and OS, obtaining hazard ratios and 95% confidence intervals (CI). The analyzes were carried out with SPSS Software (IBM corporation, Armonk, NY, USA).

Results

Patients and tumor characteristics

A total of 147 patients with advanced EGFR positive NSCLC treated with upfront EGFR TKIs between January 2015 and January 2020 were identified. Fourteen patients were not eligible due to lack of diagnostic tissue for analysis, presence of exon 20 resistant mutations, or treatment other than single-agent first-line EGFR TKIs. Therefore, 133 patients treated with first-line single-agent EGFR TKI were included, 106 of them with complete molecular information (Figure 1).

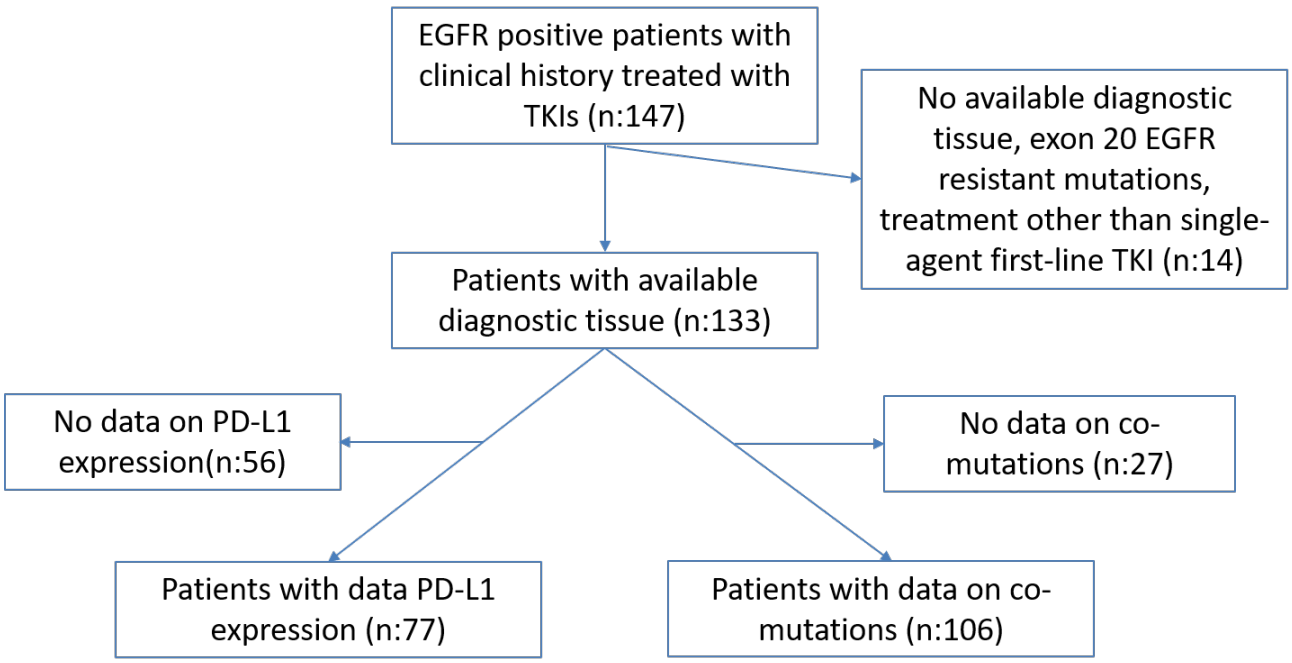

Figure 1. Consort diagram.

Majority of patients were women (69/106; 65.1%) and never smokers (61/106; 57.5%). Median age was 69.8 years (range 32–90.7). All but one patient had adenocarcinoma histology. Most patients had stage IV disease at diagnosis (94/106, 88.7%) with one (35, 33.0%), two (29, 27.4%) or more (41, 38.7%) metastatic sites. Lymph nodes, bone and pleura were the most frequent metastatic sites at diagnosis (50%, 48.1% and 44.3%, respectively). ECOG PS at diagnosis was 0 in 48 patients (45.3%) and 1 in 50 (47.2%). Most patients were diagnosed with EGFR exon 19 deletion (66, 62.3%) or L858R exon 21point mutation (33, 31.1%), while the other had uncommon or double mutations.

Sixty-five patients (61.3%) were treated with upfront first generation (gefitinib or erlotinib) or second-generation (afatinib) TKIs, while 41 (38.7%) received first line osimertinib. Patients' characteristics are reported in Table 3.

	Total (%) (n= 106)	**Patients without concomitant pathogenic mutations (%) (n=59)**	**Patients with concomitant pathogenic mutations (%) (n=47)**
Gender			
Male	37 (34.9%)	19 (32.2%)	18 (38.3%)
Female	69 (65.1%)	40 (67.8%)	29 (61.7%)
Age (median, range)	69.8 (32.0 – 90.7)	72.5 (34.6 – 90.7)	66.6 (32.0 – 85.5)
ECOG Performance Status			
PS 0	48 (45.3%)	25 (42.4%)	23 (48.9%)
PS 1	50 (47.2%)	27 (45.8%)	23 (48.9%)
PS 2	7 (6.6%)	6 (10.2%)	1 (2.1%)
PS 3	1 (0.9%)	1 (1.7%)	0
Smoking status			
Never	61 (57.5%)	35 (59.3%)	26 (55.3%)
Former/current	45 (42.5%)	24 (40.7%)	21 (44.7%)
Stage at diagnosis			
Stage I	1 (0.9%)	0	1 (2.1%)

Stage II	6 (5.7%)	3 (5.1%)	3 (6.4%)
Stage III	5 (4.9%)	2 (3.4%)	3 (6.4%)
Stage IV	94 (88.7%)	54 (91.5%)	40 (85.1%)
Histology			
Adenocarcinoma	105 (99.1%)	58 (98.3%)	47 (100.0%)
Non-adenocarcinoma	1 (0.9%)	1 (1.7%)	0
EGFR mutation			
Ex19 del	66 (62.3%)	33 (55.9%)	33 (70.2%)
Ex21 L858R	33 (31.1%)	23 (39.0%)	10 (21.3%)
Rare or double	7 (6.6%)	3 (5.1%)	4 (8.5%)
Treatment			
Gefitinib/Erlotinib/Afatinib	65 (61.3%)	34 (57.6%)	31 (66.0%)
Osimertinib	41 (38.7%)	25 (42.4%)	16 (34.0%)
Number of metastatic sites			
0 sites	1 (0.9%)	0	1 (2.1%)
1 site	35 (33.0%)	20 (33.9%)	15 (31.9%)
2 sites	29 (27.4%)	13 (22.0%)	16 (34.0%)
3 sites	22 (20.7%)	14 (23.7%)	8 (17.0%)
More than 3 sites	19 (18.0%)	12 (20.3%)	7 (14.9%)
Metastatic sites at diagnosis			
Lung	32 (30.2%)	19 (32.2%)	13 (27.7%)
Pleural	47 (44.3%)	33 (55.9%)	14 (29.8%)
CNS	35 (33.0%)	20 (33.9%)	15 (31.9%)
Liver	18 (17.0%)	12 (20.3%)	6 (12.8%)
Bone	51 (48.1%)	29 (49.2%)	22 (46.8%)
Adrenal	14 (13.2%)	7 (11.9%)	7 (14.9%)
Nodes	53 (50%)	24 (40.7%)	29 (61.7%)

Table 3. Patient characteristics. ECOG: Eastern Cooperative Oncology Group; EGFR: Epidermal Growth Factor Receptor; Ex: exon; CNS: Central Nervous System.

Using diagnostic specimen, 57 patients (53.8%) showed co-occurring genetic alterations. The most common co-mutated genes were: TP53 (n: 36, 34.0%), CTNNB1 (n: 8, 7.5%), PIK3CA (n:6, 5.7%), while the others included NRAS, MET, PTEN, AKT, SMAD4, RET, DDR2, FGFR3 (Figure 2). Double co-mutations occurred in 4 cases. According to the COSMIC database, 28 pathogenic TP53 mutations were found, while the others were benign or of neutral/unknown significance. Therefore, as for survival analysis, patients with concomitant mutations were defined by the presence of pathogenic mutations only. Using such definition, 47 patients were considered co-mutation positive and 59 co-mutation negative.

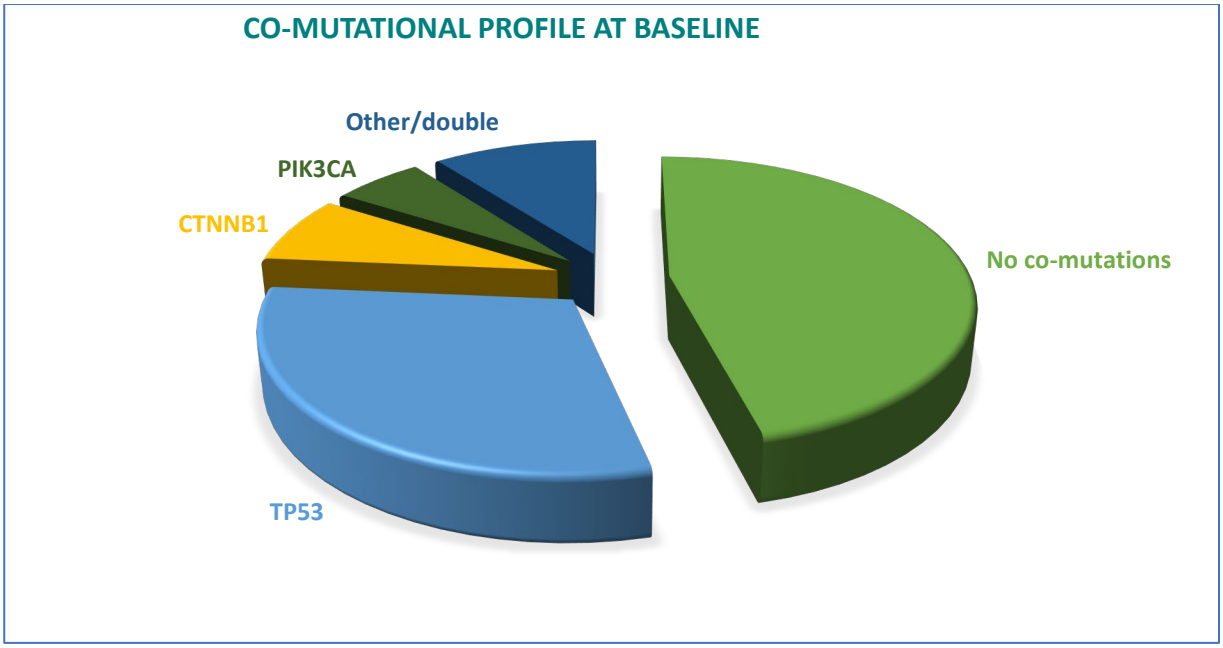

Figure 2. Baseline co-mutations

A greater proportion of patients with EGFR exon 19 deletion harbored concomitant mutations (n:33; 70.2%) as compared with those with EGFR exon 21 L858R mutation (n:10; 21.3%), although the difference was not statistically significant (p=0.138) (Table 4). No associations were found between the presence of concomitant mutations and gender or smoking habits. However, patients with concomitant mutations were younger than those without concomitant mutations (p=0.018). (Table 5).

Interestingly, the presence of concomitant mutations was associated with the presence of lymphnodes metastases at baseline (p=0.032), while patients without concomitant mutations were more likely to present with pleural metastases (p=0.007) (Table 6).

	Patients without concomitant pathogenic mutations (n=59)	Patients with concomitant pathogenic mutations (n=47)	All patients (n=106)
Main mutation			
Exon 19	33 (55.9%)	33 (70.2%)	66
L858R	23 (39.0%)	10 (21.3%)	33
Other	3 (5.1%)	4 (8.5%)	7
	Chi square p=0.138		

Table 4. Co-mutational status by EGFR type of mutation. EGFR: epidermal growth factor receptor.

	Patients without concomitant pathogenic mutations (n=59)	Patients with concomitant pathogenic mutations (n=47)	Chi square
Age			
Younger than 70	24 (40.7%)	30 (63.8%)	**p=0.018**
70 and older	35 (59.3%)	17 (36.2%)	

Median (range)	72.5 (34.6 – 90.7)	66.6 (32.0 – 85.5)	
Smoking status			p=0.679
Never	35 (59.3%)	26 (55.3%)	
Ever	24 (40.7%)	21 (44.7%)	
Sex			p=0.513
Male	19 (32.2%)	18 (38.3%)	
Female	40 (67.8%)	29 (61.7%)	

Table 5. Patient characteristics according to co-mutational status.

	Patients without concomitant pathologic mutations (n=59)	**Patients with concomitant pathologic mutations (n=47)**	**Chi square**
Lung	19/59 (32.2%)	13/47 (27.7%)	p=0.613
Pleural	33/59 (55.9%)	14/47 (29.8%)	**p=0.007**
CNS	20/59 (33.9%)	15/47 (31.9%)	p=0.829
Liver	12/59 (20.3%)	6/47 (12.8%)	p=0.302
Bone	29/59 (49.2%)	22/47 (46.8%)	p=0.810
Adrenal	7/59 (11.9%)	7/47 (14.9%)	p=0.647
Nodes	24/59 (40.7%)	29/47 (61.7%)	**p=0.032**

Table 6. Co-mutational status by baseline metastatic sites.

Treatment outcomes in the whole population

At a median follow up of 27.9 months, the median PFS and OS were 11.2 (95% CI 9.2 – 13.1) and 26.5 (95% CI: 21.1 – 31.8) months in the population with complete molecular data, respectively (Figure 3). PFS and OS were also analyzed by EGFR mutation type in the whole population (n:133) (figure 4). Median PFS was 11.2, 12.1 and 11.6 months for ex19 deletion, ex21 L858R and other mutations, respectively. Median OS was 30.8, 29.0 and 31.6 months for ex19 deletion, ex21 L858R and other mutations, respectively. The overall response rate was 68.1% in the whole population, 61.8% in patients treated with 1st or 2nd generation TKIs and 70.35 in those treated with osimertinib. By analyzing the type of treatment, mPFS was 10.3, 11.9, and 11.6 months in patients with exon 19 deletion, exon 21 L858R mutation, and other EGFR mutations treated with old-generation TKIs, respectively. The mPFS of patients treated with first-line osimertinib was 16.8 months and not reached in those with exon 19 deletions and L858R mutation, respectively. No significant differences were observed when comparing patients treated with first- and second-generation TKIs with those treated with upfront osimertinib (mPFS 10.6 vs 16.8 months; HR 0.61, 95% CI 0.35-1.06; p=0.081).

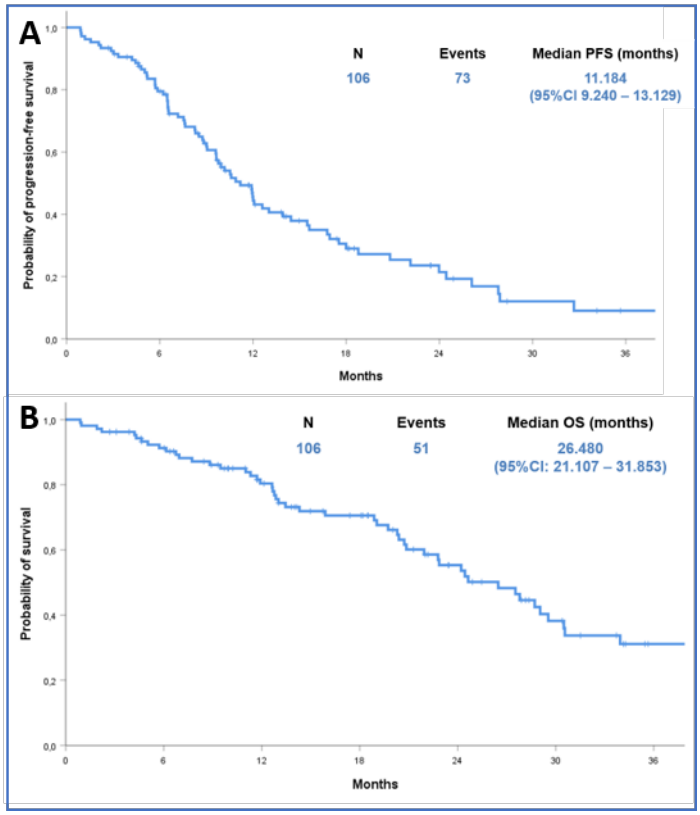

Figure 3. Progression-free survival (A) and overall survival (B) in patients with known co-mutational status.

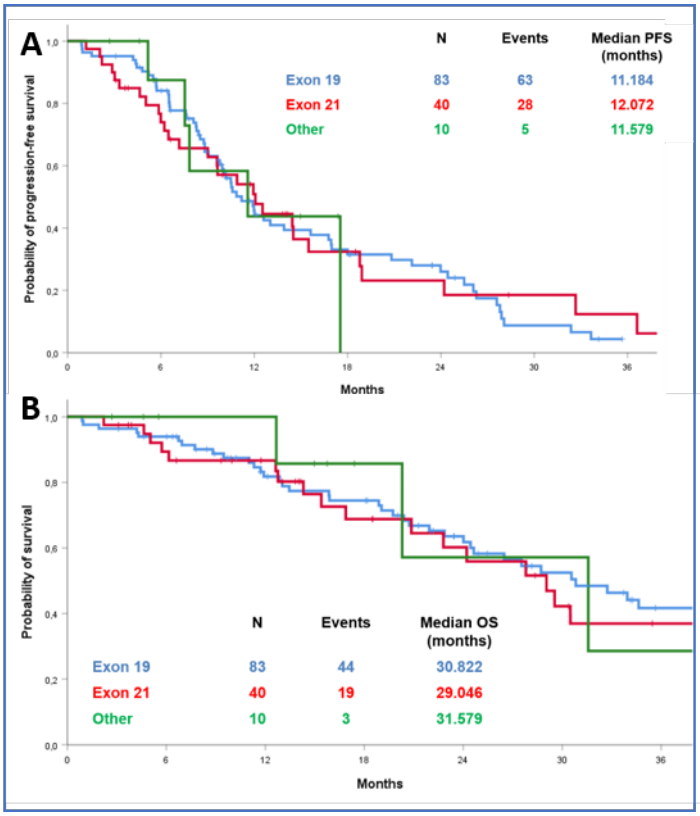

Figure 4. Progression-free survival (A) and overall survival (B) by mutation type in the whole population.

Association between concomitant alterations and treatment outcomes

No association was found between survival and co-mutational status. The median PFS was 11.2 months in patients with concomitant alterations versus 10.9 months in patients without [Hazard Ratio (HR) 0.88 (95% CI 0.56–1.40) p = 0.597]. No differences were found even when analyzing by treatment (1st and 2nd generation TKIs or 3rd generation TKI). Indeed, median PFS was of 9.9 versus 9.8 months in patients with or without co-alterations treated with old generation TKIs, respectively [HR 0.77 (95% CI 0.45 – 1.30) p = 0.319]. In patients treated with osimertinib, mPFS was 16.8 in patients with concomitant alterations versus 17.5 months in patients without [HR 1.01 (95% CI 0.37 – 2.73) p = 0.985] (Figure 5).

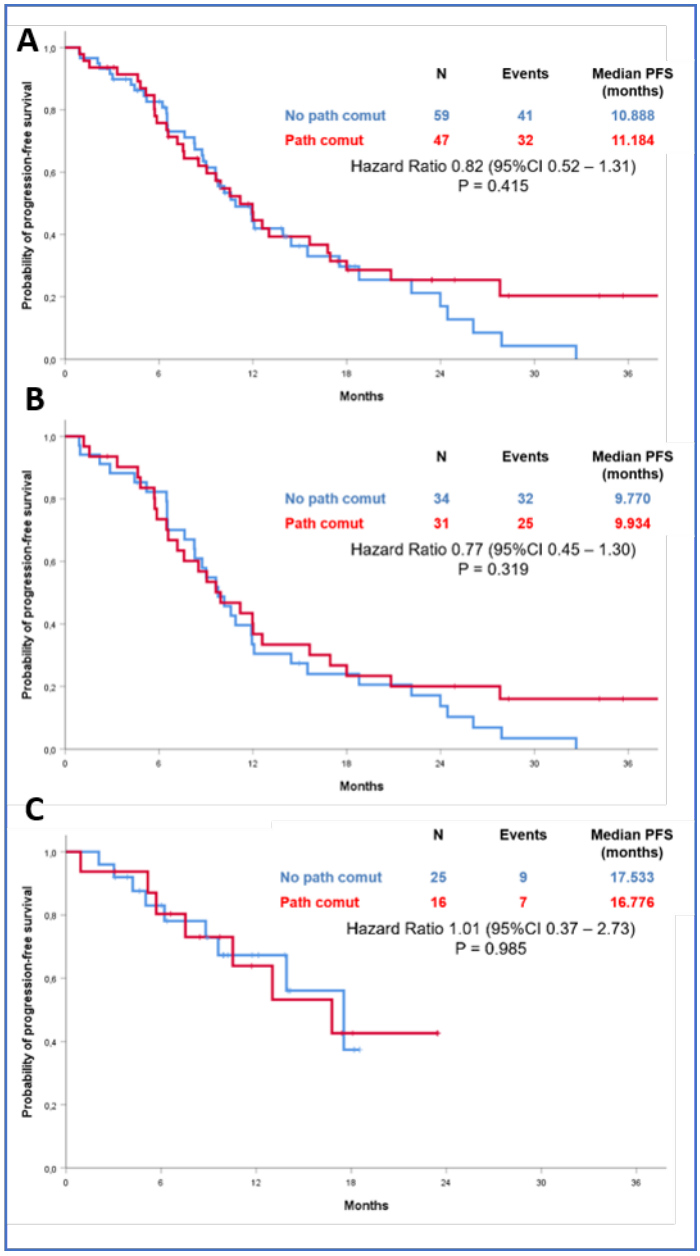

Figure 5. Progression-free survival according to co-mutational status. A. Whole population; B. Patients treated with old-generation TKIs; C. Patients treated with osimertinib.

OS survival was not statistically different according to the presence or absence of concomitant alterations [median OS 29.5 versus 22.8 months, respectively, HR 0.61 (95% CI 0.35 – 1.08) p = 0.088]. No differences were also found when analyzing patients treated with old-generation TKIs or osimertinib (Figure 6).

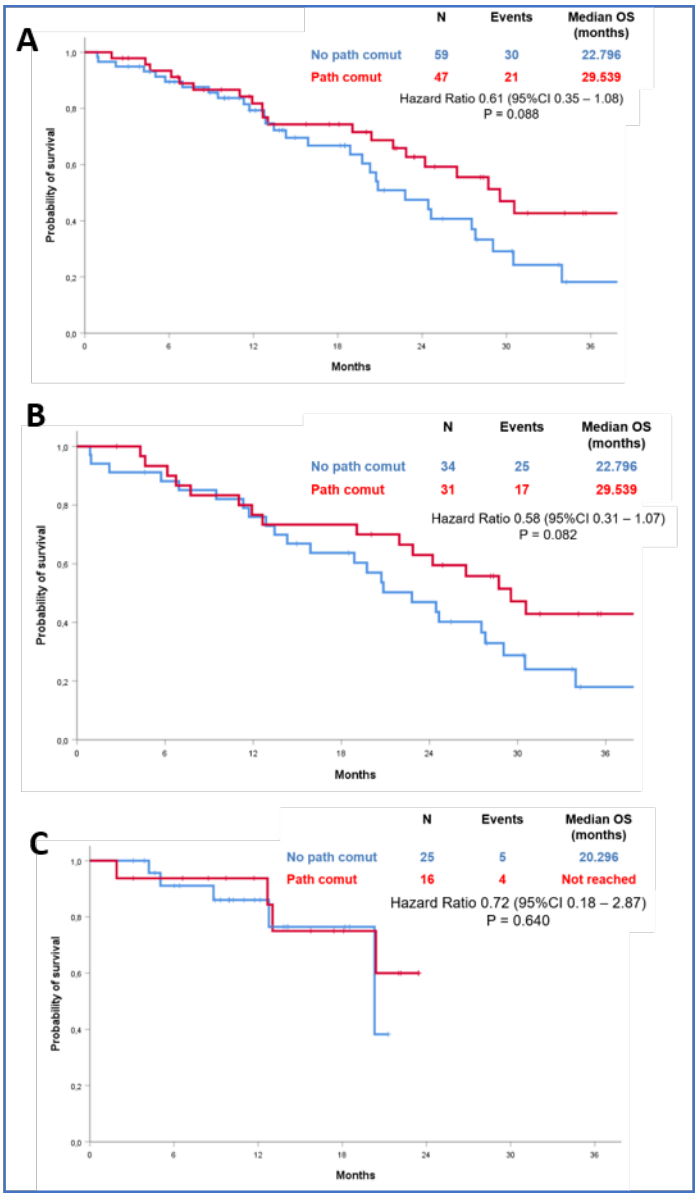

Figure 6. Overall survival according to co-mutational status. A. Patients with known co-mutational status; B. Patients treated with old-generation TKIs; C. Patients treated with osimertinib.

ORR were similar between patients with or without co-occurring mutations (68.1% vs 55.9%, p=0.202). ORR in patients with co-mutations treated with 1st or 2nd generation TKIs was 64.5% vs 50.0% in those without co-mutations (p=0.238). No differences were observed in patients treated with first-line osimertinib as well (75.0% vs 64.0%, p=0.460).

We also performed an exploratory analysis considering all co-mutations (thus including also benign, neutral mutations and those of uncertain significance). Differences remain not statistically significant: median PFS was 10.9 months in patients without co-mutations versus 11.2 in patients with all types of mutation [HR

0.88 (95%CI 0.56 – 1.40) p = 0.597]. mOS was 20.8 versus 28.7 months, respectively [HR 0.69 (95%CI 0.40 – 1.21) P = 0.199] (Figure 7).

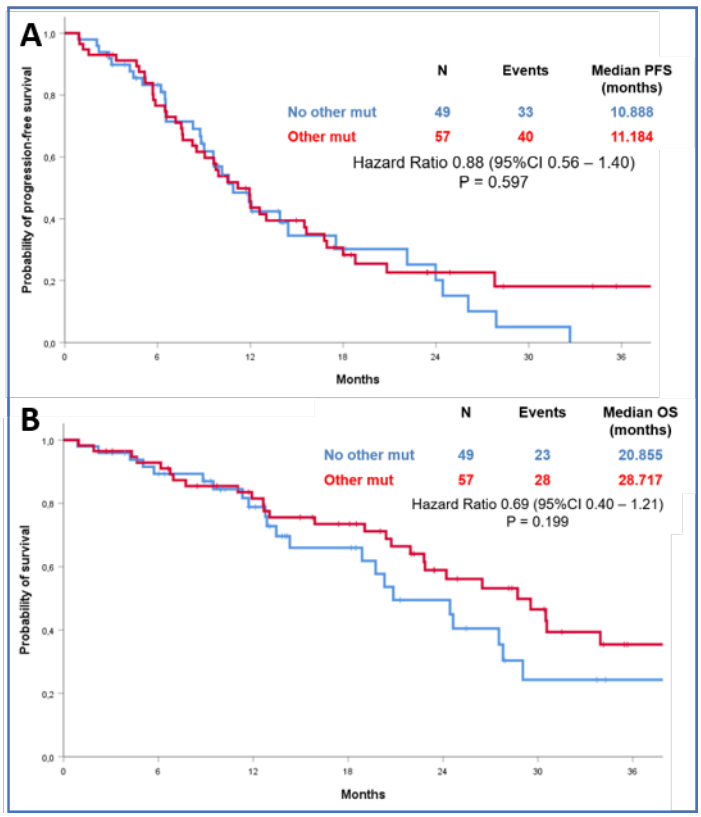

Figure 7. Progression-free survival (A) and overall survival (B) according to co-mutational status (considering all co-mutations).

Association between concomitant alterations and second-line treatment outcomes

Sitxy-four patients experienced progression in the whole cohort. The most frequent sites of progression were lung (n:31, 48.4%), central nervous system (n:24, 37.5%) and pleura (n:15, 23.4%).

Patients without co-mutations showed a significantly higher incidence of bone metastases at progression as compared to patients with co-occurring alterations (26.5% vs 3.3%; p=0.011) (Table 7).

	Patients without concomitant pathologic mutations	Patients with concomitant pathologic mutations	Chi square

	(n=34)	(n=30)	
Lung	18/34 (52.9%)	13/30 (43.3%)	p=0.443
Pleural	10/34 (29.4%)	5/30 (16.7%)	p=0.230
CNS	12/34 (35.3%)	12/30 (40.0%)	p=0.698
Liver	7/34 (20.6%)	4/30 (13.3%)	p=0.443
Bone	9/34 (26.5%)	1/30 (3.3%)	**p=0.011**
Adrenal	1/34 (2.9%)	2/30 (6.7%)	p=0.482
Nodes	4/34 (11.8%)	1/30 (3.3%)	p=0.210

Table 7. Sites of progression according to presence or absence of co-mutations.

Complete molecular information, including T790M status, was obtained either by liquid or tissue biopsy in 43 patients treated with old-generation TKIs at the time of progression. T790M resistance mutation rate was similar between patients with or without baseline co-occurring mutations (52.6% vs 70.8%; p=0.220). NGS was done both at baseline and at the time of progression in 30 patients. No differences in molecular profile were detected in 23 (76.7%). The discordant co-mutational status included loss of *TP53*, *CTNBB1* or *PIK3CA* mutations, and acquisition of *CTNBB1*, *DDR2*, *SMAD4* or *TP53* mutations. Twenty-seven patients received second-line osimertinib at progression, and 10 had co-occuring mutations at baseline.

PD-L1 expression and outcomes

PD-L1 expression level was available in 77 patients in the whole cohort, 51 of whom (66.2%) were negative. Among PD-L1 positive cases, 20 (26%) showed a TPS between 1% and 49%, and 6 (7.8%) equal or higher than 50%. An exploratory analysis on the association between PD-L1 expression and the presence/absence

of co-mutations (n:73) showed a comparable distribution of the PD-L1 expression in the two groups (Tables 8 and 9).

	Patients without concomitant pathologic mutations (n=38)	Patients with concomitant pathologic mutations (n=35)	Wilcoxon – Mann-Whitney
Median	0	0	p=0.916
Range	0 - 75	0 - 95	

Table 8. Distribution of PD-L1 expression in patients with and without co-mutations.

	Patients without concomitant pathologic mutations (n=38)	Patients with concomitant pathologic mutations (n=35)	Chi square
PD-L1 0%	25 (65.8%)	24 (68.6%)	p=0.483
PD-L1 1-49%	11 (28.9%)	7 (20.0%)	
PD-L1 >=50%	2 (5.3%)	4 (11.4%)	

Table 9. Distribution of PD-L1 expression levels in patients with and without co-mutations.

There was no significant difference in ORR between patients with different PD-L1 expression levels (PD-L1 negative vs PD-L1 positive: 62.7% vs 65.4%, p=0.820; PD-L1 negative vs PD-L1 1-49% vs PD-L1 > 50%:62.7% vs 65% vs 66.7%; p=0.972). Median PFS was 12.0 months versus 9.6 months in patients with PD-L1 of 0% and PD-L1 expression $\geq$ 1%, respectively (Figure 8). No differences in median OS were observed in the PD-L1 negative and PD-L1 positive patients (mOS 27.5 and 24.4 months, respectively) (Figure 8).

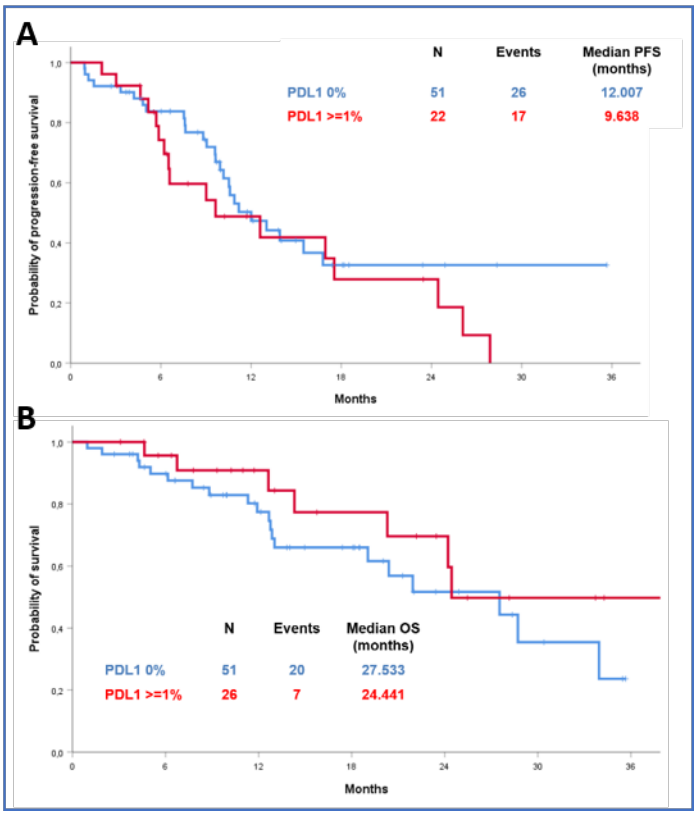

Figure 8. Progression-free survival (A) and overall survival (B) according to PD-L1 expression. PD-L1: programmed death ligand 1.

By analyzing the association between PD-L1 expression and the presence / absence of co-mutations, median PFS in PD-L1 negative patients was 13 months in patients with concomitant mutations versus 10.9 in patients without [HR 0.87 (95% CI 0.40 – 1.90) P = 0.727] (Figure 8). In the PD-L1 positive cohort (TPS $\geq$ 1%), the median PFS was 6.6 versus 17.5 months in those with and without co-mutations, respectively [HR 1.60 (95% CI 0.43 – 5.99) P = 0.484] (Figure 9).

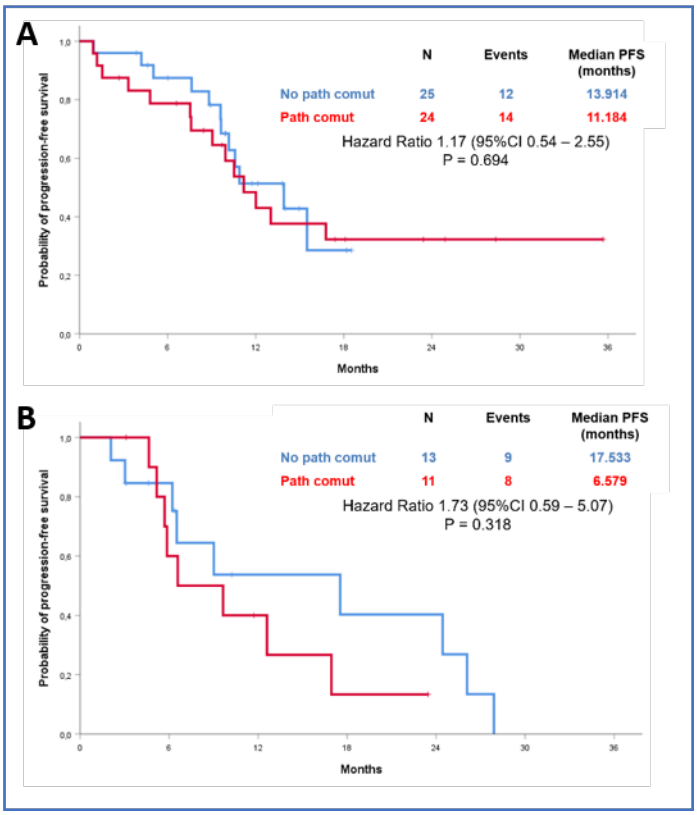

Figure 9. Progression-free survival in PD-L1 negative (A) and positive (B) patients by co-mutational status. PD-L1: programmed death ligand 1.

Discussion

This retrospective study analyzed data from all patients with advanced NSCLC and EGFR activating mutation treated with single-agent TKI in the first-line setting in the last 5 years at our Institution along with all consecutive patients treated with first-line osimertinib at Mauriziano Hospital. Patient characteristics, tumor histopathologic features, mutation types, PFS, and OS are consistent with those reported in the literature. Most patients were women (65.1%) and never smokers (57.5%), and 93.4% of them had common EGFR mutations.

Similar to other studies[44-55], we found that 53.8% of patients harbor concomitant alterations, even if some of them are of unknown significance or benign. Therefore, we decided to define as co-mutation positive patients with concomitant pathogenic mutations only. Interestingly, a significant correlation was found

between the presence of concomitant molecular alterations and age, since co-mutations occur more frequently in younger population (<70 years old) (p=0.018). This previously unreported correlation may be the epiphenomenon of the higher vulnerability of some patients to carcinogens, although we have no proof of this hypothesis.

The most common co-mutated gene in our cohort was *TP53* (n:36, 63.2%), although only 28 were pathogenic according to the COSMIC database. Other frequent mutated genes were CTNNB1 (6.87%), PIK3CA (4.9%), and others, including NRAS, MET, PTEN, AKT, SMAD4, RET, DDR2, FGFR3 (9.8%).

Our results suggest that genomic profile may not influence treatment efficacy and clinical outcomes of patients with advanced EGFR mutated NSCLC. The presence of concomitant alterations studied by our NGS panel was not associated different outcomes following treatment with first, second or third generation EGFR TKIs. To date, the predictive value, if any, of concomitant mutations for targeted therapy in advanced NSCLC is still matter of study. As previously discussed, while some studies suggest that co-mutations could define a population with lower probability of response to EGFR TKIs, others do not (see table 2). Such studies are all retrospective series, with an extreme intra- and inter-study heterogeneity when dealing with ethnicity, type of *EGFR* mutations and treatment and, more importantly, diagnostic techniques. Indeed, gene panels as well as assay varies between studies, and in some of them different patients were tested with different techniques. Moreover, the definition of co-mutations or co-alterations is different among studies. The present study analyzed only patients treated with single-agent TKI, carrying TKI-sensitive mutations and tested with the same technology and gene panel, thus limiting intra-study heterogeneity. As some mutations do not carry any pathologic significance, we decided to consider only truly pathogenic ones. While most other studies reported a higher prevalence of concomitant mutations in patients with exon 21 mutation, our cohort did not. Indeed, half of patients with exon 19 deletions had co-mutations as compared to 30.3% of those with exon 21 L858R mutation.

Consistent with literature data[58], the most common co-mutated gene was *TP53* (63.2%) also in this cohort. Considering pathogenic mutations only, patients with co-mutations represent 44.3% of those analyzed. An exploratory analysis including also benign mutations and those with unknown/neutral significance did not

show any differences in ORR, PFS and OS between patients with and without other mutations. Other clinical studies have identified TP53 co-alterations as a negative prognostic marker in EGFR mutated NSCLC and a consistent predictor of worse clinical outcomes with EGFR TKI therapy[44-47,49,51,52,54,57]. Other results revealed that concomitant concurrence of TP53 mutation at baseline is significantly associated with shorter OS in patients treated with 1st generation TKIs but not in those treated with 2st/3nd generation ones[56]. Also in the prospective randomized RELAY study, baseline *TP53* mutations were associate with shorter PFS and trend of greater efficacy of the experimental treatment (erlotinib plus ramucirumab) was observed[59]. Moreover, patients with baseline TP53 mutations had a higher likelihood of developing T790M exon 20 mutations upon progression to both treatments. However, other studies did not show any correlation between *TP53* mutations and survival[48,50,55].

To our knowledge, very limited data exist on co-mutational profile and treatment outcomes with osimertinib, both in first or second line[56,60]. The present study shows that the benefit derived from front-line osimeritinib seems independent from the co-mutation profile.

Co-mutations do not seem to predict the occurrence of T790M resistance mutations upon treatment with old-generation TKIs neither. Acquired T790M mutation was observed in 70.8% of patients without co-mutations and 52.6% of those with co-alterations (p=0.220).

Overall, NGS on tissue specimens was done both at baseline and at disease progression, without showing different molecular profiles in most cases (76.7%). This may underscore the inability of our NGS panel to detect some resistance mechanisms to TKIs.

Interestingly, patients without concomitant alterations seem to progress more frequently to the bones as compared to those with concomitant mutations (26.5% vs 3.3%, p=0.011).

To further explore our cohort, we also analyzed PD-L1 expression levels. Several studies on the predictive role of PD-L1 expression and TKIs efficacy in EGFR-mutated NSCLC have shown contrasting results[61-64]. However, none have evaluated PD-L1 expression in relation to co-mutational profile. Our cohort showed a comparable distribution of the PD-L1 expression in the two groups. No significant differences were

observed in terms of ORR, PFS and OS between patients with different PD-L1 expression levels. When dealing with PD-L1 expression and co-mutational status, neither ORR nor PFS changed according to the presence or absence of co-alterations, suggesting that PD L1 cannot be considered a predictive biomarker in this context.

The main strengths of our study are the longitudinal availability of real-world clinical data, the standardized molecular profiling that was performed in the same institution using the same technology, as well as the presence of a cohort of patients treated with first-line osimertinib.

However, several limitations must be acknowledged. The retrospective design and the relatively small sample sizes of each cohort could have affected subgroup analyses. Furthermore, the small NGS panel may have miss some important molecular alterations. Moreover, tumor genetic heterogeneity is not appropriately catched by tissue biopsy analysis[65]. Therefore, these findings require further prospective studies conducted in larger cohorts, either a validation one or, ideally, in a prospective study.

In conclusion, our study did not demonstrate any predictive or prognostic role of co-mutation in EGFR-positive advanced NSCLC patients treated with first-line TKIS. Although, from a clinical point of view, an intra-driver diversity exists, how to identify factors explaining different clinical behaviors is still an open challenge. Wider genomic studies, both on tissue specimens and liquid biopsies, may guide treatment selection in the next-future, helping clinician to deliver more intensive treatment strategies to high-risk patients, sparing useless toxicities in others. The development of such risk-adapted treatment algorithms require further translational studies, especially because genomic data without clinical ones may not bring any benefit to the patients.

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