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Histological transformation to small cell lung carcinoma in non-small cell lung tumours oncogene and non-oncogene addicted. Clinical and biological features from an international cohort of patients.

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Introduction

Incidence and epidemiology

Primary lung cancer remains the most common malignancy after non-melanocytic skin cancer, and deaths from lung cancer exceed those from any other malignancy worldwide [1]. In 2015, lung cancer was the most frequently diagnosed cancer and the leading cause of cancer death in male populations. Among females, lung cancer was the leading cause of cancer death in more developed countries, and the second one in less-developed countries [2,3]. In 2017 in the European Union (EU), lung cancer mortality prediction fell in men by 10.7% compared with 2012, while cancer death rates increased in women by 5%, thereby approaching male counterparts [4]. The prediction for the 2018 in the United Stated (US) confirms that lung cancer incidence rates continue to decline about twice as fast in men as in women, reflecting historical differences in tobacco uptake and cessation, as well as upturns in female smoking prevalence in some birth cohorts [5].

Data from 2015 revealed that in the US, lung cancer did represent the leading cause of cancer death in males from the age of 40 and in females from the age of 60 [5]. The number of cancer deaths expected to occur in 2017 in the US has been estimated, still reporting lung cancer as the leading cause of death in both genders, despite the fact that death rates declined by 45% from 1990 to 2015 among males and 19% from 2002 to 2015 among females [5].

A significantly higher proportion of lung cancer is diagnosed in patients aged 65 years and over [6], and the median age at diagnosis is around 70 years [7]. The cumulative probability of lung cancer in the general population for individuals up to 74 years of age is 10% to 15% in those who smoke one or more pack of cigarettes par day [8]. A subset of patients with non-small-cell lung cancers (NSCLCs) presents at a younger age (<40 years), but the incidence in this population has decreased in the US from 1978 to 2010 [9].

Tobacco smoking is still the main cause of lung cancer in most of the patients, and the geographic and temporal patterns of the disease largely reflect tobacco consumption during the previous decades. Both smoking prevention and smoking cessation can lead to a reduction in a large fraction of human cancers. In countries with effective tobacco control measures, the incidence of new lung cancer has begun to decline in men, and is reaching a plateau for women [10–12]. Several other factors have been described, including exposure to asbestos, arsenic, radon and non-tobacco-related polycyclic aromatic hydrocarbons. There is evidence that lung cancer rates are higher in cities than in rural settings, but many confounding factors other than outdoor air pollution may be responsible for this pattern. Interesting hypotheses about indoor air pollution (e.g. coal-fuelled stoves and cooking fumes) are available, showing a correlation with the relatively high burden of non-smoking-related lung cancer in women in some countries [13]. Evidence for a genetic predisposition to lung cancer has been difficult to establish as it is confounded by environmental exposures, but there are emerging data suggesting that single nucleotide polymorphisms in genes in certain loci – 15q24-25 (*CHRNA3, CHRNA5, CHRNAB4*), 6p21.33, 5p15.23 – have some association with lung cancer risk [15,16].

The World Health Organisation (WHO) estimates that lung cancer is the cause of 1.37 million deaths globally per year. An estimated 71% of these deaths are caused by smoking, indicating that about 400000 deaths annually are attributed to lung cancer in lifetime never smokers [1]. Prevalence of lung cancer in females without a history of tobacco smoking is estimated to represent 19%, compared with 9% of male lung carcinoma in the US [16,17]. Especially in Asian countries, an increase in the proportion of NSCLC in never smokers has been observed [18]. These new epidemiological data have resulted in ‘non-smoking-associated lung cancer’ being considered a distinct disease entity, where specific molecular and genetic tumour characteristics have been identified [19].

Classification of lung tumours

Almost all lung cancers are carcinomas (other histologies comprise well under 1%). The predominant histological types are adenocarcinoma, squamous cell carcinoma (SCC), small cell carcinoma (SCLC) and large cell carcinoma. Preinvasive lesions, benign epithelial tumours, lymphoproliferative tumours, and other miscellaneous tumours also occur, but they are relatively rare. The relative proportions of the histological types have varied considerably over the years. During the earliest period (1977-1981), squamous cell carcinoma accounted 32% of cases; but by 2006-2010, the proportion had declined to 20%. Adenocarcinoma accounted for less than 30% of cases during the earliest years, but the proportion increased to more than 40% by 2006-2010. The small cell proportion decreased from 17% to 13% and the large cell proportion from 8% to 2%. The other unspecified carcinoma proportion rose from 2% to 4%. The unspecified carcinoma proportion rose from 12% to 23% during 2001-2005, and then dropped to 18%. These recent trends reflect improvements in the determination of histological type over past decade, especially for large cell carcinoma and adenocarcinoma, after the introduction of immunohistochemical staining with TTF1 and squamous markers [20]. There is now an increased emphasis on the accurate determination of histological type, because of treatment and outcome implications.

Being this manuscript focused on a specific population of lung cancer patients, I will deliberately describe only some histologies.

Adenocarcinoma

Although most cases are seen in smokers, adenocarcinoma develops more frequently than any other histologic type of lung cancer in individuals (particularly women) who have never smoked [22,23]. Invasive adenocarcinoma is a malignant epithelial tumour with glandular differentiation, mucin production, or pneumocyte marker expression. The tumours show an acinary, papillary, micropapillary, lepidic or solid growth pattern, with either mucin or pneumocyte marker expression. Tumours are

classified according to their predominant pattern. Invasive adenocarcinomas characteristically consist of a complex heterogeneous mixture of histological subtypes, which often represent a morphological continuum rather than discrete compartments.

According to the last WHO classification of tumours of the lung, pleura, thymus and heart we can classified invasive adenocarcinomas according to different patterns: lepidic adenocarcinoma, acinar adenocarcinoma, papillary adenocarcinoma, micropapillary adenocarcinoma and solid adenocarcinoma [23] (Table 1).

The lepidic variant typically consists of bland pneumocyte cells growing along the surface of alveolar walls, similar to the morphology defined in the sections on minimally invasive adenocarcinoma and adenocarcinoma in situ. An invasive adenocarcinoma component is present in at least one focus of >5mm in greatest dimension.

The acinar variant shows a majority component of glands, which are round to oval-shaped with a central luminal space surrounded by tumour cells [21,25]. The neoplastic cells and/or glandular spaces may contain mucin. Acinar structures may also consist of rounded aggregates of tumour cells with peripheral nuclear polarization and central cytoplasm without a clear lumen.

The papillary variant shows a major component of a growth of glandular cells along central fibrovascular cores, while the micropapillary one has, as a major component, tumour cells growing in papillary tufts forming florets that lack fibrovascular cores [21,25]. The tumour cells are usually small and cuboidal, with variable nuclear atypia.

The solid variant shows a major component of polygonal tumour cells forming sheets that lack recognizable patterns of adenocarcinoma [21,25]. If the tumour is 100% solid, intracellular mucin should be present in more than 5 tumours cells in each of two high-power fields, and confirmed with histochemical stains for mucin [21,25]. Solid adenocarcinoma must be distinguished from squamous cell carcinomas and large cell carcinomas, both of which may show rare cells with intracellular mucin.

Currently, the most commonly used pneumocyte markers are TTF1 and napsin A. Approximately 75% of invasive adenocarcinomas ae positive for TTF1 [26,27]. Among the adenocarcinoma patterns, most lepidic and papillary areas are positive for TTF1, whereas positivity is less common in solid predominant cancer [27]. The sensitivity of napsin A is comparable with that of TTF1, although some reports have suggested that the former is superior for differentiating from squamous cell carcinoma if positive reactions from entrapped pneumocytes are excluded [28]. P40, which is expressed in a strong, diffuse manner in squamous cell carcinoma, is a more specific squamous marker than p63, as the latter is also positive in up to 30% of lung adenocarcinomas [26,30,31]. It is worth noting that TTF1 is also expressed in other tumours, such as small cell lung cancer, large cell neuroendocrine carcinomas, some carcinoid tumours, and thyroid carcinomas. Napsin A is sometimes expressed in other tumours such as renal cell carcinoma.

Several driver gene alterations are now known in lung adenocarcinomas, including *EGFR*, *KRAS*, *BRAF*, *ERBB2/HER2*, *ALK*, *ROS1*, *RET*, *NTRK1* and *NRG1. HER2, ROS1* and *NTRK1* share clinicopathological features with *EGFR* and *ALK* in terms of involvement that is nearly specific to adenocarcinoma in lung cancer, particularly frequent in TTF1 positive adenocarcinoma, and preferentially in never-smokers and women.

Unlike the specific alterations seen in other tumours, there are no specific histological-molecular correlations in lung cancer [21,25]. The strongest histological-molecular correlation is with the invasive mucinous adenocarcinoma, where a high percentage of these tumours have *KRAS* mutations and lack of *EGFR* mutations. *EGFR* mutations are most often seen in association with non-mucinous adenocarcinomas that are lepidic or papillary predominant, and there have been reports of an association with a micropapillary pattern [31–33]. *KRAS* mutations are reported most often in tumours with a solid pattern, and can be present in tumours producing extracellular mucin [34–36]. *ALK* rearrangement has been mostly associated with an acinar pattern, and with signet ring cell features [38,39].

Squamous cell carcinoma

Squamous cell carcinoma (SCC) is a malignant epithelial tumour that either shows keratinization and/or intercellular bridges, or is a morphologically undifferentiated non-small cell carcinoma that expresses immunohistochemical markers of squamous cell differentiation, that arises from bronchial epithelium. Like all lung cancers, but to a significantly higher degree than adenocarcinoma, SCC is strongly associated with smoking [39].

SCC may be keratinizing or non-keratinizing. Keratinizing SCC is recognized by the presence of keratinization, pearl formation, and/or intracellular bridges. These features vary with degree of differentiation; they are prominent in better differentiated tumours, where there is typically keratinization and present only focally or are less apparent in those that are poorly differentiated. In non-keratinizing SCC, immunohistochemistry is required to distinguish tumours from large cell carcinoma with a null phenotype. For such tumours, diffuse positive staining with a squamous marker (p40, p63, CK5, or CK 5/6) and negativity for TTF1 confirm their squamous phenotype and classification. The presence of intracellular mucin in a few cells does not exclude tumours from this category. Some non-keratinizing SCCs may morphologically resemble urothelial transitional cell carcinoma. Non-keratinizing SCC should show diffuse positive staining with p40, which is a more specific marker than p63, CK5, or CK5/6. In keratinizing squamous cell tumours, TTF1 also be negative. In non-keratinizing SCC, p40, p63, CK5, or CK5/6 are diffusely and strongly expressed; there may rarely be weak focal TTF1 expression [40].

A comprehensive analysis of the genome of SCC identified a very high rate of mutations per megabase (3-10 times higher than in other common cancer) [41], reflecting the mutagenic effects of cigarette smoke in this strongly smoking-associated lung cancer subtype.

SCCs are characterized by gene copy number alterations, including gain/amplification of chromosomes 3q (*SOX2, TP63*) (147,2655), 7p (*EGFR*), and 8p (*FGFR1*), as well as frequent deletion of chromosome 9p

(*CDKN2A*) (1006, 2320). Common gene mutations include *TP53, CDKN2, PTEN, PIK3CA, KEAP1, MLL2, HLA-A, NFE2L2, NOTCH1*, and *RB1* (936). With only rare exceptions, pure squamous carcinomas, as diagnosed in resection specimens, do not harbour *EGFR* and *KRAS* mutations [41, 42].

Small cell carcinoma

Small cell carcinoma is a malignant epithelial tumour that consists of small cells with scant cytoplasm, poorly defined cell borders, finely dispersed granular nuclear chromatin, and absent or inconspicuous nucleoli. Necrosis is typically extensive, and the mitotic count is high.

Combined small cell carcinoma, which is rare, has an additional component that consists of any of the histological types of NSCLC; usually adenocarcinoma, SCC, large cell carcinoma, large cell neuroendocrine carcinoma (LCNEC), or less commonly spindle cell carcinoma or giant cell carcinoma.

Among the major lung cancer subtypes, SCLC shows the strongest association with cigarette smoking; the odds ratio is estimated to be 111 in current smokers with a >30 pack-year history compared to never-smoker [43].

From a histopathological point of view, SCLC is characterised by densely packed small tumour cells that commonly form a sheet-like diffuse growth pattern, without obvious neuroendocrine morphology apart from nuclear characteristics. Architectural patterns such as nesting, trabeculae, peripheral palisading, and rosette formation are less common. There is a high mitotic rate (at least 10 mitoses per $2mm^2$, but averaging over 60 mitoses per $2mm^2$). The proliferative index is evaluated by Ki-67 antigen immunohistochemistry is >50%, averaging >80%.

Combined SCLC refers to the admixture of NSCLC element. Because of the morphological continuum between SCLC and LCNEC, at least 10% of the tumour should show large cells to be subclassified as combined SCLC and LCNEC. There is no percentage requirement for components of

adenocarcinoma, squamous cell carcinoma, or sarcomatoid carcinoma, as they are easily recognized [44].

The diagnosis of SCLC can be reliably made based on routine histological and cytological preparations, but immunohistochemistry may be required for confirmation of the neuroendocrine and epithelial nature of the tumour cells. Broadly reactive cytokeratin antibody mixtures, including AE1/AE3, CAM5.2, and MNF116, highlight epithelial differentiation in nearly all cases of SCLC, with either dot-like, paranuclear, or diffuse cytoplasmic staining pattern [44–46]. A high-molecular weight cytokeratin cocktail (recognizing CK1, CK5, CK10 and CK14) is always negative in pure SCLC [47]. A panel of neuroendocrine markers is useful, including NCAM/CD56 [48, 49]. Dense core granule-associated protein chromogranin A, and the synaptic vesicle protein synaptophysin (both with cytoplasmic labelling) are regularly expressed in SCLC [43,49]. NCAM/CD56 is the most sensitive marker, but it is also less specific, and should be interpreted in the appropriate morphological context. Synaptophysin and NCAM/CD56 can diffusely and strongly stain SCLC, while chromogranin A can be more focal and weak. However, <10% of SCLC can be completely unreactive or only very focally reactive for neuroendocrine markers, probably due to the lack of overt neuroendocrine differentiation [46]. SCLC is also positive for TTF1 in up to 90-95% of instances [44,50–52], whereas napsin A, a marker of adenocarcinoma differentiation, is consistently unreactive [54].

The neuroendocrine tumours of the lung do not form a continuous pathogenic spectrum and high-grade neuroendocrine tumours are driven by inactivating mutations in the *RB* and *TP53* genes. They contain the characteristic tobacco carcinogen-associated molecular signature (abundant numbers of mutation and high fraction of G→T transversions caused by polycyclic aromatic hydrocarbons, often occurring a methylated CpG dinucleotides) [54,55]. The early activation of *TP53* results in genomic instability, with multiple frequent sites of allelic imbalance [57], including losses as chromosome 3p, 4q, 13q and 15q. A small number of molecular changes, in particular *TP53* mutations and high total numbers of so-called

smoking signature mutations, are common to all lung cancers, but inactivating *RB1* mutations are a hallmark of SCLC. *PTEN* mutation and *FGFR1* amplifications occur in important subsets of SCLC (10% and 6%, respectively), as do *SOX2* amplification and mutations in *SLIT2*, *EPHA7*, and multiple histone modifier genes.

Many genomic and epigenomic aberrations have been identified (although clinical therapeutic targets have yet to be achieved) [58–60], such as KIT overexpression [61–63]; telomerase activation [63,64]; *RASSF1*inactivation upon hypermethylation [66]; and TTF1, BAI3, and BRN2 expression [66,67].

Molecular profile of NSCLC

In the beginning of the 20th century, Hansemann and Boveri hypothesized that cancer is the result of genetic lesions [69]. Today, we know that the epidemiology of lung cancer is strongly associated with environmental genotoxins, but our understanding of the biological implications of critical genetic alterations is still incomplete. Exposure to cigarette smoke is associated with approximately 90% of lung cancer [70]. Cigarette smoke contains more than 50 different carcinogens, that induce alterations in a large number of genes controlling the homeostasis of normal alveolar and bronchial cells [71]. With the use of cytogenetics, comparative genomic hybridization, and allelotyping, a wide array of genetic changes has been discovered in cancer. In the last years, there has been an increasing amount of new molecular alterations, identified in NSCLC including oncogenes and tumour suppressors genes [72]. Concurrent with the emergence of NSCLC subtype (histology) as an indicator of probable response to therapy, molecular biomarkers now have a similar role. Biomarkers may predict response to ‘traditional’ cytotoxic chemotherapeutic agents [73], but, more importantly, patients can be selected according to their likelihood of benefiting from molecularly targeted therapy [73,74]. The majority of these are small molecular inhibitors of a specific tyrosine kinase (TK) or a monoclonal antibody against a specific receptor. In general, these biomarkers either reflect the actual target of the specific drug or some factor that might abrogate the effect of the drug. In lung cancer medicine, this shift

towards specific treatments given to selected patients has been a revolution for oncologists and pathologists alike.

Proto-oncogenes are genes that contribute to malignant transformation when mutationally activated or overexpressed. Proto-oncogenes that have been associated with lung cancer include *EGFR, KRAS, MET, ALK, ROS1, BRAF, PIK3CA, RET* [76].

EGFR

The human epidermal growth factor (*EGF*) receptor (*HER*) gene family encodes proto-oncogenes, which belong to the class of receptor tyrosine kinase [77]. This family is also named *ERBB* after the viral erythroblastosis B oncogene and includes the EGF receptor (*EGFR/HER1*), *HER2, HER3* and *HER4.* These receptors bind ligands such as EGF and neuregulin 1. Binding of ligand induces receptor homo- or heterodimerization, activation of the intrinsic receptor tyrosine kinase, and activation of intracellular signal transduction pathways that stimulate proliferation and survival. The EGFR-HER2 heterodimer has been shown to initiate the strongest and most long-lived signalling in NSCLC models [78]. Strong *EGFR* expression is present in 40 to 80% of NSCLC tumour specimens [77,78]. High-level overexpression in lung cancer is amplification of the gene copy number [80]. Oncogenic mutations of *EGFR* and *HER2,* located in the kinase domain and leading to constitutive activation of the kinase, have been reported in up to 35% and 4% of NSCLC specimens, respectively [81]. *EGFR* mutations are found in about 10%-12% of Caucasians with adenocarcinoma and are more frequent in never smokers, females and in patients of East Asian ethnicity. Similar mutations have been detected in the normal respiratory epithelium of up to 43% of patients with *EGFR* mutant adenocarcinomas and are identical to those seen in tumour specimens from the same patient, suggesting that these mutations may be an early event of carcinogenesis, particularly in never smoker [82].

Activating *EGFR* mutations are predictive for response to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib, afatinib, dacomitinib, icotinib and

osimertinib. Such treatments result in improved response rate (RR) and progression-free survival (PFS), better tolerability and superior quality of life (QoL) when compared with platinum-based chemotherapy in the first-line setting, as demonstrated in several randomised trials [83]. *EGFR* mutation testing is recommended in all patients with advanced non-squamous cell carcinoma (NSCC), regardless of smoking history [84]. Molecular EGFR testing is not recommended in patients with a unequivocal diagnosis of squamous cell carcinoma, except in never/former light smokers (<15 pack years) [85]. EGFR mutation testing should provide an adequate coverage of all clinically relevant mutations; test methodology should have adequate coverage of mutations in exons 18–21, including those associated with resistance to some therapies. At a minimum, when resources or material are limited, the most common activating mutations (exon 19 deletion, exon 21 L858R point mutation) should be determined.

The majority of patients will progress after 9-12 months of treatment with an EGFR TKI, and various mechanisms of acquired resistance to first and second generation EGFR TKIs have been described [86]. The most common (49%-60%) mechanism of acquired resistance is the acquisition of a single recurrent missense mutation within exon 20, the T790M mutation [86,87]. This mutation leads to the substitution of threonine by methionine at position 790, which encodes part of the kinase domain of the receptor and results in increased affinity for ATP, causing resistance to competitive inhibition by EGFR TKIs [88,89]. Some third-generation EGFR TKIs, that are specifically designed to target EGFR T790M mutation have undergone clinical development. Among these, osimertinib, an oral, selective, irreversible EGFR TKI inhibitor with activity against T790M mutation is licensed for use in patients who have developed the EGFR T790M resistance mutation [89,90]. A recent phase III trial demonstrated a better PFS for *EGFR* positive patients treated with osimertinib compared to first generation TKIs in first line treatment [93]. A paper by Sequist et al., investigating the mechanism of acquired TKI resistance in *EGFR* mutated NSCLC (Figure 1), revealed that all drug-resistant tumours retained their

original activating *EGFR* mutation and some acquired the T790M mutation or *MET* amplification. Some resistant cancers showed unexpected genetic changes and surprisingly also unexpected histological changes. Another mechanism of resistance to an EGFR TKI is through *MET* amplification, and in vivo combination of EGFR and MET inhibition seems to overcome this resistance [94].

ALK

Anaplastic lymphoma kinase (ALK), also known as ALK tyrosine kinase receptor or cluster of differentiation 246 (CD246), is an enzyme that is encoded by the *ALK* gene and mutations in this gene, which has been implicated in the pathogenesis of NSCLC. This mutation is caused by fusion of the *EML4* gene with the signalling portion of the *ALK* gene resulting in the formation of the fusion protein EML4-ALK, which has been implicated as a driver of oncogenesis. An inversion on the short arm of chromosome 2 (Inv(2)(p21p63)) that joins exons 1 to 13 of EML4 to exons 20 to 29 of ALK leads to the formation of this EML4-ALK fusion oncogene. The resulting chimeric protein contains an N-terminus derived from EML4 and a C-terminus containing the entire intracellular tyrosine kinase domain of ALK. A study utilizing transgenic mouse lines that expressed EML4-ALK specifically in lung alveolar epithelial cells revealed that these mice developed hundreds of adenocarcinoma nodules in both lungs within few weeks after birth, and in vivo treatment of these EMK4-ALK transgenic mice with an oral small-molecule inhibitor of the kinase activity of ALK resulted in tumour regression, confirming the potent oncogenic activity of this fusion gene [95]. *ALK* fusion genes were first identified in anaplastic large cell lymphoma, and subsequently in NSCLC and rare tumours such inflammatory myofibroblastic tumour [95,96]. Overall, more than 20 *ALK* fusion partners have been identified. In lung cancer, aside from the major partner *EML4*, fusion with *KIF5B, TGF, KLC1,* and *HIPI* have been reported [97–103]. The breakpoints on the *ALK* gene almost always occur in intron 19 and, rarely, in exon 20, resulting in a constant inclusion of the ALK kinase

domain in the fusion gene/protein. A common feature of the fused partner genes is the presence of basic coil-coil domain, which allows the spontaneous dimerization of the fusion proteins. EML4-ALK, the most common ALK fusion found in NSCLC, is formed by an inversion occurring on the short arm of chromosome 2 and involves the genes encoding for ALK (2p23) and EML4 (2p21), with variants 1, 2, and 3a/3b being the most common fusion patterns among more than 13 variants [104]. Because the gene rearrangement involves large chromosomal inversion and translocation, FISH was the first method used for detecting all forms of ALK rearrangement, and until recently, FISH with ALK break-apart rearrangement probes was the reference criterion for the diagnosis of lung cancers with ALK rearrangement. More recently, the detection of ALK fusion protein by an ALK D5F3 IHC assay has received the Food and Drug Administration (FDA) approval for the selection of patients to be treated with an ALK inhibitor in the US. ALK fusion is encountered more frequently, but not exclusively, in never smokers, adenocarcinoma subtype and in younger patients, with a prevalence of around 5% in adenocarcinomas [104,105]. Patients ALK-rearranged can be treated in first line setting with crizotinib, a dual ALK and MET tyrosine kinase inhibitor, which activity was initially demonstrated in two multicentre single-arm studies with significant ORR and PFS advantages [107], as well as a survival advantage compared with other treatment [108]. Similar to the case with EGFR-mutated lung cancer, almost all ALK-rearranged tumours develop resistance to crizotinib treatment. Sequencing of the resistant tumour DNA has led to the identification of resistant point mutations on the ALK gene in 20% to 40% of patients [109–111]. These mutations result in decreased binding of the inhibitor or increased ATP binding affinity [109]. Other resistance mechanisms have also been identified, including the activation of EGFR and KRAS pathways by their respective mutations, and ALK and KIT gene amplification [108,109]. Second-generation ALK inhibitors (ceritinib and alectinib) that may overcome some of these resistances have recently

received approval in various parts of the world, including the United States, Europe, and Japan [112].

ROS1

The human *ROS1* gene is located on chromosome 6p22 and encodes a tyrosine kinase receptor that is evolutionally related to the ALK receptor. It is a homologue of the chicken c-ros, proto-oncogene of v-ros from UR2 avian sarcoma virus [112,113]. The first *ROS1* fusion gene discovered was FIG(GOPC)-ROS1 in human glioblastoma cell line U-118 MG; it resulted from a 6p deletion between the FIG and ROS1 genes [114,115]. The FIG-ROS1 fusion subsequently was found in cholangiocarcinoma and lung adenocarcinoma [117]. Other ROS1 fusion partners that have been identified in lung cancer include SLC34A2, CD74, TPM3, SDC4, EZR, LRIG3, KDEL R2, LIMA1, MSN, CLTC, CCDC6, TMEM106, and TPD52L1 [118–121]. With more widespread profiling of tumours with NGS, the number of ROS1 fusion partners likely will continue to grow. The mechanism by which ROS1 fusion proteins become oncogenic remains unclear. Unlike ALK-fusion oncogenes, a majority of ROS1 fusions lack coil-coil domain that promotes spontaneous dimerization and kinase activation [122]. Nevertheless, some ALK inhibitors inhibit proliferation of the cell line HCC-78, which harbours *ROS1* rearrangement [121,122], probably as a result of evolutional correlation of both molecules. Furthermore, in the expansion cohort of the PROFILE 1001 trial, the response rate to crizotinib was 72% among patients with lung cancer in which ROS1 rearrangement was identified by break-apart FISH assay [125]. This result formed the basis for approval of crizotinib for the treatment of ROS1-rearranged lung cancer in the US and EU. However, several resistance mutations in *ROS1* fusion genes acquired during the treatment of ROS1-rearranged lung cancer with crizotinib have already been identified. Considering the rapid evolution of ALK inhibitor therapies, one can expect that a strategy for overcoming the resistance mechanism in ROS1-rearranged lung cancer will soon be forthcoming.

KRAS

The rat sarcoma viral oncogene homolog (*RAS)* family of proto-oncogenes (*KRAS, NRAS*, and *HRAS*) encodes membrane-bound GTPases, which link signalling from growth factor receptors to the mitogen-activated protein (MAP) kinase proliferation pathway [126]. The *RAS* oncogene was first isolated from a virus causing rat sarcoma. *KRAS* is activated by point mutation in up to 35% of lung adenocarcinomas and *KRAS* mutations are associated with smoking [127]. Oncogenic mutations of *KRAS* is sufficient to expand murine bronchioalveolar stem cells in culture and in vivo [128]. The mutational and stem cell model-derived data indicate that KRAS is a strong oncoprotein of the lung. The function of KRAS is reliant on membrane-anchoring of the protein, which in turn is facilitated by several posttranslational modifications, including farnesylation, geranylation, methylation, and palmitoylation. Despite KRAS being one of the earliest known oncogenic drivers in NSCLC, effective targeting remains a therapeutic challenge.

MET

The *MET* gene is located on the long arm of chromosome 7 at position 31 [129]. This oncogene encodes for a tyrosine kinase receptor (hepatocyte growth factor receptor), which activates multiple signalling pathways, that play fundamental roles in cell proliferation, survival, motility, and invasion [130]. Pathologic activation of *MET* includes mutation, gene amplification, and protein overexpression [131]. MET alterations were first reported in patients with renal papillary carcinoma and mutations in the MET kinase domain leading to constitutive activation of the receptor [132]. In lung cancer, *MET* mutations are found in the extracellular semaphorin and juxtamembrane domains, occurring in 3% of squamous cell lung cancers and 8% of lung adenocarcinomas [131]. *MET* amplifications are found in 4% of lung adenocarcinomas and 1% of squamous cell lung cancers and are associated with sensitivity to MET inhibitors [131]. In NSCLC, MET and hepatocyte growth factor protein expression, along with high *MET* gene

copy number, have been described as poor prognosis factors [132,133]. *MET* amplification in NSCLC is implicated in acquired resistance to EGFR inhibitors and has been reported in approximately one-fifth of cases with EGFR inhibitor resistance [135].

Activating point mutations affecting splice sites of exon 14 of the *MET* gene (*MET*ex14), which occur in 4% of lung adenocarcinomas, represent a possible oncogenic driver and identify a subset of patients who may benefit from MET inhibitors, such as capmatinib and crizotinib [131]. This novel alteration is usually assayed by NGS methodology.

B-RAF

The B-RAF proto-oncogene, serine/threonine kinase (*BRAF*) oncogene is located on the long arm of chromosome 7 at position 34. It encodes for a serine/threonine kinase, which is involved in the RAS/RAF/MEK/ERK signalling pathway [136]. When activated by oncogenic mutations, BRAF phosphorylates MEK and promotes cell growth, proliferation, and survival. The highest incidence of *BRAF* mutation is in malignant melanoma (27%–70%), followed by papillary thyroid cancer, colorectal cancer, and serous ovarian cancer [137]. *BRAF* mutations have also been reported in 1% to 3% of NSCLCs [136]. In contrast to melanoma, only half of *BRAF* mutations in NSCLC are V600E. Other non-V600E mutations reported in NSCLC include G469A (35%) and D594G (10%). All BRAF mutations are mutually exclusive with other driver alterations, such as those of *EGFR*, *KRAS*, and *ALK* [135,137]. *BRAF*-mutated NSCLC has been reported to be mostly adenocarcinoma, and in contrast to patients with *EGFR* mutations or *ALK* rearrangements who are mostly never-smokers, patients with *BRAF* mutations are mostly current or former smokers [138]. Nevertheless, patients with NSCLC and *BRAF* V600E mutations have a worse prognosis and lower response to platinum-based chemotherapy than patients with wild-type *BRAF*. These patients have benefited from treatment with BRAF and MEK inhibitors [138,139]. BRAF inhibitors, such as vemurafenib and dabrafenib, have high and selective activity against the V600E-mutant BRAF kinase, with overall responses rates from 33% to 42% [138,140].

RET

The *RET* proto-oncogene is located on the long arm of chromosome 10 at position 11.2. It encodes for a tyrosine kinase receptor for the glial cell line–derived neurotrophic factor family of ligands and is involved in cell proliferation, migration, and differentiation, and neuronal navigation [142]. *RET* chromosomal rearrangements were originally described in papillary thyroid carcinoma [142]. Approximately 1% to 2% of NSCLCs harbour *RET* fusions, and several fusion partners, including kinesin family member 5B (*KIF5B*; 90%), coiled-coil domain containing 6 (*CCDC6*), nuclear receptor coactivator 4 (*NCOA4*), and tripartite motif-containing 33 (*TRIM33*), have been described [142,143]. RET-rearranged NSCLC typically occurs in adenocarcinomas with more poorly differentiated solid features in young never-smokers, and it is mutually exclusive with known driver oncogenes [142,144]. In vitro studies showed that *RET* fusions lead to oncogenic transformation, which can be inhibited by multitargeted kinase inhibitors, such as vandetanib, sorafenib, and sunitinib [145]. Preliminary studies with cabozantinib (MET and vascular endothelial growth factor receptor 2 inhibitor) in *RET*-rearranged lung adenocarcinoma are promising [144]. FISH is currently the standard diagnostic assay for detection of *RET* chromosomal rearrangements. RT-PCR is usually insufficient for the detection of new partners or isoforms, and *RET* IHC has shown low sensitivity and specificity for *RET* rearrangements [142,144]. Sequencing approaches, including NGS methodologies, are also frequently used to detect *RET* translocations.

Her2

The human EGFR 2 gene *HER2 (ERBB2)* is a proto-oncogene located on chromosome 17 at position 12.46 It encodes for a tyrosine kinase receptor member of the ERBB receptor family [146]. HER2 lacks a specific ligand. Nevertheless, it can be combined with other ERBB receptors to form a heterodimer [147]. This allows for the activation of important signal transduction pathways, including the MAPK and PI3K pathways, involved in

cell proliferation, differentiation, and migration [146]. HER2 expression and/or amplification are found in many cancers including breast and gastric cancer [147]. Overexpression of HER2 has been reported in 7% to 34.9% of NSCLCs and has been associated with poor prognosis in patients with these tumours [146]. Activating mutations of *HER2* have been found in 1.6% to 4% of lung cancers [80,145]. These mutations occur in the four exons of the tyrosine kinase domain (exons 18–21) and are found more often in adenocarcinomas in female, Asian, never-smokers, or light smokers. *HER2* mutations are almost always mutually exclusive with other driver oncogene alterations in lung cancer described previously [81]. Different studies reinforce the importance of screening lung adenocarcinomas for *HER2* mutation as a method to select patients who could benefit from *HER2*-targeted therapies (afatinib and trastuzumab), which have shown response rates of approximately 50% [148]. Several clinical trials of targeted agents, such as trastuzumab, neratinib, and pyrotinib, among others, are being conducted in patients with *HER2* mutation [148]. HER2 mutations are usually assessed via sequencing approaches.

PIK3CA

PI3Ks are heterodimeric lipid kinases composed of catalytic and regulatory subunits and are part of several downstream pathways involved in cell growth, transformation, adhesion, apoptosis, survival, and motility [149]. The *PIK3CA* gene is located on the long arm of chromosome 3 at position 26.3. It encodes for the catalytic subunit p110 alpha of P13Ks [150]. *PIK3CA* amplifications, deletions, and somatic missense mutations have been reported in many tumours including lung cancers. In fact, *PIK3CA* is one of the most commonly mutated oncogenes, along with *KRAS*, in human cancers [151]. Mutations are found in 1% to 4% of patients with NSCLC, usually affecting exons 9 and 20 (80%) [129,148,150–152]. These mutations are not mutually exclusive with other driver alterations and have been reported more frequently in lung squamous cell carcinoma compared with adenocarcinoma (6.5% versus 1.5%) [153]. However, *PIK3CA* mutations have not shown association with any clinicopathologic features

[148,151,152]. Squamous cell carcinomas with *PIK3CA* gains are not accompanied by other genetic alterations, suggesting that this gene may play an important role in the pathogenesis of squamous cell cancers [149]. Studies have shown that *PIK3CA* mutations in *EGFR*-mutated lung cancer confer resistance to EGFR-TKIs and are a negative prognostic predictor in patients with NSCLC treated with EGFR-TKIs [154]. *PI3KCA* alterations and their downstream effectors, such as phosphatase and tensin homolog, mTOR, and AKT, are potential therapeutic targets for NSCLC therapy and are being evaluated in clinical trials for lung cancer [155]. Alterations in *PIK3CA* are detected using sequencing approaches, mostly NGS assays.

NTRK1

The neurotrophic receptor tyrosine kinase 1 (*NTRK1*) proto-oncogene is located on chromosome 1q21 to 22 and encodes for a receptor tyrosine kinase, also known as tropomyosin-related kinase (TRK) A, belonging to the TRK superfamily of receptor tyrosine kinases [156]. *NTRK1* is involved in the regulation of cell growth and differentiation via activation of several signal transduction pathways including MAPK, PI3K, and phospholipase C-gamma. *NTRK1* rearrangements have been found in colon cancer, thyroid cancer, and glioblastoma multiforme [156]. In lung cancer, approximately 3% of adenocarcinomas harbour *NTRK1* fusions, and some fusion partners, including myosin phosphatase RHO-interacting protein *(MPRIP)-NTRK1* and *CD74-NTRK1*, have been reported [157]. All of these fusions result in constitutive TRKA kinase activity, which has been reported to be oncogenic [157]. In early phase 1 studies, NTRK inhibitors, such as entrectinib and LOXO-101, have shown promising results in patients with solid tumours harbouring NTRK fusions [158].

Treatment strategies for the metastatic disease

The treatment strategy should take into account factors like histology, molecular pathology, age, PS, comorbidities and patient's preferences. Treatment decisions should ideally be discussed within a multidisciplinary

tumour board, who can recommend additional investigations and changes in treatment modality [159].

First-line and second line treatments for EGFR and ALK-negative disease

Chemotherapy with platinum-doublets should be considered in all stage IV NSCLC patients with *EGFR* and *ALK* negative disease, without major comorbidities and PS 0-2. Benefits of chemotherapy versus best supportive care (BSC), are observed irrespective of age, sex, histology and PS in two meta-analyses [159,160]. The survival benefit of two-agent over one-agent chemotherapy regimens was reported in a meta-analysis in 2004, with no survival benefit seen for three-agent over two-agent regimens [162]. Based on a 2006 meta-analysis, revealing a statistically significant reduction (equal to 22%) in the risk of death at one year for platinum over non-platinum combinations, without induction of unacceptable increase in toxicity, platinum-based doublets are recommended in all patients with no contraindications to platinum compounds [163]. Neither a large individual trial nor a meta-analysis found an overall survival (OS) benefit of six versus fewer cycles of first-line platinum-based doublets, although a longer PFS coupled with significantly higher toxicity was reported in patients receiving six cycles [162,163]. Therefore, four cycles of platinum-based doublets followed by less toxic maintenance monotherapy, or four up to a maximum of six cycles in patients not suitable for maintenance monotherapy, are currently recommended.

Several platinum-based regimen with third-generation cytotoxics (cisplatin/paclitaxel, cisplatin/gemcitabine, cisplatin/docetaxel, carboplatin/paclitaxel) have shown comparable efficacy [166].

Any platinum-based doublets with a third-generation agent, including gemcitabine, vinorelbine or taxanes, might be used in NSCLC. However, the incorporation of pemetrexed and bevacizumab into individual treatment schedules should be considered in NSCC. Pemetrexed-based combination chemotherapy represents a therapeutic option, based on the results of a

meta-analysis that showed a slight but significant survival benefit compared with gemcitabine- or docetaxel-based combinations and of a pre-planned subgroup analysis of a large randomised phase III trial [165,166].

Two randomised clinical trials revealed that bevacizumab, a monoclonal antibody against the vascular endothelial growth factor (VEGF), improves OS when combined with paclitaxel/carboplatin regimens in patients with NSCC and PS 0–1, and, therefore, may be offered in the absence of contraindications in eligible patients with advanced NSCC [167,168]. While one trial of non-taxane, gemcitabine/cisplatin combination with or without bevacizumab demonstrated an objective RR (ORR) and modest PFS advantage, but no OS benefit [171], two meta-analyses showed a consistent significant improvement of RR, PFS and OS for the combination of bevacizumab and platinum-based chemotherapy, compared with platinum-based chemotherapy alone in eligible patients with NSCC [171,172]. Treatment with bevacizumab also delayed the incidence of brain metastases in a retrospective analysis [174].

After four cycles of platinum-based doublets, decision making about maintenance therapy must take into account histology, residual toxicity after first-line chemotherapy, response to first line treatment, PS and patient preference. Several trials have investigated the role of maintenance treatment in patients with good PS (0-1) either as ‘continuation maintenance’ or as ‘switch maintenance’. ‘Continuation maintenance’ and ‘switch maintenance’ therapies refer, respectively, to either the maintained use of an agent included in first-line treatment or the introduction of a new agent after four cycles of platinum-based chemotherapy.

A randomised phase III switch maintenance trials have reported improvements in PFS and OS with pemetrexed [175] versus placebo following four cycles of platinum-based chemotherapy in NSCC.

A large phase III randomised trial of continuation maintenance with pemetrexed versus placebo after four induction cycles of cisplatin plus pemetrexed chemotherapy demonstrated a PFS and OS improvement in patients with a PS 0–1, confirmed at long-term follow-up [175,176]. Median

OS was 13.9 months (95% CI, 12.8 to 16.0 months) for pemetrexed and 11.0 months (95% CI, 10.0 to 12.5 months) for placebo, with 1-year and 2-year survival rates significantly longer for patients given pemetrexed (58% and 32%, respectively) than for those given placebo (45% and 21%).

Another phase III study comparing maintenance bevacizumab, with or without pemetrexed, after first-line induction with bevacizumab, cisplatin and pemetrexed showed a benefit in PFS for the pemetrexed-bevacizumab combination but no improvement in OS [178], although a trend towards improved OS was seen when analysing 58% of events of 253 patients randomised for this study [179]. Continuing pemetrexed following completion of four cycles of first-line cisplatin/pemetrexed chemotherapy is, therefore, recommended in patients with non-squamous histology, in the absence of progression after first-line chemotherapy, and upon recovery from toxicities from the previous treatment.

Chemotherapy is not the only therapeutic approach available for *EGFR* and *ALK* negative patients in first line setting. Lung cancer has been historically considered poorly immunogenic, with no established benefit from cytokine modulation or vaccines. Nevertheless, the recent development of checkpoint inhibitors provided a promising new approach for immunotherapy in patients with NSCLC. Immune checkpoints are inhibitory pathways that maintain self-tolerance and protect peripheral tissues by restricting the immune responses. The two checkpoint targets that have been studied more extensively in lung cancer are the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the programmed death ligand 1 (PD-1) receptor.

Pembrolizumab is an anti-PD-1 monoclonal antibody that has recently received an approval in first line treatment for patients with NSCLC and a tumour expression of PD-L1 on at least 50% [180]. The KEYNOTE-04 study was an open-label, phase III trial, for untreated advanced NSCLC with PD-L1 expression on at least 50%, that randomised to receive either pembrolizumab (at a fixed dose of 200 mg every 3 weeks) or platinum-based chemotherapy. Median PFS was 10.3 months (95% CI, 6.7 to not

reached) in the pembrolizumab group versus 6.0 months (95% CI, 4.2 to 6.2) in the chemotherapy group (HR for disease progression or death, 0.50; 95% CI, 0.37 to 0.68; $P < 0.001$). The estimated rate of OS at 6 months was 80.2% in the pembrolizumab group versus 72.4% in the chemotherapy group (HR for death, 0.60; 95% CI, 0.41 to 0.89; $P = 0.005$). The RR was higher in the pembrolizumab group than in the chemotherapy group (44.8% versus 27.8%), the median duration of response was longer (not reached versus 6.3 months), and treatment-related adverse events of any grade were less frequent (occurring in 73.4% versus 90.0% of patients), as were grade 3, 4, or 5 treatment-related adverse events (26.6% versus 53.3%).
The recommended dose and schedule of pembrolizumab in first line is 200 mg (flat dose) administered as an intravenous infusion every 3 week.
Combination of chemotherapy in association with anti PD-1 or anti PD-L1 treatments are under investigations in several phases II- III trials.
The FDA approved the use of pembrolizumab in combination with carboplatin and pemetrexed for the first-line treatment of metastatic NSCC, irrespective of PD-L1 expression based on the results of a randomized phase II trial (KEYNOTE-021) [181].
Atezolizumab, an anti-PD-L1 monoclonal antibody, recently demonstrated its benefit, in a phase III trial, in terms of PFS in association with carboplatin-paclitaxel and bevacizumab compared to carboplatin-paclitaxel in all patients independently from the PD-1 status[182].

For several decades only three options were available in second line treatment: pemetrexed and docetaxel monotherapy, or erlotinib [183–185].
Treatment choice was guided by: first line treatment combination, residual toxicities from first line, histology and PS.
A phase III second-line study demonstrated non-inferiority for OS between pemetrexed and docetaxel (8.3 versus 7.9 months, HR 0.99, 95% CI: 0.8–1.2). However, pemetrexed showed a better toxicity profile with a significantly lower rate of neutropenia and alopecia as well as lower rates of gastrointestinal adverse events [183]. In a retrospective analysis a

predictive impact of histology on outcome by pemetrexed was reported favouring those patients with NSCC (median OS: 8.0 versus 9.3 months, docetaxel versus pemetrexed, HR 0.78, 95% CI: 0.61-1.0, *P* = 0.004) [186]. Erlotinib improved OS in second-line or in third-line in all NSCLC histological subtype patients not eligible for further chemotherapy, including patients with PS 3 [185]. Erlotinib was shown to be equivalent to pemetrexed or docetaxel in refractory (progression during the four cycles of a standard platinum-based chemotherapy doublet) and in unselected patients in a randomised trial [187]. Finally, a large randomised phase III trial showed comparable outcome with pemetrexed or erlotinib [188].

Erlotinib still represents a potential second-line treatment option in pre-treated patients with unknown or wild type (WT) EGFR status and preferably in patients not suitable for chemotherapy, with, however, limited efficacy in WT EGFR patients when compared with chemotherapy.

While registration trials of pemetrexed, docetaxel and erlotinib did not limit therapy to a set number of treatment cycles, second-line treatment duration should be individualised, and treatment may be prolonged if disease is controlled and toxicity acceptable.

The development of antibodies against PD-1 has dramatically changed the treatment strategies in second line in both SCC and NSCC.

Among the antibodies against PD-1, nivolumab, a fully IgG4 PD-1 immune checkpoint inhibitor, was the first to be investigated in phase III trials and it is approved after a platinum-based chemotherapy in both squamous and non-squamous tumours independently from the PD-L1 expression.

A phase III trial (CheckMate-017) in patients previously treated for a SCC, compared 3 mg/kg of nivolumab given every 2 weeks to docetaxel, showing an improvement in terms of PFS of 3.2 months [193,194] leading to its approval by the FDA and EMA. At the data cut-off, median OS was 9.2 months (95% CI 7.3–13.3 months) on nivolumab compared with 6.0 months (95% CI 5.1–7.3 months) on docetaxel, with a 41% reduction in the risk of death in the nivolumab arm (HR 0.59, 95% CI 0.44–0.49; *P* < 0.001). An updated follow-up reported an 18 months-OS of 28% and 13% in the

nivolumab and docetaxel arm respectively and an 18 months-PFS equal to 17% for nivolumab and 2.7% for docetaxel [190].

In the phase III trial, nivolumab was better tolerated than docetaxel, the experimental arm also showed a positive impact on QoL and a longer time to symptom deterioration compared to the standard arm [191]. The expression of PD-L1 was neither prognostic nor predictive of clinical benefit in a retrospective analysis using various cut-points in this study.

Nivolumab also led to a significant prolongation of OS compared with docetaxel in 582 pretreated patients with NSCC, who were recruited to the Checkmate-057 trial (median OS 12.2 versus 9.4 months, HR 0.73, 95% CI: 0.59-0.89; *P* = 0.002), although a small excess of early progression and/or death events were observed for nivolumab, compared with docetaxel. In addition, RR (19% versus 12%, *P* = 0.021) and duration of response (17.2 versus 5.6 months) were in favour of nivolumab, while no significant difference has been reported for PFS (median PFS 2.3 versus 4.2 months, HR 0.92, 95% CI: 0.77-1.1). An exploratory retrospective analysis revealed an association of efficacy by nivolumab and the level of tumour membrane expression of PD-L1. However, this analysis is limited by the retrospective and unplanned nature of this biomarker assessment. In the nivolumab arm, compared with docetaxel, a lower frequency of both severe adverse events (CTCAE grade 3/4 events: 10% versus 54%) and adverse events leading to treatment discontinuation (5% versus 15%) were observed. The most frequent selected adverse events were rash, pruritus, diarrhea, hypothyroidism, elevation of liver enzymes and pneumonitis [192].

Pembrolizumab received accelerated FDA and subsequently EMA approval for the treatment of any histological type of NSCLC after failure of first-line therapy in patients with tumours expressing PD-L1 on at least at 1%.

The phase III KEYNOTE-010 trial randomised 1034 patients with previously treated NSCLC with PD-L1 expression to receive pembrolizumab 2 mg/kg, pembrolizumab 10 mg/kg or docetaxel 75 mg/m² every 3 weeks. The primary end points were OS and PFS both in the total population and in patients with PD-L1 expression on at least 50% of tumour cells [193]. In the

entire population, OS was significantly longer for pembrolizumab 2 mg/kg versus docetaxel (HR 0.71, 95% CI: 0.58–0.88; *P* = 0.0008) and for pembrolizumab 10 mg/kg versus docetaxel (HR 0.61, 95% CI: 0.49–0.75; *P* < 0.0001), with median OS of 10.4, 12.7 and 8.5 months in the three arms, respectively. Grade 3–5 treatment-related adverse events were less common with pembrolizumab than with docetaxel. The recommended dose and schedule of pembrolizumab in second line is 2 mg/kg administered as an intravenous infusion every 3 week.

Immunotherapy should be the reference second line treatments in both squamous and non-squamous NSLC, while patients not eligible to this treatment can receive chemotherapy (docetaxel or pemetrexed) or erlotinib.

EGFR-mutated NSCLC patients

Among EGFR TKIs we can identify three generations of drugs. The first-generation *EGFR* TKIs (gefitinib, erlotinib, icotinib), 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) compared to platinum-based chemotherapy. The second-generation irreversible *EGFR/HER* TKIs (afatinib, dacomitinib) were developed to treat resistant disease, targeting not only the exon 20 T790M mutation, but also the *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 WT *EGFR*. The third-generation *EGFR* TKIs selectively and irreversibly target *EGFR* T790M and activating *EGFR* mutations (with limited activity against *EGFR* WT), showing efficacy in NSCLC resistant to the first- and second-generation *EGFR* TKIs and also in frontline treatment comparing to first generation TKIs [194].

Several studies have consistently demonstrated that the EGFR TKIs produce higher RR, longer PFS and improve QoL when compared with standard platinum-based doublet chemotherapy in patients with good PS (PS 0-2), whose tumour harbours an activating (sensitising) EGFR mutation

[195–202]. Patients with PS 3–4 may also be offered an EGFR TKI, as they are likely to receive a similar clinical benefit to patients with good PS [203]. The phase IIb study LUX-LUNG 7 showed that afatinib achieves higher RR and longer PFS than gefitinib as first-line treatment for patients with advanced NSCLC with common activating mutations (del19 or L858R). Data on OS (co-primary end point) showed no difference between the two treatments [204].

EGFR TKIs represent the standard-of-care as first-line treatment for advanced *EGFR* mutant NSCLC. All patients with advanced NSCC, and patients with squamous histology who are never/former light smokers (<15 pack years), should have their tumour tested with EGFR mutational analysis at diagnosis, and results should preferably be available before initiation of first-line treatment.

Notably, none of the above studies have shown any benefit in OS for an EGFR TKI over platinum-based chemotherapy, likely due to the high level of crossover. However, an unplanned pooled OS analysis of patients who have been recruited to either the LUX-Lung 3 or the LUX-Lung 6 trial revealed an OS benefit for afatinib compared with chemotherapy in patients with EGFR del-19 mutations (median OS: 27.3 months versus 24.3 months; HR 0.81, 95% CI: 0.66-0.99; *P* = 0.0374), whereas this improvement was not observed in patients with EGFR Leu858Arg mutations [205]. This data was not confirmed in a prespecified subgroup analyses of the LUX-Lung 7 trial, that showed similar OS trends in patients with exon 19 deletion (30.7 versus 26.4 months; HR, 0.83, 95% CI 0.58–1.17, *P* = 0.2841) and L858R (25.0 versus 21.2 months; HR 0.91, 95% CI 0.62–1.36, *P*= 0.6585) mutations [204].

Should the information on the presence of an EGFR-sensitising mutation become available during first-line platinum-based chemotherapy, it is recommended to continue chemotherapy for up to four cycles, and then to offer the EGFR TKI as maintenance treatment in those patients achieving disease control [206], or as second-line treatment at the time of progression.

In a Japanese randomized trial 154 *EGFR* mutated patients were

randomized to receive erlotinib and bevacizumab or erlotinib alone (75 patients in the combination arm and 77 in the erlotinib alone arm were included in the efficacy analyses) [207]. Median progression-free survival was 16.0 months (95% CI 13.9–18.1) with erlotinib plus bevacizumab and 9.7 months (5.7–11.1) with erlotinib alone (HR 0.54, 95% CI 0.36–0.79; log-rank test *P* = 0.0015). A similar PFS was described in a European phase II trial also using the combination of erlotinib and bevacizumab [208]. This treatment combination is an option in EGFR mutated patients but not available in many European countries.

Osimertinib showed efficacy superior to that of standard EGFR-TKIs in the first-line treatment of EGFR mutation–positive advanced NSCLC, with a similar safety profile and lower rates of serious adverse events. In a phase 3 trial, 556 patients with previously untreated, *EGFR* mutation–positive (exon 19 deletion or L858R) advanced NSCLC were randomised to receive either osimertinib (at a dose of 80 mg once daily) or a standard EGFR-TKI (gefitinib at a dose of 250 mg once daily or erlotinib at a dose of 150 mg once daily). The median progression-free survival was significantly longer with osimertinib than with standard EGFR-TKIs (18.9 months versus 10.2 months; HR for disease progression or death, 0.46; 95% confidence interval [CI], 0.37 to 0.57; *P*<0.001). The objective response rate was similar in the two groups: 80% with osimertinib and 76% with standard EGFR-TKIs (OR, 1.27; 95% CI, 0.85 to 1.90; *P*=0.24). The median duration of response was 17.2 months (95% CI, 13.8 to 22.0) with osimertinib versus 8.5 months (95% CI, 7.3 to 9.8) with standard EGFR-TKIs. Data on overall survival were immature at the interim analysis (25% maturity). The survival rate at 18 months was 83% (95% CI, 78 to 87) with osimertinib and 71% (95% CI, 65 to 76) with standard EGFR-TKIs (hazard ratio for death, 0.63; 95% CI, 0.45 to 0.88; *P*=0.007 [nonsignificant in the interim analysis]) [93]. Osimiertinib is not still available in clinical practice in this setting of patients.

The majority of patients will progress after 9-12 months of treatment with an EGFR TKI, and various mechanisms of acquired resistance to first-and second generation EGFR TKIs have been described (Figure 1) [86]. The

most common (49%-60%) mechanism is the acquisition of a single recurrent missense mutation within exon 20, the T790M mutation [86,87]. This mutation leads to the substitution of threonine by methionine at position 790, which encodes part of the kinase domain of the receptor and results in increased affinity for ATP, causing resistance to competitive inhibition by reversible EGFR TKIs [89, 90].

A number of third-generation EGFR TKIs that are specifically designed to target EGFR T790M mutation have undergone clinical development. Among these, osimertinib, is licensed for use in patients who have developed the EGFR T790M resistance mutation as previously described [90,91].

Patients who progress after an EGFR TKI should undergo a re-biopsy to perform molecular analysis specifically looking for EGFR T790M mutation, as this could influence the next therapeutic step or reveal alternative EGFR TKI resistance mechanisms such as transformation to SCLC or bypass tracks that could potentially be addressed in clinical trials.

At the present time, when re-biopsy is not feasible or when the EGFR T790M mutation is not detected as resistance mechanism, the standard of care is represented by platinum-based chemotherapy alone. There is no data to support continuation of the EGFR TKI with platinum-based chemotherapy [209].

A good alternative to tissue re-biopsy is represented by liquid biopsy, which has been validated [210] and represents a surrogate source of DNA and a new strategy for tumour genotyping, mainly at the time of progression for *EGFR*-mutated patients [211–213]. In case a T790M mutation in peripheral blood is observed treatment with 3rd generation EGFR TKI's is justified [214]. In case of a T790M negative liquid biopsy is observed, a re-biopsy is recommended if feasible and accepted by the patient.

A phase II study has demonstrated benefit in PFS in patients who continued first-line erlotinib beyond radiological progression [215]; therefore, this strategy could be considered in patients with asymptomatic progression. Evidence from retrospective series and case reports suggests that, in

patients where there is evidence of radiological progression in a single site (i.e. central nervous system metastasis or adrenal gland), but with ongoing dependence on the driver oncogene addiction and without rapid systemic progression, the combination of continuing the EGFR TKI with local treatment (radiotherapy or surgery) may represent a reasonable option and could be considered on an individualised basis [216].

The treatment strategy and the evolution of *EGFR* positive patients could substantially change in case of approval of osimertinib in first line. Mechanisms of resistance to osimertinib that have been identified in patients with T790M-positive NSCLC after EGFR-TKI treatment include acquired *EGFR* mutations (e.g., C797S), *MET* and *HER2* amplification, and SLCC [217–219]. Mechanisms of resistance to osimertinib when used as first-line therapy remain to be fully characterized, although an analysis of genomic mechanisms of resistance in nine patients with previously untreated EGFR mutation–positive advanced NSCLC who received osimertinib in the phase 1 component of the AURA trial showed no cases of acquired T790M mutation [220].

ALK-rearranged NSCLC patients

The anti-tumour activity of crizotinib, a dual ALK and MET tyrosine kinase inhibitor, was initially demonstrated in two multicentre single-arm studies with significant ORR and PFS advantages [107], as well as a survival advantage compared with other treatment [108].

The phase III PROFILE 1007 study confirmed the benefit of crizotinib over chemotherapy, pemetrexed or docetaxel (investigator´s choice), as second-line treatment with better ORR and PFS [221]. The median PFS, as determined by independent radiologic review, was 7.7 months (95% CI, 6.0 to 8.8) in the crizotinib group, as compared with 3.0 months (95% CI, 2.6 to 4.3) in the chemotherapy group (HR for disease progression or death with crizotinib, 0.49; 95% CI, 0.37 to 0.64; $P < 0.001$). Crizotinib also showed an advantage over both pemetrexed and docetaxel with regards to the improvement in symptoms and QoL [222].

Based on these data, any patient with NSCLC harbouring an ALK fusion and previously treated should receive crizotinib in second line, if this was not previously administered.

Subsequently, the phase III study PROFILE 1014 compared crizotinib with cisplatin-pemetrexed without maintenance pemetrexed as first-line treatment in ALK-positive advanced NSCC [223], and demonstrated a significantly longer PFS (median, 10.9 months versus 7.0 months; HR for progression or death with crizotinib, 0.45; 95% CI, 0.35 to 0.60; $P < 0.001$) and higher ORR with crizotinib compared with chemotherapy (74% and 45%, respectively; $P < 0.001$). Median OS was not reached in either group. First-line treatment with crizotinib is the standard treatment of patients with ALK- rearranged NSCLC.

As for the *EGFR* mutated, also for the ALK rearranged NSCLC patients the combination of continuing the EGFR TKI with local treatment (radiotherapy or surgery) may represent a reasonable option and could be considered on an individualised basis [216].

Despite improved outcome in patients with tumours harbouring ALK rearrangements and treated with crizotinib, all patients will eventually experience disease progression through primary or acquired resistance. Furthermore, crizotinib penetration into the cerebrospinal fluid (CSF) is negligible and this pharmacologic limitation is extremely relevant in treatment decisions, taking into account the high propensity of ALK-rearranged NSCLC to metastasise to the brain [224]. Various resistance mechanisms to ALK inhibitors have been identified, resulting in the development of new therapeutic approaches and novel TKIs.

Two phase I studies, including the multicentre open-label ASCEND-1 study, showed a significant activity of ceritinib, based on an ORR of 56% and 6.9 months of PFS in patients with ALK-rearranged NSCLC with crizotinib resistance [225]. The benefit also included intracranial responses in patients with brain metastasis. These results were confirmed in two phase II trials (ASCEND-2 and ASCEND-3) [222,223], in which the ORR was 38.6% and 63.7% respectively with a median OS of 14.9 months and not reached at

the data cut -off in the ASCEND-3 trial (the estimated 24-month OS rate was 67.5%).

In the open-label, randomized, phase III ASCEND-5 study, ceritinib was compared with chemotherapy (docetaxel or pemetrexed) in locally advanced or metastatic, ALK-rearranged NSCLC patients who had received previous crizotinib and chemotherapy (including a platinum doublet). [228] Results showed that median PFS was significantly improved with ceritinib compared to chemotherapy (5.4 versus 1.6 months; HR 0.49, 95% CI: 0.36–0.67; *P* = 0.001). The improvement in PFS was robust, demonstrating consistency across a number of subgroups, and clinical benefit was further supported by ORR (39.1% versus 6.9%) and DCR (76.5% versus 36.2%). Based on this data, ceritinib is recommended in patients with ALK-positive advanced NSCLC who progress on treatment with or are intolerant to crizotinib.

The efficacy of ceritinib was also investigated in first line in the ASCEND-4 trial, in which patients were randomly assigned to receive ceritinib 750 mg/day or pemetrexed-platinum chemotherapy, followed by maintenance pemetrexed [229]. Ceritinib treatment significantly improved median PFS compared to chemotherapy, with a risk reduction of 45% in PFS (16.6 versus 8.1 months for ceritinib and chemotherapy, respectively; HR 0.55, 95% CI: 0.42–0.73; *P* = 0.00001). Ceritinib was also associated with improved median PFS compared to chemotherapy both in the subgroup of patients without brain metastases (26.3 versus 8.3 months, HR 0.48, 95% CI: 0.33–0.69) and with brain metastases (10.7 versus 6.7 months, HR 0.70, 95% CI: 0.44–1.12). In addition, significantly higher and durable responses were attained with ceritinib compared to chemotherapy (ORR and DOR: 72.7% and 23.9 months versus 27.3% and 16.6 months, respectively). The median OS was not reached in the ceritinib group and was 26.2 months in the control group. Following the results of this phase III trial ceritinib received an expands approval in first line by the FDA and EMA.

Alectinib is another second-generation ALK inhibitor, which has been approved in Japan for all patients with advanced ALK-positive NSCLC. Two

phase II studies have also demonstrated RR between 45%-50% and PFS of 8.9 months. Alectinib was also effective for brain metastases [230].

The ALEX trial was a phase III trial comparing alectinib versus crizotinib in untreated ALK-positive advanced NSCLC patients. The primary end point was investigator-assessed PFS. Secondary end points were independent review committee–assessed PFS, time to CNS progression, ORR, and OS. A total of 303 patients were enrolled in this trial; the rate of investigator-assessed PFS was significantly higher with alectinib than with crizotinib (12-month event-free survival rate, 68.4% (95% CI, 61.0 to 75.9 (with alectinib versus 48.7% (95% CI, 40.4 to 56.9) with crizotinib; HR for disease progression or death, 0.47 (95% CI, 0.34 to 0.65; $P < 0.001$); the median PFS with alectinib was not reached. The results for independent review committee–assessed PFS were consistent with those for the primary end point. A total of 18 patients (12%) in the alectinib group had an event of CNS progression, as compared with 68 patients (45%) in the crizotinib group (cause-specific HR, 0.16; 95% CI, 0.10 to 0.28; $P < 0.001$). A response occurred in 126 patients in the alectinib group (response rate, 82.9%; 95% CI, 76.0 to 88.5) and in 114 patients in the crizotinib group (response rate, 75.5%; 95% CI, 67.8 to 82.1). Grade 3 to 5 adverse events were less frequent with alectinib (41% versus 50% with crizotinib) [231]. As compared with crizotinib, alectinib showed superior efficacy and lower toxicity in primary treatment of ALK-positive NSCLC.

Several alternative ALK inhibitors are currently in clinical development, which are all more potent than crizotinib, with broader activity against a number of mutated ALK genes and mainly characterised by higher brain activity [232].

First-line and second line treatments for metastatic small cell lung cancer

Treatment of stage IV SCLC is palliative, and combination chemotherapy has been the main treatment option for more than three decades. Despite RRs close to 70%, outcomes remain poor with a median PFS of only 5.5

months and a median OS of less than 10 months [229,230]. A meta-analysis of 19 randomised trials with a total of 4054 patients demonstrated prolonged OS of patients receiving a cisplatin-containing regimen compared with older chemotherapy combinations [235]. Another meta-analysis of 36 trials reported an OS benefit in favour of etoposide alone or in combination with cisplatin compared with regimens that did not contain one of the two drugs [236]. These results led to the adoption of etoposide–cisplatin as a standard treatment regimen. A recent individual patient data meta-analysis including four randomised clinical trials comparing cisplatin versus carboplatin-based combination chemotherapy demonstrated no difference in efficacy outcomes including RR, PFS and OS [237]. In the carboplatin group, increased haematological toxicity rates were observed, whereas higher renal and neurotoxicity was seen with cisplatin. According to these results, cisplatin can be substituted by carboplatin in patients with metastatic SCLC. Due to the limited number of only 663 patients included in this analysis, there was limited statistical power to draw conclusions in important subgroups such as patients with localised disease and young patients. In these subgroups, etoposide–cisplatin is recommended.

Studies with 3-drug regimens and the administration of increased dose intensity regimens, using increased dose or non-cross-resistant regimens, have not consistently reported improvement in OS. In addition, they have frequently been associated with significant toxicity in this usually co-morbid patient population and they are not recommended as first-line treatment [238].

A recent literature-based meta-analysis of seven randomised studies showed an improved OS, but not PFS with irinotecan–platinum compared with etoposide–platinum. Irinotecan led to more gastrointestinal toxic effects, while more haematological toxic effects were observed with etoposide [239]. The results, however, were primarily driven by Asian studies, and pharmacogenomic differences between Asian and Western populations possibly contributing to these differential outcomes have previously been described [240]. No chemotherapy doublet has yet been

shown to be superior to i.v. etoposide–platinum in a Western population. Randomised phase III trials which compared irinotecan–cisplatin, gemcitabine–carboplatin (in poor prognostic patients only) or i.v. or oral topotecan–cisplatin to etoposide–platinum have demonstrated non-inferiority for survival [241–244]. These regimens are recommended as alternative treatment options in the case of contraindications to etoposide.

Continuation of chemotherapy beyond 4–6 cycles has been assessed in at least 14 randomised, controlled trials. Although a significant OS benefit was reported in a literature-based review including 11 trials (HR 0.89, 95% CI: 0.81–0.92; *P* = 0.02), the benefit was small and high heterogeneity among the included trials was observed [245]. Similarly, a previous meta-analysis found a small OS benefit of 4% at 2 years with maintenance therapy [246]. However, the majority of the randomised, controlled trials did not show any significant OS benefit, and a properly designed large clinical trial to address this question is lacking. In addition, there is a considerable risk of increased toxicity with prolonged platinum-based chemotherapy.

Prophylactic cranial irradiation (PCI) significantly decreases the risk of symptomatic brain metastases (from 40.4% to 14.6% at 1 year) and increases OS (HR 0.68; 95% CI, 0.52–0.88) [247]. PCI is associated with adverse effects such as fatigue and hair loss, and health-related quality of life may be negatively affected as well [248]. Patients with any response to first-line treatment and who have a reasonably good PS should be evaluated for PCI. The PCI dose may be 25 Gy in 10 daily fractions or 20 Gy in 5 fractions.

Due to the often centrally located primary tumours, symptoms such as dyspnoea, infections due to atelectasis, chest pain or superior vena cava syndrome are frequent and make the incorporation of thoracic radiotherapy into the initial treatment algorithm an appealing concept. A four-arm randomised phase III trial has demonstrated a survival benefit of concurrent thoracic radiotherapy in patients whose primary tumours have responded after three cycles of cisplatin–etoposide and whose metastatic sites were in complete remission (OS: 17 versus 11 months, *P* = 0.041) [249]. This single

centre trial was however small (54 patients per arm), and the concurrent chemoradiotherapy treatment used does not correspond to the current standard approach.

RRs to second-line treatment depend on the treatment-free interval and are usually in the order of 10% in resistant disease (i.e. progression-free interval <3 months) and 20% in sensitive disease (i.e. interval >3 months). In refractory patients (i.e. patients not responding or progressing during chemotherapy) and resistant patients with early relapse (<6 weeks), outcomes are poor and the clinical benefit of further systemic therapy is uncertain. For these patients, participation in a clinical trial or best supportive care is recommended. Oral topotecan led to better symptom control including slower time to quality of life deterioration and improved survival compared with best supportive care in a study in which half of the patients had resistant disease [250]. Prior to topotecan development, anthracycline-based regimes have been commonly used, including cyclophosphamide, doxorubicin and vincristine (CAV). In 1999, a trial of i.v. topotecan and CAV demonstrated equal efficacy, with similar RRs, time-to-progression, and OS, and better tolerance when compared with CAV [251]. Oral and i.v. topotecan have shown to be equally effective [252], but with differing toxicity profiles. Either oral or i.v. topotecan are recommended for patients having resistant or sensitive relapse with CAV being an alternative option. Only patients with sensitive disease derive benefit from rechallenge with first-line therapy (usually platinum–etoposide).

The TransCPC and TransBio projects

Rational

Phenotypic transformation from NSCLC to SCLC is a resistance mechanism in TKI treated *EGFR* mutant tumours, although some cases of SCLC transformation from WT *EGFR* tumours are also described in literature [249,250]. This transformation is a rare event and little is known about the clinical and therapeutic characteristics of these patients. In *EGFR* mutant lung cancers, mostly adenocarcinoma, phenotypic transformation to SCLC has been described as one of the resistance mechanisms aside major events, such as the secondary exon 20 T790 mutation or the *c-Met or Her2* amplifications [93,249,251–256]. In the original description of resistance mechanisms and in published clinical cases there were anecdotal description of sensitivity to platinum etoposide chemotherapy but neither response rate nor survival characteristics after treatment were reported.

A documented transformation from adenocarcinoma to SCLC in non-EGFR-mutant cancers treated with chemotherapy is a well-known, but rarely diagnosed event [254]. The low incidence might be partially explained by the limited use of re-biopsy in the oncological history of these patients.

A recent systematic review and pooled analysis described clinical/pathological features and prognosis of oncogene addicted adenocarcinoma transformed in SCLC. Thirty-nine patients were eligible for the analysis and the median time from initial diagnosis of lung adenocarcinoma to the transformation to SCLC was 19 months (range 1–61 months). The median survival after SCLC diagnosis was 6 months; female gender was significantly associated with longer transformation to SCLC at the multivariable analysis and smoking status seemed to be associated with worse prognosis after the diagnosis of SCLC [261]. One limitation of this analysis is that the patient data were retrospectively collected from published article.

The development of a SCLC by the transformation of an adenocarcinoma is a surprising phenomenon, considering the radically different characteristics of these cancers and the clinical characteristics of the

patients developing them. The classic histomolecular dogma is that these cancers develop from the transformation of different cellular precursors (epithelial for adenocarcinoma, neuroendocrine for SCLC). However, some murine experiences showed that mice with a *TP53* and *Rb1* loss in all alveolar type II cells might have the potential to develop SCLC, even if at lower frequency than for neuroendocrine cells [262].

Patients developing primary SCLC are almost all heavy smokers, whereas *EGFR* mutant adenocarcinoma is more frequent in never or light smoker. From a molecular level, these two cancers are also distinct entities, characterized by radically different genetic alterations (apart from the usual mutation of *TP53* gene).

Several hypotheses try to explain the mechanism of this phenomenon: one is that these patients have a combined histology at the time of initial diagnosis, which is not apparent on the diagnostic sample, and at progression SCLC become predominant due to activity of an EGFR TKI on the adenocarcinoma counterpart [253]. Series published about this population show that most of patients initially respond to EGFR inhibitors and have a tumour growth at the time when SCLC was diagnosed. If we consider tumor heterogeneity as the mechanism of SCLC development in these patients we could expect a less important response to EGFR TKIs and an earlier acquired resistance. A paper from Roca et al. confirmed that these patients have a benefit in terms of PFS with an EGFR TKI that is similar to a population of adenocarcinoma that do not become a SCLC [261].

In contrast with this first hypothesis, a series of patients published by Sequist at al. shows that the *EGFR* genomic sequencing from both the diagnostic and re-biopsy samples at the time of resistance retained the original EGFR-activating mutation, which suggests that these were not independent de-novo cancers, but a transformed phenotype as a mechanism of resistance to treatment [94].

The phenomenon of SCLC relapse is not unique to lung cancer and has also been documented in prostate cancer [263]. These observations lead to

a radical reconsideration about our knowledges on the lung cancer histopathogenesis.

On the basis of the present knowledge, three major and joint molecular events seem to preside over the conversion of an adenocarcinoma into a SCLC: the *TP53* mutation, RB1 inactivation and EGFR repression. However, it is unknown whether these events are necessary or sufficient, nor in what context of evolutive tumor heterogeneity they appear.

A recent paper published by Lee et al. revealed that the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)–induced hypermutation was frequent in the branches toward small-cell transformation [264]. The APOBEC cytidine deaminase has a function in innate immunity as well as in RNA editing in addition to the activation-induced cytidine deaminase (AID) [265]. Several cancer types displayed high levels of the APOBEC mutation pattern as well as a wide variation among individual samples, which could reflect different biological pathways leading to carcinogenesis [266]. In the current study we describe and compare the characteristics of SCLC arising from mutant or non-mutant NSCLC in a large cohort of patients.

Objectives

The aims of this project are:

For the transCPC study:

- Primary objective: to analyze survival data of the population after the transformation to SCLC.
- Secondary objectives: to define the epidemiological characteristics at the diagnosis of NSCLC, the histomolecular characteristics at the diagnosis and at the transformation; the clinical characteristics at the time of diagnosis of NSCLC and SCLC and the treatment characteristics before and after transformation.

For the transBio study:

- Primary objective: to develop an extensive histopathological, clinical and molecular characterization of this phenomenon, in order to better

characterize the histological and genetic tumor dynamics that preside over this conversion.

- Secondary objectives: the research of sensitivity biomarkers to targeted agents; the comparison of the SCLC genomic profiles with phenotypic transformation and classical SCLC, and the comparison of genomic profiles of *EGFR* mutated tumor transformed into mutated SCLC versus *EGFR* tumors that never switch.

Material and Method TransCPC study

We performed an international multicentric retrospective collection of cases between 2005 and 2017. Thirty Italian and French centers, after a global e-mailing to a network of thoracic oncology centers, decided to participate.

Consecutive NSCLC patients with stage III or IV NSCLC (according to the 7th TNM Lung Cancer Stage Classification) with or without initial *EGFR* mutation with a transformation to SCLC were included. We excluded in our collection patients with a previous history of SCLC or neuroendocrine tumor of the lung as well as patients with combined SCLC/ NSCLC on the initial pathology sample.

Study ethics approval was obtained both in Italy (in each participating center) and in France (CECIC Rhône-Alpes-Auvergne, Clermont-Ferrand, IRB 5891).

Anonymized data were collected at each center then centrally analyzed at the Albert Bonniot Institute, Inserm U 823, Grenoble Alpes University, in Grenoble, France.

Continuous variables were described as median (25%-75% interquartile range [IQR]) and categorical variables as number. Associations between categorical variables were compared using the chi-2 test or Fisher's exact test and those between continuous variables using the Wilcoxon test.

Patients were followed until 26th July 2017 and no patients were lost to follow-up. Overall survival is considered from the initial diagnosis of lung cancer to death and survival after SCLC transformation is from the re-biopsy to death. Kaplan-Meier plots of survival curves were compared between groups using the log-rank test. All tests were two-sided, and *P* values <0.05

were considered statistically significant. All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA).

TransBio study

Following the data collection phase, we started the second part of the project that is still ongoing.

The first step of the project consisted in a centralized histological revision of the samples to confirm the presence of the transformation and to select the samples for the molecular analysis. On the basis of these analysis and the pathologist' agreement, selected tissues were recut, alternating 2 thick sections (10 mM) for DNA extraction and molecular biology (total 8 sections) and 5 thin sections (5 mM) for histomolecular analysis and FISH (total: 20 sections) (Figure 2).

The exome sequencing of six samples (three couples) was performed using the DNA extracted from the thick sections. The definition of the regions to be analyzed has been done based on the selection of a representative and sufficiently abundant area of initial adenocarcinoma and relapse SCLC, constituting a "before / after" doublet. The sequencing is carried out by a specialized provider, IntegraGen Genomics (Campus Génopole, Evry, France). Exome sequencing is performed at a depth of 145X on Illumina HighSeq4000.

If enough tissue will be available the later step of the study will be the histochemical analysis, carried out by the Molecular Diagnostic Platform of the Grenoble University Hospital (PDMiS Grenoble). The markers selected for these analyzes will be: TTF-1, p63, p53, pRb, phosphatase and tensin homolog (PTEN), EGFR expression, phospho-AKT, chromogranine A, synaptophysine, enolase neurone-specific (NSE), PD-L1, DDL3, vimentin, E-cadherin.

Results

Sixty-two cases of SCLC transformation were registered, but one was not considered for the final analysis because it did not respect the inclusion

criteria (absence of transformation to SCLC at re-biopsy). Forty-eight patients were EGFR mutant and 13 non-EGFR mutant NSCLC. Median age in the overall population was 62 [range 52-70] without any differences between EGFR mutant and non-mutant patients (*P* = 0.70). Sixty-nine percent of the patients in the EGFR mutant group were female and 46% in the non-mutant one (*P* = 0.19), most of them with a PS between 1 and 2 (54% in the overall population). About smoking status, in the overall population 53 (91%) patients were former or never smokers, with a statistical difference between the two groups (93% in EGFR mutant versus 85% in non-mutant, *P* = 0.03). Most of the patients (53; 87%) had stage IV disease, whereas the others were stage III unresectable lung tumors; adenocarcinoma was the most prevalent histology (55; 90%) and any patients in the *EGFR* mutated group had a squamous cell carcinoma (Table 2).

Forty one out of sixty-one (67%) of initial biopsies were performed on the lung primary tumor site, whereas in 8 cases (13%) the site of the biopsy was not recorded. Eight cases had a pleural biopsy (13%), 2 cases (3%) had lymph-node biopsy, one case had a brain or a bone biopsy respectively (Table 3).

Overall, 57 (93%) tumors had molecular analyses at the time of initial diagnosis; in the EGFR mutant group 36 patients presented an exon 19 mutation (in one case associate to an exon 20 T790M one), 10 a L858R one and 2 case an exon 18 or 20 mutation (Table 4).

The median time to SCLC transformation was 16 months [IQR 25%-75%, 11-27] in the EGFR mutant group and 26 months [IQR 25%-75%, 23-36] in the non-EGFR mutant one (*P* = 0.01). The number of different treatment lines administered before SCLC transformation are shown in Figure 3A and 3B. As first line treatment in the EGFR mutant group, 38 (79%) patients received a TKI: gefitinib (n=16), erlotinib (n=12) and afatinib (n=10). The last line of treatment before SCLC transformation was a TKI in 39 cases (81%) in the EGFR mutant group and in 3 patients (23%) in the non-EGFR one.

Erlotinib was prescribed in the non-EGFR-mutated group according to its label in second or third line of treatment.

Site of re-biopsy was lung in 24 cases (39%), lymph-nodes or liver in 11 cases (18%) respectively, pleura in 2 cases (4%) or adrenal gland in 1 case (2%), while in 12 cases (19%) re-biopsy site was not reported. (Table 3).

Thirty-eight cases (79%) had a tumor molecular analyses after SCLC transformation in the EGFR mutant group, whereas as expected no molecular analyses were performed in the non-EGFR mutant one. In the EGFR mutant group, 38/48 (79%) had a driver mutation, mostly the same mutation as the one observed initially (Table 4). In 3 cases, initially mutated exon 19 patients lost their starting mutation and the transformation into SCLC was associated with PI3K mutation and C-Met amplification (1 case), ALK fusion alone (1 case) and ALK fusion with exon 21 and 18 mutations (1 case).

After the SCLC transformation, the number of lines received are shown in Figure 4. In the EGFR mutant group 42 (88%) patients were treated and received at least one line of therapy; in most of cases the treatment choice was a “classical” SCLC treatment with platinum and etoposide doublet (38 cases, 79%), while in 2 cases (4%) the chemotherapic regimen with platinum and etoposide was associated with a TKI and in one case the patient received nivolumab or paclitaxel respectively (Table 5). The response rate was available in 31 out of these 42 cases (72%), with partial response (PR) achieved in 14 (45%) cases, stable disease (SD) in 5 (16%) and progressive disease (PD) in 12 (39%). In the non-EGFR mutant group, 11 of 13 patients (85%) received at least one line of treatment, in all cases a combination of etoposide and either carbo or cisplatin. The response rate was available in 10 of these cases, with PR achieved in 4 (40%) cases, SD in 2 (20%) cases and PD in 4 (40%) cases. There was no statistical difference in the type of response between the two groups ($P = 1$).

The median follow-up duration after the initial diagnosis was 30 [IQR 25%-75%, 17-40] months among 50 patients who have died at the time of last follow up and 25 months [IQR 25%-75%, 17-44] months among 11 alive

patients. In the EGFR mutant group, the median OS were respectively 28 [IQR 25%-75%, 17-40] months after the initial diagnosis and 9 [IQR 25%-75%, 3-13] months after transformation. In the non-EGFR mutant group, the median OS were respectively 37 [IQR 25%-75%, 32-49] months after the initial diagnosis and 10 [IQR 25%-75%, 6-15] months after transformation. The Kaplan Meier survival curves from the initial diagnosis and after SCLC transformation are shown in Figure 5 and 6. There was a tendency towards a worse overall survival after SCLC transformation in the EFGR mutant group ($P = 0.56$), and a trend of statistically significant difference in survival estimated after the initial diagnosis, was observed in the non-EGFR mutant one ($P = 0.06$).

Centralized histological revision of the initial diagnosis was performed for 22 out of 61 patients (36%) before and 33/61 (54%) after transformation. Histological revision was performed on 26/48 (54%) EGFR mutant patients and on 7 out of 13 EGFR-non-mutant ones (54%). Of the 19 initial adenocarcinoma at diagnosis, all of them were confirmed after revision and 1 carcinoma NOS was reclassified as adenocarcinoma after control. About the 3 SCC only 2 were available for revision and the initial diagnosis was confirmed.

Twenty-two of the 33 revised SCLC (66%) were confirmed after revision, while 4 (13%) were classified as large cell neuroendocrine carcinoma, 5 (16%) as mixed adenocarcinoma-SCLC and 2 (6%) as mixed small/ large cell lung cancer.

We compared clinical characteristics between cases that were revised and not and we did not find any statistically difference between the two populations (Table 6).

Discussion

In NSCLCs, the TKIs erlotinib, gefitinib and afatinib are used to treat patients with EGFR mutations. While these drugs are associated with high response rates and improved survival characteristics, patient inevitably develop resistance during treatment. Among the different mechanisms of resistance, 50 to 60 % of cases are associated with the occurrence of T790M

gatekeeper mutation and *c-Met* amplification is the second one. In 4–14% of EGFR-mutant, histological transformation to SCLC occurs [92, 251,260]. The biological mechanism underlying transformation to SCLC is not well understood, although 2 possibilities have been proposed [253]: (1) a phenotypic switch from NSCLC to SCLC or (2) a combination of SCLC and adenocarcinoma may be present at baseline, with SCLC becoming the main histological component after an EGFR- TKI treatment. In published case reports of transformation to SCLC [252,260,262], these tumors were treated with the standard etoposide cisplatin combination and achieved tumor regression. However, to date no response rate nor survival characteristics are available on a significant number of patients.

Furthermore, combined SCLC and non EGFR mutant NSCLC are described in the WHO classification of lung tumors and rarely a transformation to SCLC can occurs in non EGFR mutant NSCLC treated with chemotherapy [253, 259].

In the current series of 61 patients, 48 cases came from EGFR mutated tumors and 13 from non-mutated NSCLC. We hypothesize that the latter 13 cases might be considered as genuine classical SCLC. Indeed, these tumors occur mostly in current of former heavy smokers. In contrast and as expected most of the cases (93%) occurring in EGFR mutant tumors came from former and never smokers.

Neither EGFR mutant nor non-EGFR mutant NSCLC displayed SCLC features in the primary biopsy specimen; this data was confirmed for the specimens available at the centralized histological revision. In agreement with previous reports on a smaller number of patients we observed identical EGFR mutations in both primary tumor and SCLC in 31 out of 38 cases with molecular analyses. Interestingly, one case had an exon 19 mutation and a T790M resistance mutation at diagnosis, one other case loss the EGFR mutation and presented an ALK fusion at transformation.

The median time to SCLC transformation was significantly shorter (16 months [IQR 25%-75%, 11-27]) in the EGFR mutant group when compared to the non-EGFR mutant one (26 months [IQR 25%-75%, 23-36]) ($P = 0.01$).

The median number of lines of treatment before transformation was 2 in both groups. As expected, the last line of treatment before SCLC transformation was more frequently a TKI in the EGFR mutant group (81%) than in the non-EGFR one (23% treated with erlotinib in the last line).

Fifty-three among the 61 SCLC cases were treated after transformation, and 49 cases received a platinum based with etoposide regimen. Most of the patients achieved good disease control rate response rates as usually observed in SCLC (61% in the EGFR mutant group and 60% in the non-EGFR, *P* =1). The median number of lines after transformation was identical in the 2 groups.

Despite high response rate to chemotherapy the 48 cases of SCLC occurring in EGFR mutant seem to behave more aggressively with a tendency to a shorter survival (median OS after transformation of 9 months) than those occurring in non-EGFR mutant NSCLC that behave more classical SCLC (median OS of 10 months). It should be emphasized that in the latter group the survival characteristics were comparable with those regularly observed for SCLC [269]. Furthermore, the survival after the initial diagnosis of NSCLC was also shorter in the EGFR mutant group. Therefore, we hypothesize that the usual indolent course of EGFR mutated tumors is strongly modified by the transformation into a very different aggressive entity. Our series of patients seem in contrast with the series published by Sequist et al. that displayed a more indolent natural history compared with classical SCLC [94].

In classical SCLC, the Rb is altered in the vast majority of cases [266,267] and this is also observed in tumor samples and cell lines derived from SCLC transformed cases [272] suggesting that this subset of resistant tumors adopt the key molecular characteristics of classical SCLC. However, the mechanisms underlying the transformation remain to be discovered. The vast majority of these tumors keep on expressing the same EGFR mutation as observed in the initial NSCLC. These mutations are possibly present in the SCLC transformed cells or may also be present in a NSCLC residual subclone. The observation of an ALK fusion in one case probably reflects

probably more the coexistence of different subclones than a SCLC with an ALK fusion. Recently, some cases of SCLC transformation after treatment with alectinib of an NSCLC with an ALK fusion have been described [273–275]. These observations together with the observation of SCLC transformation after third generation EGFR TKI osimertinib and rociletinib, suggests that transformation into SCLC is a rare but common mechanism of resistance to TKI. One case with a T790M resistance mutation also demonstrates that distinct mechanisms of resistance to EGFR TKI may be present within the same tumor. Osimertinib will be probably available in the next future, as an option in first line setting for EGFR positive NSCLC; the advent of a third generation TKI in first line open some questions about the possible mechanisms of resistance of this drug. Following the results of a phase I trial in first line, none of the patients treated with osimertinib developed the T790M mutation at progression [220]. Instead, the EGFR C797S mutation is described as a mutation inducing resistance in T790M mutant patients treated with osimiertinib in. We do not know exactly what will be the most frequent mechanisms of resistance to osimiertinib in first line and it will be fundamental to perform re-biopsies as much as possible, in order to understand if more cases of SCLC will be diagnosed in this setting of patients.

This cohort is at the moment the largest described in scientific literature, demonstrating how the phenomenon of phenotypic transformation in SCLC is not only anecdotal and limited to a single case. The project is still ongoing, data about sequencing will be available in the near future, and they could give us some answers about the molecular mechanisms related to this phenomenon. The objective of these sequences is also to compared the genome of this population with a series of adenocarcinoma that never become a SCLC, with the idea to identify some predictive biomarkers.

With the greater development of molecular targeting, this phenomenon will probably be more and more frequent by opening up new scenarios for the histological classification of pulmonary carcinomas, including small cell carcinomas that are derived from a non-small cell tumor. The creation of a

collaboration between Italy and France has allowed us to collect a very interesting case from both epidemiological and biological point of view, demonstrating the importance of cooperation between different centers and nations.

Tables and Figures

Table 1. 2015 WHO Classification of Lung Adenocarcinomas [40]

Histologic Type and Subtypes	ICDO Code
Adenocarcinoma	8140/3
Lepidic adenocarcinoma	8250/3
Acinar adenocarcinoma	8551/3
Papillary adenocarcinoma	8260/3
Micropapillary adenocarcinoma	8265/3
Solid adenocarcinoma	8230/3
Invasive mucinous adenocarcinoma	8253/3
Mixed invasive mucinous and nonmucinous adenocarcinoma	8254/3
Colloid adenocarcinoma	8480/3
Fetal adenocarcinoma	8333/3
Enteric adenocarcinoma	8144/3
Minimally invasive adenocarcinoma	
Nonmucinous	8256/3
Mucinous	8257/3
Preinvasive lesions	
Atypical adenomatous hyperplasia	8250/0
Adenocarcinoma in situ	
Nonmucinous	8250/2
Mucinous	8253/2

Table 2. TransCPC and TranBio patients' characteristics

	All (n=61)	**EGFR mutant** (n=48)	**Non EGFR mutant** (n=13)	**chi2 test or Wilcoxon**
Age	62 [52-70]	61 [51-72]	62 [56-64]	0.70
Female gender	39 (64%)	33 (69%)	6 (46%)	0.19 (Fisher)
PS (MD=2)				0.86
0	27 (46%)	21 (46%)	6 (46%)	
1-2	32 (54%)	25 (54%)	7 (54%)	
Tobacco smoking (MD=3)				0.03
Active	5 (9%)	3 (7%)	2 (15%)	
Former	22 (38%)	14 (31%)	8 (62%)	
Never	31 (53%)	28 (62%)	3 (23%)	
Pack/year (MD=7)				
≤20	7 (35%)	6 (46%)	1 (14%)	
>20	13 (65%)	7 (54%)	6 (86%)	
Stage IV/III	53 (87%)/ 8 (13%)	43 (90%)/ 5 (10%)	10 (77%)/ 3 (23%)	0.35 (Fisher)
Histology				0.02 (Fisher)
Adenocarcinoma	55 (90%)	45 (94%)	10 (77%)	
Squamous	3 (5%)	0	3 (23%)	
NOS	3 (5%)	3 (6%)	0	
Median number of lines of treatment before transformation	2 [1-3]	2 [1-2]	2 [1-3]	0.33

MD: Missing Data

Table 3. Sites of biopsy and re-biopsy

	Biopsy	**Re-biopsy**
Lung	41 (67%)	24 (39%)
Pleura	8 (13%)	2 (4%)
Lymph node	2 (3%)	11 (18%)
Liver	-	11 (18%)
Adrenal gland	-	1 (2%)
Bone	1 (1%)	-
Brain	1 (1%)	-
Missing data	8 (13%)	12 (19%)

Table 4. Different types of EGFR mutations

	Mutation at the time of initial diagnosis		
n=48	exon 19, n=36	L858R n=10	exon 18 or 20, n=2
	Mutation at the time of SCLC transformation		
No molecular analyses	6/36 (17%)	3/10 (30%)	1/2 (50%)
Molecular analyses performed	30/36 (83%)	7/10 (70%)	1/2 (50%)
Exon 19	24 (80%)[a]	0	0
L858R	1 (3%)	6 (86%)	0
Exon 18 or 20	0	0	1 (100%)
No mutation	5 (17%)	1 (14%)	0

[a]1 associated with a second mutation in exon 20 T790M

Table 5. First line treatment after SCLC transformation

	EGFR Mutant (n=48)	**Non-EGFR Mutant** (n=13)
No treatment	6 (13%)	2 (15%)
Platinum etoposide	38 (79%)	11 (85%)
Nivolumab	1 (2%)	0
Paclitaxel	1 (2%)	0
Platinum etoposide and TKI	2 (4%)	0

Table 6. TransCPC and TranBio patients' characteristics comparison between revised and not-revised cases

	All (n=61)	**Revised** (n=33)	**Not-revised** (n=28)	**chi2 test or Wilcoxon**
Age	62 [52-70]	61 [55-68]	63 [50-70]	1.00
Female gender	39 (64%)	18 (55%)	21 (75%)	0.1
PS (MD=2)				0.39
0	27 (46%)	13 (41%)	14 (52%)	
1-2	32 (54%)	19 (59%)	13 (48%)	
Tobacco smoking (MD=3)				0.06
Active	5 (9%)	5 (16%)	0	
Former	22 (38%)	9 (29%)	13 (48%)	
Never	31 (53%)	17 (55%)	14 (52%)	
Pack/year (MD=7)				
≤20	7 (35%)	4 (40%)	3 (30%)	
>20	13 (65%)	6 (60%)	7 (70%)	
Stage IV/III	53 (87%)/ 8 (13%)	27 (82%)/ 6 (18%)	26 (93%)/ 2 (7%)	0.27 (Fisher)
Histology				0.43 (Fisher)
Adenocarcinoma	55 (90%)	28 (85%)	27 (96%)	
Squamous	3 (5%)	2 (6%)	1 (4%)	
NOS	3 (5%)	3 (9%)	0	
Median number of lines of treatment before transformation	2 [1-3]	2 [1-2]	2 [1-3]	0.34

Figure 1. The frequency of observed drug resistance mechanisms after an EGFR TKi treatment [94]

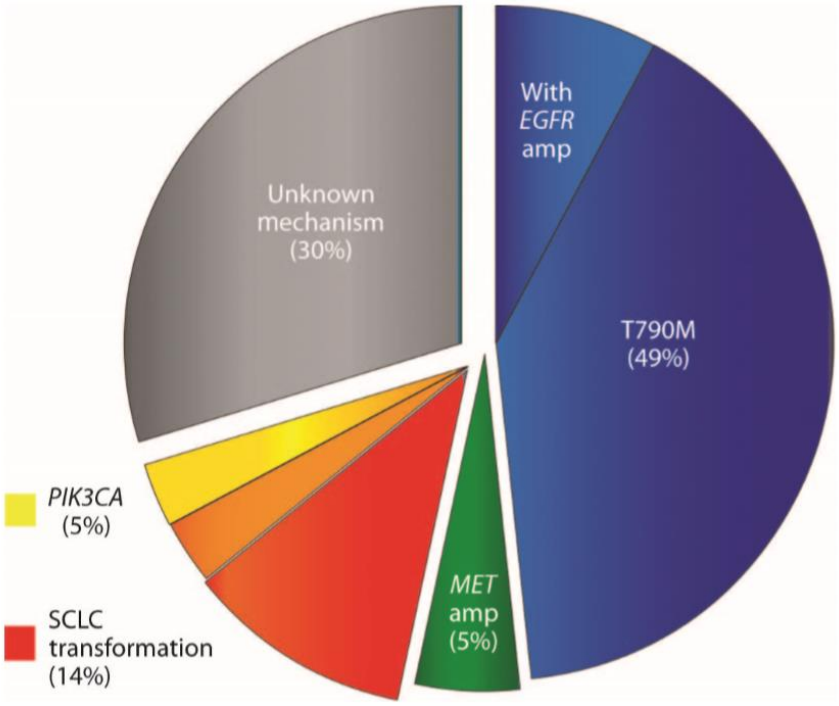


Figure 2. TransBio study design

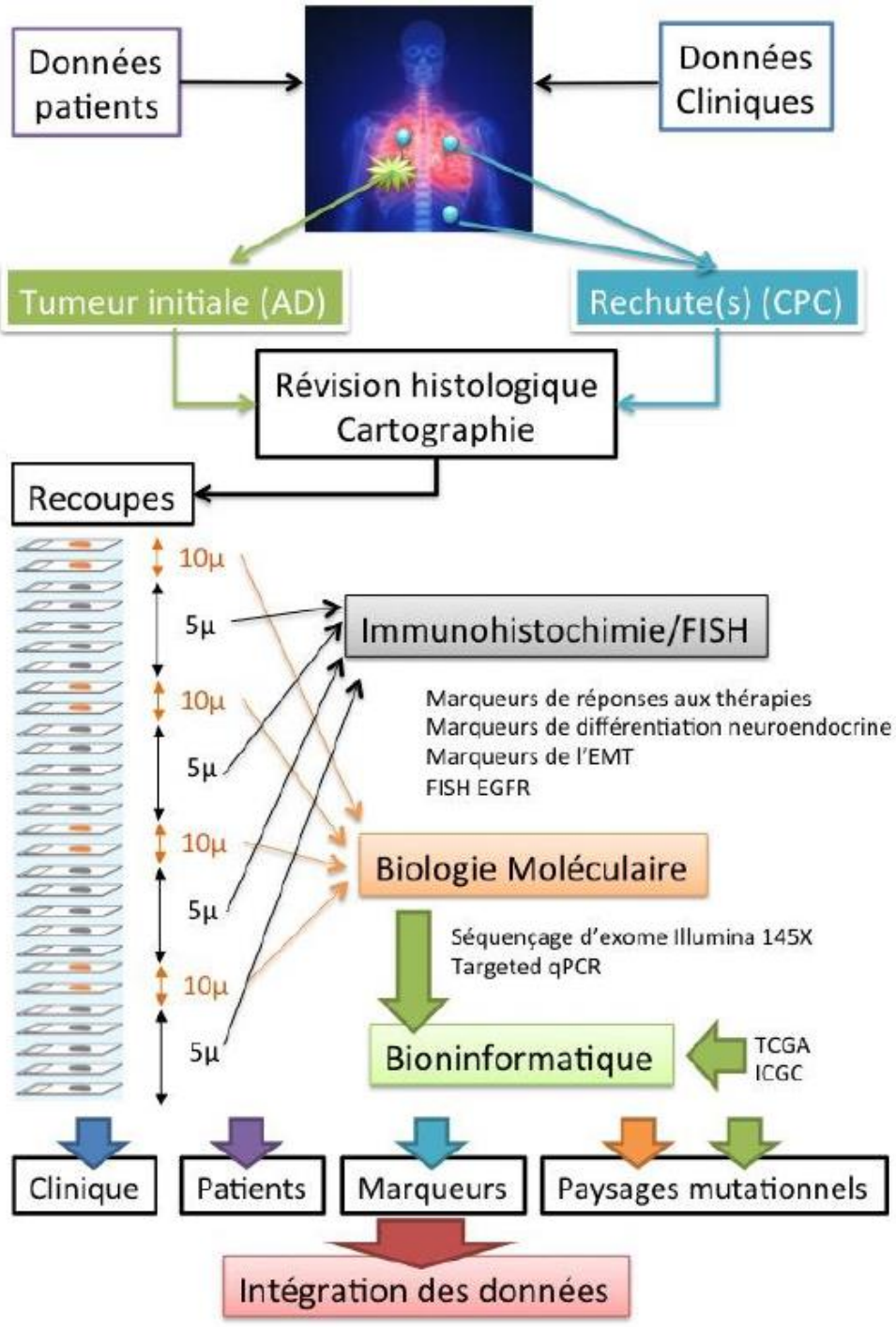


Figure 3A. Type and number of different treatment lines administered before SCLC transformation in EGFR mutant patients (n=48).

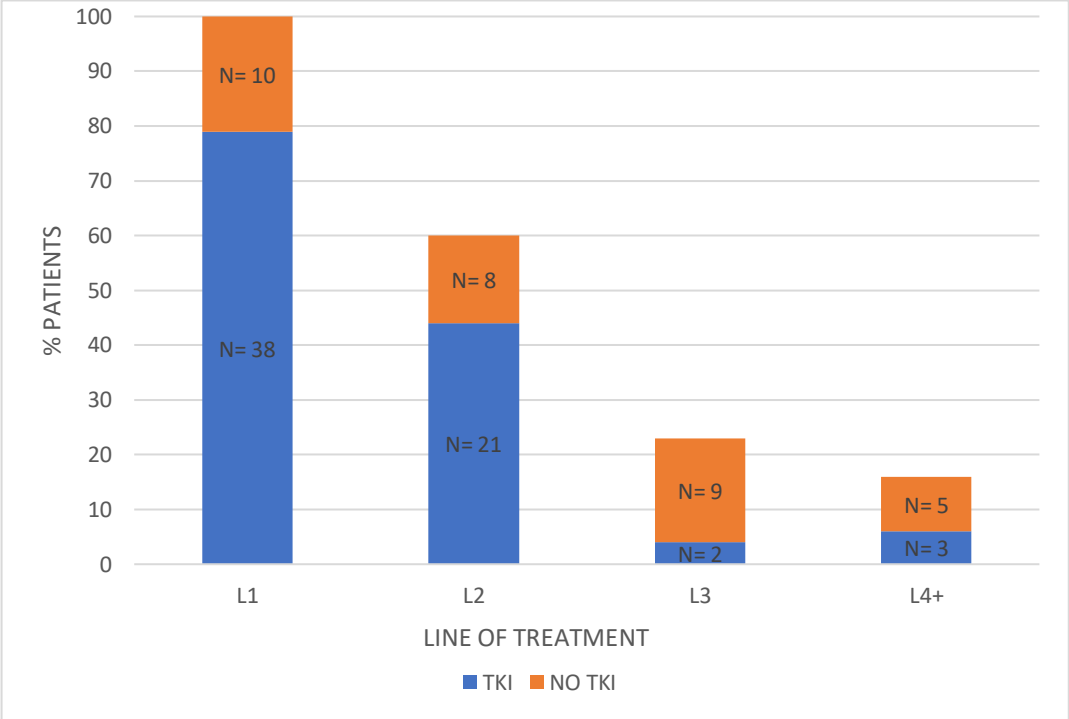


Figure 3B. Type and number of different treatment lines administered before SCLC transformation in non- EGFR mutant patients (n=13).

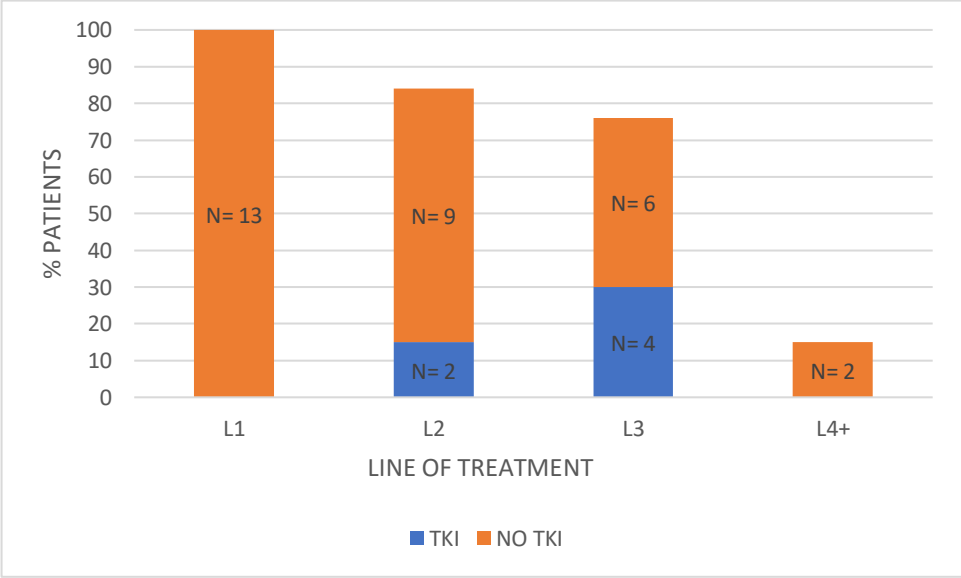


Figure 4. Type and number of different treatment lines administered after SCLC transformation

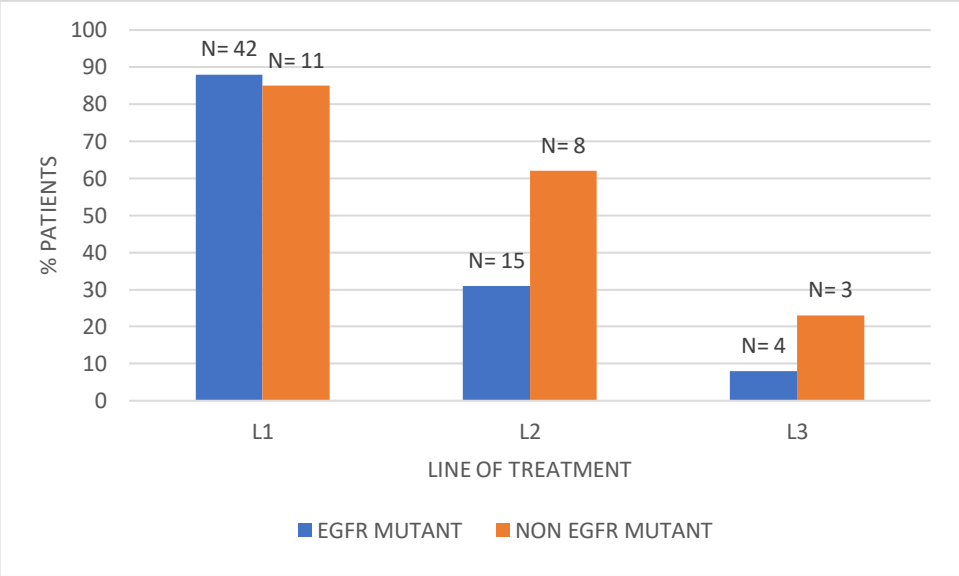
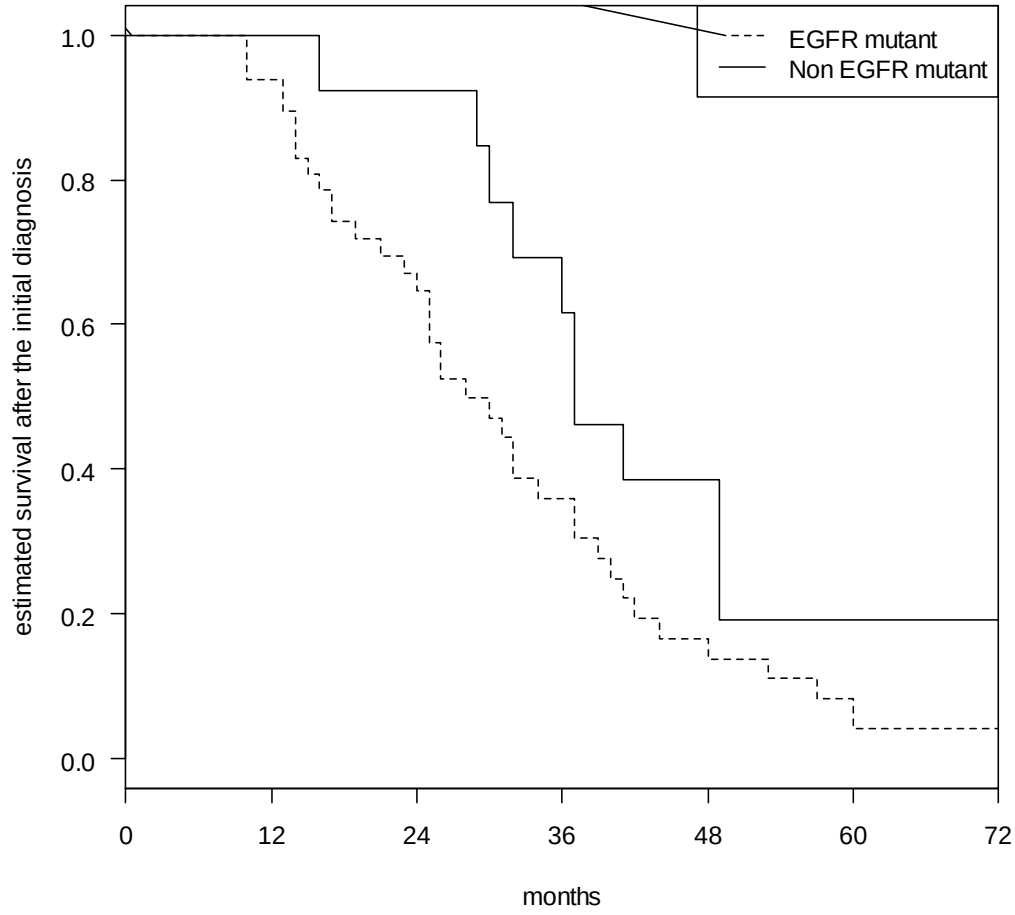
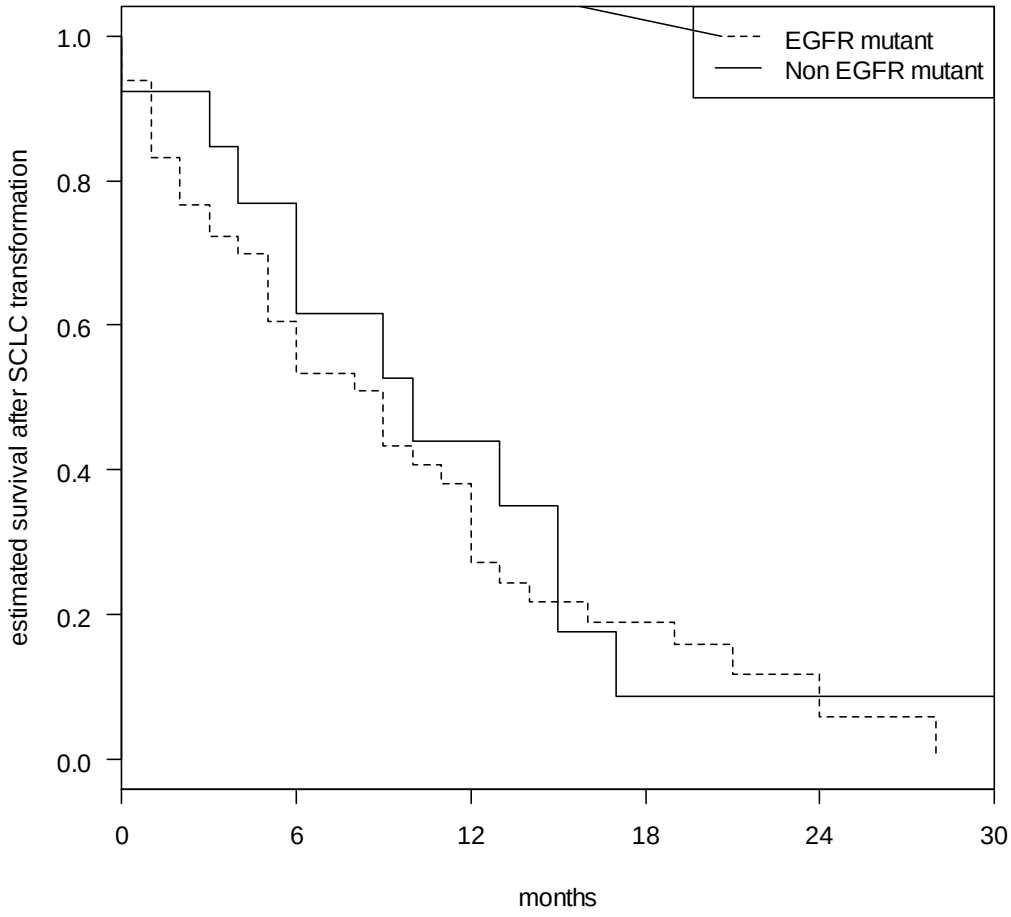


Figure 5. The Kaplan Meier overall survival from the initial diagnosis



Time (months)	0	12	24	36	48	60	72
N at risk	61	57	40	22	10	4	2
EGFR mutant	48	44	28	13	6	1	0
Non-EGFR mutant	13	13	12	9	4	2	2

Figure 6. The Kaplan Meier overall survival from the SCLC transformation



Time (months)	0	12	18	24	30
N at risk	61	35	7	3	1
EGFR mutant	48	25	6	2	0
Non-EGFR mutant	13	10	1	1	1

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