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EGFR signaling pathway as therapeutic target in human cancers

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ABSTRACT

Epidermal Growth Factor Receptor (EGFR) enacts major roles in the maintenance of epithelial tissues. However, when EGFR signaling is altered, it becomes the grand orchestrator of epithelial transformation, and hence one of the most world-wide studied tyrosine kinase receptors involved in neoplasia, in several tissues. In the last decades, EGFR-targeted therapies shaped the new era of precision-oncology. Despite major advances, the dream of converting solid tumors into a chronic disease is still unfulfilled, and long-term remission eludes us. Studies investigating the function of this protein in solid malignancies have revealed numerous ways how tumor cells dysregulate EGFR function. Starting from preclinical models (cell lines, organoids, murine models) and validating in clinical specimens, EGFR-related oncogenic pathways, mechanisms of resistance, and novel avenues to inhibit tumor growth and metastatic spread enriching the therapeutic portfolios, were identified. Focusing on non-small cell lung cancer (NSCLC), where *EGFR* mutations are major players in the adenocarcinoma subtype, we will go over the most relevant discoveries that led us to understand EGFR and beyond, and highlight how they revolutionized cancer treatment by expanding the therapeutic arsenal at our disposal.

1. Deregulation of EGFR signaling in lung cancer and other solid malignancies

Lung cancer is the leading cause of oncological deaths worldwide. Regardless of the continuous advances in diagnosis and multimodality therapies, the prognosis remains poor with 5-year overall survival rates [1]. Lung cancer is characterized by the accumulation of multiple genetic and epigenetic alterations, including somatic mutations, and gene copy number gains, that result in the activation of oncogenes or inactivation of tumor suppressor genes [2]. Lung cancer is subdivided into non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC, which is further divided into three subtypes (adenocarcinoma, squamous cell carcinoma, and large cell carcinoma), presents with frequent deregulation of the Epidermal Growth Factor Receptor (EGFR) signaling [3] particularly in the adenocarcinoma subtype, which is instead rarely observed in SCLC [4].

Historically, EGFR derives its name from studies initiated in the early 1960s by Cohen, who first discovered EGF as a protein that stimulated proliferation of epithelial cells [5]. It was not until a decade later that Carpenter identified a specific binding receptor for EGF on target cells [6], soon after termed the EGFR, that was classified as a receptor tyrosine kinase (RTK) [7]. The EGFR pathway normally provides a robust signal for epithelial cell proliferation/survival during organogenesis and tissue repair [8–10]. However, when deregulated, its consequences are deleterious. *EGFR* is indeed one of the most frequently mutated oncogenes in lung cancer and other cancer types [11]. *EGFR* somatic mutations are clonal, and they have been described not only in full-blown NSCLCs, but also in lungs with preneoplastic lesions [12]. EGFR can switch on pulmonary tumorigenesis ab origine by activating pro-survival and anti-apoptotic cellular responses, including increased proliferation, motility, angiogenesis, vascular mimicry, and invasiveness [13–17]. Both activation and overexpression of EGFR result in increased expression of stem cell markers (Octamer-binding Transcription Factor 4, Oct4; Nanog; B cell-specific Moloney murine leukemia virus Integration site 1, BMI1; C-X-C Motif Chemokine Receptor 4, CXCR4; CD44; Stromal-Derived Factor 1, SDF1) [18], and $EGFR^{+}$ tumor-initiating cells from glioblastoma multiforme (GBM) enter a dormancy-like state by just downregulating EGFR [19], signifying its relevance in CSC maintenance.

The *EGFR* gene, frequently referred to as *HER1* (Human Epidermal growth factor Receptor 1) or *ERBB1* (avian ErythroBlastic leukemia viral

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(v-erbB) oncogene homolog 1), is located on chromosome 7p11.2 and encodes a 170-kDa glycoprotein. EGFR is one of four members of the membrane-bound ErbB/HER family of RTKs, also comprising HER2 (neu/ErbB2), HER3 (ErbB3) and HER4 (ErbB4).

Under normal conditions, EGFR is found as monomer on the cell surface in a structurally compact autoinhibited state, displaying minimal kinase activity [20]. Binding of ligand at the cell surface, leads to either receptor homo- or hetero-dimerization. The four members of the ErbB family are highly promiscuous, and their heterodimers are more potent in signal transduction than homodimers [21], which provides a means for signal amplification and diversification, with significant implications for their signaling capacity [22]. HER2, that has lost the capacity to bind ligands, and functions primarily by forming heterodimers with other family members [21], such as EGFR [23,24] is the preferred dimerization partner of all other HER receptors [25,26]. HER3 has lost robust kinase activity [27], and can only signal through heterodimerization [28]. Similarly to EGFR, HER4 is instead a fully functional RTK, capable of signaling as both homo- or heterodimer [29], however HER4 is the only family member capable of displaying growth inhibiting properties [30].

Seven growth factors have been identified as EGFR ligands, namely: EGF, Amphiregulin (AREG), Epiregulin (EREG), Heparin-Binding EGF-like Growth Factor (HBEGF), Betacellulin (BTC), Epigen (EPGN) and Transforming Growth Factor-α (TGF-α) [31]. Shifting from monomeric to dimeric state converts the receptor from the inactive to the active form [32]. EGFR signal transduction portion, located in a cytoplasmic area that contains the tyrosine kinase domain (TKD), has 5 regions: The N- and C-lobes, the ATP site (a highly reactive site of the TKD, and major target of anti-EGFR therapeutics), the hinge/inactive region and the allosteric site. Upon ligand interaction with the N-lobe, major conformational changes occur in the kinase structure that activate the ATP site for auto-phosphorylation. The newly formed phospho-tyrosine residues behave as docking sites for various adaptor molecules that subsequently initiate intracellular signaling cascades, leading to activation of downstream effectors, such as PI3K/AKT, MAPK, Ras/Raf/Mek/Erk, JAK/-STAT, and PLCγ1/PKC pathways, to name the most relevant [33–35].

Phosphorylation also initiates mechanisms of internalization of the dimerized complex, aiming mainly at maintaining the homeostasis, by interrupting the ligand-mediated signaling. Signal termination typically goes through endocytosis, followed by endosomal recycling back to the membrane, lysosomal degradation, or transport to various intercellular organelles [36–40] (Fig. 1). The regulation of EGFR, however, is more complex than initially assumed. While endocytosis was considered the event that immediately shuts down receptor signaling, there is now a consensus that signaling continues throughout endocytosis [38,39]. Novel unexpected functions of this receptor continue to emerge, some of which are linked to previously unrecognized subcellular localizations. Recent years have witnessed expansions of our mapping of the EGFR cellular trafficking odyssey from the plasma membrane to the nucleus and the mitochondria [41–43]. EGFR can translocate directly to the nucleus, and activate gene expression either alone or in conjunction with a transactivator like STAT3 [44]. Translocation of EGFR proteins to the mitochondria is significantly associated with resistance to cell death [11,45], and it is mediated by the ability of EGFR to bind to proapoptotic PUMA (p53 Upregulated Modulator of Apoptosis), which is mainly localized in the mitochondria [11,46].

2. EGFR-mediated oncogenesis

Dysregulation of EGFR is mainly initiated by an altered triggering of its TKD activity, caused by point mutations at the genomic *locus*, that will be described in the next section; however, also receptor amplification, transcriptional upregulation or ligand overproduction have been reported [47]. Although some cases of adenocarcinoma show both *EGFR* mutations and increased copy number, these are usually mutually exclusive events [48,49]. Amplification of *EGFR* is often associated with structural alterations, cataloged as class I-VIII, depending on the regions being affected. *EGFR-variant III* (carrying an in-frame deletion of 801 base pairs from exons 2–7 of the extracellular domain) is the most renown class, being the most common mutation associated with highly invasive GBM [50], besides being expressed in ~40% of head and neck squamous cell carcinomas (HNSCCs) [51]. Presence of EGFR-vIII in other tumor types remains controversial, and it is mainly attributed to technical artifacts [52]. GBM's cell of origin is currently believed to be a central nervous system (CNS) CSC in which EGFR signaling is altered [53], most probably by initial amplification of *EGFR-wt*, to which

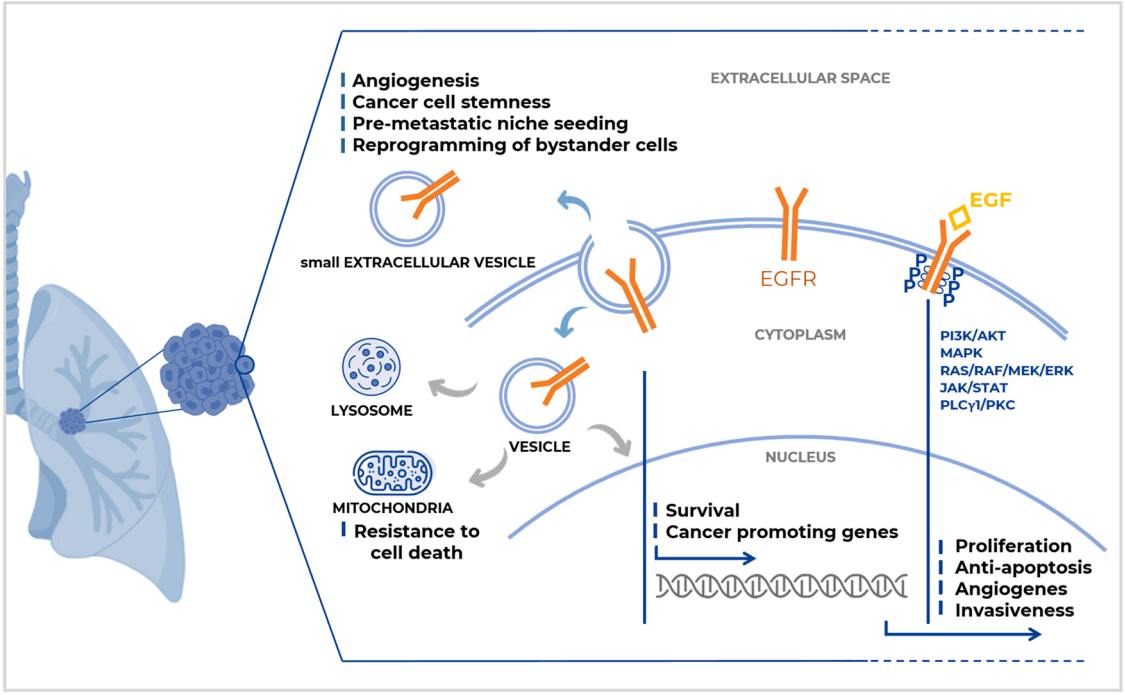

Fig. 1. Schematic of EGFR trafficking.

EGFR-vIII expression follows, as a late event [54]. EGFR-vIII is double trouble in that it is constitutively active given deletion of autoinhibitory residues, and because its abnormal conformation evades endocytic down-regulation. Consequently, EGFR-vIII does not get efficiently recycled, prolongs its level of activity at the cell surface that, even if quantified as displaying only 10% signaling intensity as compared to EGFR-wt, is enough to enhance tumorigenicity [55].

This notion, introduces an important aspect of dysfunctional EGFR regulation, which is linked to its altered protein turnover and subcellular distribution. Nuclear EGFR (nEGFR) localization in cancer cells, including NSCLC, has been described to promote survival, by functioning as nuclear kinase or co-transcriptional activator by kinase-independent interactions [56–61]. nEGFR drives expression of several cancer promoting genes, including *cyclin D1* [61], *nitric oxide synthase* (iNOS) [44], *B-Myb* [62], *Aurora Kinase A* (AurkA) [63], *cyclo-oxygenase-2* (COX-2) [64], *c-Myc* [65], and *Breast Cancer Resistant Protein (BCRP)/ATP-binding cassette subfamily G member 2* (ABCG2) [63]. Evidence supporting direct binding of EGFR to a specific DNA sequence is lacking. Thus, EGFR DNA-binding partners must accomplish such function. RNA helicase A (RHA) is one such partner [66], as well as STAT3 [44], STAT5A [63], E2F [62], DNA-dependent Protein Kinase (DNA-PK) [67], and Proliferating Cell Nuclear Antigen (PCNA) [68]. The relationship between nEGFR and cancer prognosis has been investigated in multiple solid tumors. In 2005, Lo et al. provided the first evidence in breast carcinoma patients that high levels of nEGFR were associated with poor overall survival (OS), as compared to patients with undetectable nEGFR [69]. Similar observations followed in NSCLC, where nEGFR enhances resistance to EGFR-targeted therapies, chemotherapy and radioresponse [70–74].

Dual Oxidase 1 (DUOX1), an enzyme involved in innate host defense in healthy lung epithelium, controls EGFR localization in lung cancer [75]. DUOX1 activation normally regulates EGFR internalization and recycling; its loss is instead associated with poor prognosis, given altered EGFR internalization favors its nuclear translocation, thus promoting nEGFR-associated tumorigenic functions [75]. Similarly, the pseudo-kinase Tribble 3 (TRIB3) promotes tumorigenesis by impairing autophagic and proteasomal degradation. Depletion of TRIB3 results in drastically decreased expression of several tumor-promoting factors, including EGFR, across cancers [76]. Conversely, elevated TRIB3 participates in the pathogenesis and progression of NSCLC by enhancing EGFR signaling [77]. In addition, growing evidence suggests that ubiquitination does not always serve as degradation signal; E3 ubiquitin ligase SMURF2 (SMAD Ubiquitination Regulatory Factor 2)-induced ubiquitination, for instance, enables EGFR stabilization [78]. Conversely, the E3 ubiquitin ligase CHIP selectively interacts with and degrades EGFRm, sparing the wt form [79]. ZNRF1 (Zinc and Ring Finger 1), a ring-type E3 ubiquitin ligase, associates with and ubiquitinates EGFR, thus promoting its endocytic trafficking and degradation [80]. ZNRF1 also promotes ubiquitination and degradation of Caveolin-1 (CAV1) [81], which is involved in regulating EGFR trafficking from early to late endosomes [82], thus positioning ZNRF1 as a key molecule involved in EGFR regulation. Depletion of ZNRF1 in lung cancer does indeed result in decreased EGFR ubiquitination, increased accumulation of EGFR in the early endosomes, and prolonged activation of AKT and ERK signaling [80].

Another aspect of EGFR trafficking is related to its ability to travel away from $EGFR^{+}$ cancer cells, that secrete it in small extracellular vesicles (sEVs) [83]. EGFR acts as a major cargo of sEVs and participates, with co-expressed proteins and small RNAs, in reprogramming of by-stander recipient cells, contributing to impacting immunity, pre-metastatic niche preparation, angiogenesis, cancer cell stemness and horizontal oncogene transfer [84].

Dysregulated EGFR signaling can also result from deficiency of one of its negative regulators, such as the E3 ubiquitin-protein ligase Casitas B-lineage Lymphoma (CBL), Leucine-rich Repeats and ImmunoGlobulin-like domains 1 (LRIG1), Cellular Communication Network factor 2 (CCN2), Mitogen-Inducible Gene 6 (Mig6), Mucin 15 (Muc15), and G Protein-coupled Receptor C5A (GPRC5A) that have been shown to suppress EGFR activation through various mechanisms [85–90].

The microRNA (miR) regulatory system is involved in eukaryotic gene expression [91], and miRs contribute to keep *EGFR* expression under control in healthy pulmonary epithelial cells [92]. One such regulator is miR-128b, located on chromosome 3p, whose allelic loss is one of the most frequent and earliest genetic events in lung carcinogenesis [93]. Another non-coding-related and somehow unexpected mechanism of aberrant *EGFR* activation has been recently described in GBM. Circular EGFR RNA (circ-EGFR) codes for a novel aggressive EGFR variant [94], named rolling-translated rtEGFR, which is undetectable in normal brains. rtEGFR sustains aberrant EGFR activation via its cross-membrane amino-acid sequence that, by acting as a "screw washer", interacts and reinforces EGFR membrane localization [94].

3. The EGFR mutations scenario in NSCLC

Approximately 10–40% of NSCLC patients display *EGFR* TKD activating mutations [2]. Prevalence is 10–30% in Caucasians, which increases up to 40–60% in Asians;[95,96] its incidence is approximately three times higher in non-smokers vs smokers, and in women vs men [97–99]. The reason/s for such disparities are still unknown.

The entire TKD is encoded by exons 18–24. EGFR kinase activating mutations most commonly cluster around the region encoding the ATP binding pocket in exons 18–21. There are two major types of "common mutations" that account for ~ 80–85% of *EGFR* genetic alterations: i) the "gain-of-function" mutations (~ 45% of *EGFR* mutations) occurring as short in-frame deletions in exon 19 (Ex19Del), of which there are at least 30 variants and ii) the point mutations in exon 21 (~ 40%), mainly resulting in arginine replacing leucine at codon 858 (L858R) [100–105]. Both mutations destabilize the EGFR inactive conformation, leading to increased receptor dimerization and activity [106].

The remaining 15–20% of *EGFR*-mutated tumors contain "uncommon mutations", that also display constitutively activate signal transduction, whose frequencies are almost evenly distributed among the involved exons (18–21) [106–110]. Despite their low frequency, in view of the high prevalence of lung cancer, ~30,000 newly-diagnosed NSCLCs per year harbor rare mutations [106].

EGFR exon 20 insertions (Ex20-Ins) may occur among NSCLC patients with a frequency of ranging from 0.1% to 4.0% among all patients. Exon 20 insertions appear at the TKD and include in frame insertions and three to 21 bp duplications within amino acids 762 and 774. Ex20-Ins seem to be not sensitive to first/second gen TKIs, therefore, their identification represents a critical issue for an adequate management of NSCLC patients [111]. EGFR Ex20-Ins do not directly affect the EGFR ATP-binding pocket, however, they seem to affect the C-helix domain, determining a constitutive activation of the TKI [112]. Moreover, it has been demonstrated that EGFR Ex20-Ins mutations determine an increasing affinity for ATP while reducing the affinity for first-gen EGFR TKIs [113].

Excluding mutations found as Ex20-Ins, that encompass a heterogeneous group with more than 100 mutations identified [114], the uncommon mutations involving codons G719 (exon 18), S768 (exon 20), and L861 (exon 21) are the most prevalent [108, 115–118]. The G719X point mutations, where "X" represents any of the possible substitutions (glycine to alanine G719A, glycine to cysteine G719C, and glycine to serine G719S) account for ~ 2–5% of *EGFR*-mutated tumors. Mutations affecting G719 can occur alone or paired with other uncommon mutations, such as S768I (serine to isoleucine) or L861Q (leucine to glutamine) [108, 115–118]. Mutations at E709 (exon 18) account for ~ 1.5% of rare *EGFR* mutations and frequently appear as "complex mutations" together with *L858R*, *Ex19Del*, or *G719X* [119,120]. Exon 19 insertions (~ 1%) result from the addition of a 6 amino-acid heterogeneous sequence, with a 4 amino-acid sequence (PVAI) shared by all [121]. To complete the picture, also unusual exon 25 and 26 deletions truncating

C-terminus of the *EGFR* [122], and Kinase Domain Duplications (KDDs) have been described in exons 18–25 and 18–26, although cases of duplication of exons 14–26 and 17–25 have been reported as well [123, 124], that appear to be among the rarest *EGFR* mutations in NSCLC [116,123].

Many unanswered questions remain, such as whether such myriad of different rare mutations differentially activate their downstream signaling pathways, considering mutation-specific signaling patters have been observed, for instance, when comparing EGFR-wt with EGFR-vIII or L858R [125]. Future work focusing on developing cell lines and organoids harboring rare *EGFR* mutations is necessary, and should be followed by generation of genetically-engineered murine models (GEMMs) to slice and dice their functional significance and test relevant drugs.

High-throughput comprehensive genomic screenings have also identified multiple *EGFR* co-mutations or compound mutations with non-*EGFR* genes within the same tumor [118, 119, 126–134]. The spectrum and prevalence of *EGFR* co-mutations (currently quantified as affecting 4–14% of patients), is relatively similar across the three most common subtypes (*Ex19Del*, *L858R* and *Ex20-Ins*) [135]. About non--*EGFR* compound mutations, inactivation of *RB1* is an early genetic event in ~10% of *EGFR*-mutant adenocarcinomas; alterations in other regulators of cell cycle, such as deletions in *CDKN2A* and *CDKN2B* genes, are observed in ~ 20–25% of cases; *PIK3CA* mutations characterize ~ 10% of advanced stage *EGFRm* adenocarcinomas; activating mutations in *CTNNB1* are rare in early-stage adenocarcinomas (1.8% in the TCGA cohort) and increase in late-stage tumors (~ 5–10%); while *KEAP1/NFE2L2/CUL3* (Kelch-like ECH-associated protein-Nuclear factor (erythroid-derived 2)-like 2-Cullin 3) pathway mutations have been recently identified in adenocarcinomas with activating *EGFR* mutations at diagnosis [132, 136–138].

4. EGFR personalized medicine: a serendipity story

Almost 20 years ago, when first-generation EGFR-tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib, were tested on NSCLCs, surprisingly only an unselected subset displayed significant survival advantage, advocating for the presence of a unique (and at the time unknown) predictive factor, in tumors that specifically responded to EGFR inhibition [139–142]. Thorough analyzes showed that non-smoking, young Asian women with adenocarcinoma reported dramatic response to treatment [2,143,144], and three landmark studies in 2004 finally clarified they uniquely carried mutations in the *EGFR* gene [145–147]. Such unforeseen discovery, suddenly shifted the therapeutic landscape of NSCLC from a purely histology-based approach, to treating molecular subtypes according to their distinct genetic alterations.

Currently, in patients affected by advanced NSCLC harboring "classic" EGFR mutations (Ex19Dels and L858R), tyrosine kinase inhibitors represent the standard first-line treatment. First- (gefitinib, erlotinib), second- (afatinib) and third- (osimertinib) generation of EGFR-TKIs showed to significantly improve PFS [148–156].

Within clinical trials, direct comparisons were performed between TKIs, demonstrating that osimertinib significantly prolonged both PFS and OS [156].

Regarding the "uncommon" EGFR alterations, EGFR mutations occurring in exon 18–21 may be sensitive to gefitinib, erlotinib or afatinib, whereas exon 20 insertions or *de novo* T790M mutation are considered not to be responsive to gefitinib, erlotinib or afatinib treatment [157–162]. Moreover, in patients treated as first line with first or second-generation EGFR TKIs, and developing the EGFR T790M resistance mutation at progression of disease, treatment with osimertinib can be administered as second-line therapy [163,164].

Historically, several anti-EGFR therapeutics were developed that can be subdivided into two categories: i) small molecule TKIs that inhibit EGFR TKD activity (Table 1), and ii) artificially-synthesized EGFR monoclonal antibodies (mAbs) that inhibit the activation of the EGFR ligand-binding domain.

4.1. The evolving TKI arsenal

First-gen EGFR-TKIs are reversible ATP-binding inhibitors, that decrease dimer auto-phosphorylation and intracellular signaling. Ex19Del and L858R, to place the results in historic context, were quickly identified as alterations that sensitized NSCLCs to such inhibitors. Early studies oversimplified the complexity of tumor genotypes by merely subdividing patients into *EGFRm* positive or negative, depending on the sole presence (or absence) of either of these mutations, that soon became "the" predictive biomarkers for anti-EGFR therapy [145–147]. However, differences exist among *EGFRm* genotypes, and care must be placed to not put everything under the same umbrella indiscriminately. For instance, patients carrying EGFR Ex20-Ins were usually not included in randomized clinical trials assessing EGFR TKIs activity in metastatic disease, and available clinical data suggest very low efficacy of first-gen TKIs, with an overall response rate of 5% and a disease control rate of 15% [165].

Instead it was noted that increased *EGFR* gene copy number could be associate with a better response to first-gen EGFR-TKIs [166], given their ability to inhibit *EGFR-wt*. While EGFR-TKIs initially elicited major tumor shrinkage, such astonishing response was regrettably not durable, and most patients relapsed after months [167]. The culprit was

Table 1
EGFR-therapeutics.

Drug	Mechanisms of action	Targets	Refs.
Gefitinib, erlotinib	First generation tyrosine kinase inhibitor	Ex19Del/L858R	[139–142,148–153,161, 179]
Afatinib, dacomitinib	Second generation tyrosine kinase inhibitor	Ex19Del/L858R	[154,155,157,176–178]
Osimertinib	Third generation tyrosine kinase inhibitor	Ex19Del/L858R/Ex19Del + T790M/ L858R + T790M	[114,156,163,164,182,184, 185,222,230]
Lazertinib	Third generation tyrosine kinase inhibitor	Ex19Del+T790M/L858R + T790M	[199]
ZN-e4	Third generation tyrosine kinase inhibitor	Ex19Del/L858R/Ex19Del + T790M/ L858R + T790M	[189]
Mobocertinib	Third generation tyrosine kinase inhibitor	Ex20Ins	[191]
EAI045 and EAI001	Fouth generation tyrosine kinase inhibitor	L858R + T790M/ L858R + T790M + C797S	[193–195]
JBJ-0412502	Fouth generation tyrosine kinase inhibitor	L858R + T790M + C797S	[196]
BI-4020	Fouth generation tyrosine kinase inhibitor	Ex19Del + T790M + C797S	[198]
Amivantamab	Bispecific antibody	Ex20Ins EGFR, MET	[200]
MRG003	Anti-EGFR IgG1 monoclonal antibody drug conjugate to a microtubule disrupting agent monomethyl auristatin E (MMAE)	EGFR overexpression or amplification	[201]
ABBV-321	Anti-EGFR IgG1 monoclonal antibody-drug conjugate to a pyrrolobenzodiazepine (PBD)	EGFR overexpression or amplification	[202]

identified as a frequent secondary *EGFR* mutation in exon 20, known as the "gatekeeper mutation" *T790M*, located at the entrance to a hydrophobic pocket within the ATP binding cleft [168,169]. Since the ATP site dictates the modus operandi efficacy of first-gen EGFR-TKIs, substitution of a threonine with a bulkier methionine side chain in the ATP pocket sterically hindered drug binding, eventually resulting in receptor activation, despite TKI treatment [168,170]. Interestingly, T790M displays a structural change reminiscent to the BCR-ABL imatinib resistance mutation T315I [171]. Besides *T790M*, additional mechanisms of resistance have been reported, including activating point mutations in genes involved in different pathways (i.e. RAS, PIK3CA), MET or HER2 amplification, and SCLC transformation [172–174].

To overcome these drawbacks, second-gen EGFR-TKIs (afatinib and dacomitinib) were developed, that irreversibly bind EGFR residue C797, and also act as multitarget TKIs, blocking signaling from all possible ErbB/HER-family homo- and hetero-dimers [175,176]. Second-gen TKIs showed improved progression-free survival (PFS) [154,155,177,178]. However, they displayed poor selectivity for L858R/T790M or Ex19Del/T790M mutants over EGFR-wt, leading to dose-limiting toxicities [179–181]. Only many years later these TKIs were revived to target uncommon *EGFR* mutations, after the LUX-Lung clinical trials (enrolling patients carrying *G719X*, *S768I*, and *L861Q*) showed a slightly higher PFS and ORR, as compared to later-developed third-gen EGFR-TKIs. However, afatinib use in real-world settings is still tempered by significant toxicities and adverse effects often necessitating dose reductions and/or treatment discontinuation.

Evolution towards third-gen TKIs, which proved active against exon 19 and 21 mutations, as well as the (in)famous *T790M* mutation, and concurrently displayed low avidity for EGFR-wt [182] and effective CNS penetration [156,183], revolutionized the therapeutic approach in *EGFRm* NSCLCs. Osimertinib was the first to receive FDA approval in 2015 for EGFRm-metastatic and T790M+-patients progressing on or after EGFR-TKI therapy. The AURA trial investigated the efficacy of osimertinib [182], and AURA2, AURA3 and AURA-extension trials uncovered its high clinical activity, as evidenced by a disease control rate (DCR) of $>$ 90% and an ORR of nearly 70% [163,184,185]. A phase III clinical trial (FLAURA) demonstrated that upfront use of osimertinib prevents early acquisition of *T790M* and prolongs PFS (17.2 months vs 8.5 months with first-gen TKIs) [156]. This important study represented the historic basis for establishing osimertinib as a first line EGFR-TKI option, which was approved by the FDA in 2018 for all patients with classical *EGFR* mutations.

By interacting with *C797*, osimertinib can target *T790M*; however, not surprisingly, tumors promptly counterattack by frequently developing *C797S* mutations, that adversely affects its therapeutic benefits [186]. EGFR *C797S* by impacting the covalent binding site of osimertinib [187], mechanistically parallels the acquired Bruton tyrosine kinase (BTK) *C481S* mutation seen in lymphomas, following progression on BTK inhibitors [188], suggesting cysteine-point mutations may be a recurring vulnerability for a broad range of kinase inhibitors. In essence, also with osimertinib, despite the initial major anti-tumor responses, acquired resistance inevitably occurs, whose mechanisms are being massively studied to identify targetable vulnerabilities.

Currently, a newer third-gen compound (ZN-e4) is being tested in phase I clinical trial including patients with EGFR G719X, Ex19Del, Exon 21 L858R, and L861Q or with osimertinib naïve T790M mutation osimertinib naïve, which, given its 20–40-fold selectivity ratio for EGFRm forms over the wt form, is expected to minimize potential toxicity [189]. Another compound, afatinib, a potent small molecule irreversible TKI that selectively targets mutant forms of EGFR while sparing EGFRwt is being tested for safety and tolerability (NCT02330367) in previously treated T790M^{+} NSCLC patients. Additionally, new EGFR TKIs (i.e. poziotinib, mobocertinib) and bispecific antibodies (i.e. amivantamab, see below) are currently under evaluation for *Ex20-Ins* patients in order to provide novel therapeutic options and to identify a new standard of care [190–192]. In details, poziotinib, is a TKI specifically designed to target the small kinase pocket generated by the EGFR/HER2 exon 20 mutations [113], which showed very promising preliminary results with an overall response rate (ORR) of 58% [190]. Mobocertinib, an irreversible small-molecule EGFR-TKI, designed to specifically target EGFR/HER2 exon 20 insertions, with selectivity over *EGFR-wt* [191], received FDA breakthrough therapy designation for pre-treated metastatic NSCLC patients harboring *EGFR Ex20-Ins*. In September 2021 it was approved as the first oral therapy specifically designed for patients with *EGFR Ex20-Ins* NSCLCs, based on the phase 1/2 trial results showing an ORR of 43% and favorable median progression free survival (PFS) of 7.3 months, having demonstrated potent activity against the uncommon activating mutations *G719A*, *G719S*, *S768I*, *L861Q*, and *L861R* [191].

After almost two-decades of adjusting therapeutic regimens, the seemingly never-ending-story of having to continuously identify next-gen EGFR inhibitors continues. Most of the currently available drugs act on the EGFR ATP-binding site, whereas the allosteric site has just recently been postulated as a better target for novel wt-sparing anti-EGFRm inhibitors. Design of potent allosteric fourth-gen compounds was expedited by efficient high-throughput screening methods capable of predicting synthetic effects. These efforts led to identify two compounds (EAI045 and EAI001), that can inhibit the ATP activity and stop its auto-phosphorylation, while concurrently overcoming resistance mechanisms [193,194]. EAI045 induced marked tumor shrinkage in transgenic mice carrying *L858R/T790M* and *L858R/T790M/C797S* tumors, in combination with cetuximab [193,195]. Its clinical utility was however limited by the potential toxicity of such combination [193]. More recently, a novel allosteric inhibitor (JBJ-0412502), which is active as monotherapy, appears to have greater therapeutic potential when combined with osimertinib, and even higher than osimertinib alone. However, these benefits only apply in the setting of *L858R* mutations [196]. Currently, brigatinib, a novel dual-target ALK-EGFR inhibitor, seems effective against *C797S/T790M/Ex19Del* triple mutant cells [197] and the fourth-gen EGFR-TKI BI-4020 appears to work on both the *Ex19Del/T790M* and *Ex19Del/T790M/C797S* background [198].

4.2. The anti-EGFR antibodies approach

Having EGFR become the first rationally-selected molecule for targeted therapy, also anti-EGFR antibodies were developed, that however are seldom used in routine clinical care. Amivantamab, targeting both EGFR and MET, demonstrated activity in NSCLC patients with several EGFR mutations, including C797S, exon 20 insertion and MET amplification, being the first bispecific antibody approved in patients with Ex20-ins, and is currently being tested in combination with the third-gen EGFR-TKI lazertinib [199] (NCT04077463) to increase efficacy in upfront treatment [200]. Other options being pursued include the antibody-drug conjugate (ADC) strategy, linking mAbs to cytotoxic drugs, to specifically limit their delivery to cells that express the target antigen of the selected mAb. MRG003, for instance, which is composed of an anti-EGFR IgG1 mAb conjugated to a microtubule disrupting agent, was able to stabilize disease in four EGFR-expressing head and neck (n = 2), esophageal (n = 1) and nasopharyngeal (n = 1) cancer patients [201]. A similar phase I study of the ADC ABBV-321, combining an IgG1 anti-EGFR antibody with a pyrrolobenzodiazepine dimer (PBD), via a cleavable linker, is active in EGFR-overexpressing tumors, including NSCLC patients (NCT03234712). Once bound, ABBV-321 gets internalized, the linker undergoes proteolytic cleavage, and the cytotoxic PBD is released, causing DNA cross-links and cell death [202]. ADCs built upon mAbs that specifically target proteins harboring truncal oncogenic driver mutations (such as certain mutant forms of EGFR), can also be envisioned, to maximize tumor specificity [203]. Several anti-EGFR AbDCs carrying cytotoxic radioisotopes are being studied, particularly for GBM therapy [204]. Other approaches exploit feedback mechanisms; for example, HER3 upregulation is often observed in

EGFRm tumors that are resistant to EGFR-TKIs, that may sensitize such cells to HER3-targeted ADCs [205]. HER3-DXd is indeed one such antibody that demonstrated antitumor activity in heavily pretreated advanced *EGFR*m NSCLCs [206], and is currently tested in a phase II study (NCT04619004).

Although less clinically developed, the use of nanoparticles as anti-EGFR immuno-conjugates has emerged as a strategy for selective delivery to EGFR-overexpressing cancer cells, with some advantages over ADCs, such as higher drug load, improved stability, controlled drug release, better pharmacokinetics and immunogenicity. By adopting a bacterially derived nanocell loaded with a cytotoxic agent (PNU-159682), and then coated with an anti-EGFR antibody, the new EDV (EnGeneIC Dream Vector)-D682 minicell achieved radiographic disease control in 8/9 patients, including response in 4/5 evaluable patients, in a recent phase I pancreatic cancer trial. A phase II study is currently enrolling patients [207]. Last, while use of miR therapy in the treatment of NSCLC is still a moving target [91], a nanocellular TargomiR delivery vehicle, loaded with miR-16–5p mimics, was designed to target EGFR^{+} tumors. The system adopted EDV minicells with surface-attached bispecific EGFR-targeting antibodies, for intravenous injection [208]. Preliminary data from phase I clinical trials for patients with EGFR^{+} Malignant Pleural Mesothelioma and advanced NSCLC showed manageable safety profiles supporting additional studies of TargomiRs, even in combination with chemotherapy or immune checkpoint inhibitors (ICI) [208]. Currently though, miR therapy is still brewing in the preclinical stage, and more data and validations must be accrued before eventually moving in the clinical settings.

5. Open questions in treatment strategies

Although several EGFR inhibitors are currently available, the optimal sequence to administer them has continued to be debated [209]. Recently, a cancer organoid model was established from a biopsy harboring an *EGFR* double mutation (*Ex19Del* and *L643V*), to evaluate drug responses to a range of different EGFR-TKIs [210], hinting this technology can assess the relevance of drug responses obtained directly from bedside to bench-side and back. Questions remain regarding which EGFR-TKI is most appropriate in respect to each specific *EGFR* mutation, disease characteristics, and likely availability of subsequent targeted treatment options following disease progression [211]. Even *Ex19Del* and *L858R,* that were initially considered en bloc as "the EGFR mutations" to screen for, should be treated as distinct types of NSCLC [99, 146,156,212]. Overall, rare mutations tend to display reduced response to early-generation EGFR-TKIs, as compared to common mutations [174, 213–215]. This may also underlie some cases of intrinsic/primary resistance, and explain why some of the patients believed to only carry classic mutations immediately displayed lack of treatment response to EGFR-TKI treatment in the past. Uncommon mutations present with a heterogeneous response; some have sensitivity to second-gen EGFR-TKIs (i.e., exon 18 mutations and exon 20 *S768I* point mutation) [157,216], whereas others are unresponsive to first-/second-gen TKIs, but sensitive to Osimertinib and Poziotinib (i.e., *Ex20-Ins*) [113, 114, 217–222]. Exon 20 mutations high heterogeneous nature encompass the complete spectrum of sensitivity to EGFR-TKIs [107,112,223,224], suggesting each of these rare variants should be carefully considered, and that clinical recommendations should be made on a case-by-case basis.

Overall, these data highlight the need to tailor targeted therapies not just to the gene which is mutated, but also toward the specific mutation within the gene, to be able to optimally pair patients with the best available therapies. This is a crucial aspect, since treating with EGFR-TKIs that have limited efficacy against certain mutations, may drive clonal expansion of the cells carrying the insensitive mutations, potentially causing an even more difficult-to-treat resistant milieu. In the future, with the promise of highly specific, possibly mutation-specific drugs, it will be possible to develop personalized drug treatments targeted to the molecular constitution of individual tumors [225]. In addition, state-of-the-art technologies, such as liquid chromatography coupled to tandem mass spectrometry, currently capable of simultaneously measuring multiple drugs in cell extracts [226], could also be adopted for in vivo follow-ups of specific drugs' uptakes in both primary and metastatic tumors, to better dose therapeutic treatments in a personalized manner.

Another major challenge is offered by the CNS being a pharmacological sanctuary site for metastases, because the blood-brain-barrier (BBB) restricts transit of TKIs into the brain parenchyma [227]. The poor ability of TKIs to penetrate the BBB may be caused by the ATP binding cassette subfamily B member 1 (ABCB1) and breast cancer resistance protein (BCRP) efflux transporters, which are involved in removing toxins, drugs and chemotherapeutics from the CNS [228,229]. Osimertinib showed statistically and clinically significant results in the FLAURA brain metastasis subgroup, as compared to standard treatment [230], and the risk of CNS progression was significantly reduced [156, 231]. However, novel EGFR-TKIs with proven efficacy in CNS are clinically needed. Pharmacokinetic studies in mice showed that almonertinib, a novel third gen TKI, has a good BBB penetration ability [232], and early clinical evidence of almonertinib activity in a patient with advanced EGFRm NSCLC and BMs has been reported, showing intracranial and extracranial response [232].

Last, despite targeted therapies are not characterized by the toxicity profile associated to other anti-neoplastic agents, anti-EGFR are typically characterized by gastrointestinal and cutaneous adverse events [233,234]. More data are needed to determine how to manage cutaneous and other dose-limiting toxicities (i.e. diarrhea) associated with EGFR inhibitors. As the use of these agents becomes more widespread, evidence-based guidelines for management of adverse reactions is imperative. Improved control of adverse reactions including dermatologic and gastro-intestinal toxicities, may enable patients to tolerate higher doses of cancer therapies for longer durations, and this may lead to better control of their illness.

6. Tumor acquired resistance: an eternal issue in cancer therapy

The tractability of EGFR as druggable target is too frequently eclipsed by our current inability to manage acquired resistance to its inhibitory therapy. World-wide efforts to address the underlying mechanisms, made EGFR-TKI-based therapy the paradigm of how constantly revise therapeutic management.

Since EGFRm NSCLCs are "addicted" to oncogenic EGFR signaling, it

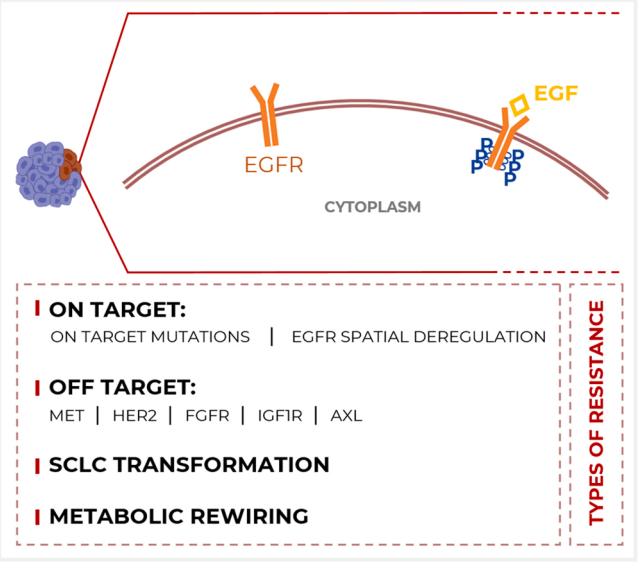

Fig. 2. Mechanisms of resistance to anti-EGFR therapy.

is not surprising that its blockade forces tumors to adapt, by turning on a series of molecular/cellular mechanisms (Fig. 2) that are classified as i) *EGFR*-dependent mechanisms of resistance, ii) activation of parallel pathways or EGFR-independent mechanisms of resistance, and iii) histologic transformation [235]. We propose adding to this growing list also EGFR spatial deregulation (included in i) and iv) metabolic rewiring.

6.1. EGFR-dependent mechanisms of resistance

Frequently, *EGFR* modifications occur as self-directed/on-target secondary mutations in the *EGFR* gene itself (exemplified by *T790M* and *C797S*) that, by affecting drug binding and ATP binding pocket, reactivate the TKI-silenced EGFR signaling. The initial sensitizing *EGFR* mutations seem to bias the on-target variant that emerges upon drug treatment. For instance, both *T790M* and *C797S* are more frequent in *Ex19Del*$^{+}$, rather than *L858R*$^{+}$ tumors [236,237], and *G724S* appears to be uniquely present in *Ex19Del*$^{+}$ cells [238], suggesting continued EGFR-dependence in these tumors. On-target mechanisms are more commonly described upon first/second-gen TKIs, whereas parallel/by-pass signaling is prevalent with osimertinib [239–241], where only ~ 10% of patients develop on-target mutations, i.e. *C797S* [242]. The reason why EGFR-dependent mechanisms of resistance are more frequent with first/second-gen TKIs may be explained by the potency and the binding affinity of the drugs themselves [173].

EGFR amplifications and copy number alterations are additional on-target mechanisms [239,243,244].

While discussing on-target events, we would like to make an *excursus* on the debate over *T790M* intrinsic nature. It is still controversial whether T790M-mediated acquired resistance derives from selection (or not) of pre-existing clones [245]. By culturing EGFRm cells with escalating concentrations of gefitinib, early-resistant clones (emerging within 6 weeks) were *T790M*$^{+}$, and derived from pre-existing *T790M*$^{+}$ cells [246], as shown by tracking cancer evolution via DNA barcoding [247]. Conversely, late-resistant clones (24 weeks) were initially *T790M*$^{-}$, arose in drug-tolerant persister cells, and could gradually expand to become *de novo T790M*$^{+}$ dominant clones [246].

World-wide screenings reported an intrinsic *T790M* frequency ranging ~ 3–80% [248–253], that was attributed to potential sequencing artifacts caused by tissue processing [254]. In a paired experiment, comparing mass spectrometry with standard direct sequencing, the frequency of T790M shifted from 31.5% to 2.7% [255], implying detection methods must be carefully standardized.

By using paired pre- and post-treatment tissues, a cohort of first-line patients with short-time osimertinib exposure, showed on-target resistance was uncommon, whereas off-target mechanisms, including histologic transformation, were seen frequently [256]. *C797S*, commonly acquired on later-line osimertinib, was not identified, suggestive of first- and later-line osimertinib displaying different resistance spectra [256].

Another nuance of on-target resistance is mechanistically achieved through EGFR spatial deregulation. Such alteration is responsible for increasing EGFR availability on the plasma membrane, thus inducing a persistent signaling output [257]. We recently identified a novel mechanism that EGFRm kinase disrupts microtubule organization and results in defective endosomal/lysosomal pathways [258]. This prevents efficient degradation of phosphorylated proteins that become trapped within the endosomes, and continue to signal, abnormally amplifying downstream proliferative/survival pathways, such as AKT and MAPK [258]. Lysosomal-inhibitors such as hydroxychloroquine (HCQ) and the microtubule-stabilizing agent Paclitaxel were able to re-sensitize resistant cells to EGFR-TKIs, thus identifying the endosome-lysosome pathway and microtubule dysfunction as a mechanism of TKI resistance [258]. Interestingly, a combination of osimertinib and chloroquine, markedly decreased tumor growth, as compared with osimertinib alone, in xenograft mice carrying *EGFRm* gefitinib-resistant cells [259], warranting further exploration of these therapies in the acquired resistance arena.

Being osimertinib a third-gen TKI, it is characterized by an increased potency and an irreversible covalent binding to mutant EGFR with specificity for EGFR T790M mutants [260]. Therefore, its spectrum of resistances shows a reduced incidence of EGFR-dependent mechanisms, which includes only a 6% of third EGFR mutations, and a higher incidence of EGFR-independent events [242].

6.2. Activation of parallel pathways or EGFR-independent mechanisms of resistance

Off-target resistance frequently manifests through activation or amplification of other RTKs, that work synonymously to EGFR, by activating in a snow-ball effect, common downstream effectors. A plethora of mechanisms have been described: MET amplification, HER2 amplification, HER3 augmentation, oncogenic fusion/chromosomal rearrangements, including RET, BRAF, NTRK, ROS1, and FGFR; additional mutations (i.e. RAS, PIK3CA, FGFR) [261].

MET is one of the most frequently altered pathway at the resistance after EGFR treatment, resulting in bypassing of the EGFR downstream signaling through STAT, MAPK and PI3K pathways [262]. MET amplification was found in a percentage ranging from 5% to 22% of patients progressing to EGFR TKIs, with increasing incidence in third vs first gen TKIs [242,260,263]. Combinatorial approaches with double inhibition of EGFR and MET are currently under investigation in patients with EGFR-mutant NSCLC with MET amplification with promising results [264,265]. Moreover, other in vitro evidences reported additional mechanisms of resistance linked to MET, including also MET activation achieved through overexpression of its ligand, hepatocyte growth factor (HGF), is linked to decreased response to TKIs [266].

Similarly, HER2 amplification mediates EGFR TKIs resistance by alternative activation of the MAPK/PI3K pathways in about 12% of patients progressing to first-gen EGFR TKIs with no coexisting T790M [267]. In patients progressed to second line osimertinib, HER-2 amplification was detected in 5% of patients, while in patients treated with osimertinib as front-line HER-2 amplification was found in 2% of patients [268,269]. Interestingly, HER2 amplification and the EGFR T790M mutation seems to be mutually exclusive in all of the observed findings [268,269].

A recent study has just demonstrated that formation of the heterodimer EGFR/HER2 can be induced by tumor-associated macrophages, via secretion of the EGFR ligand Epiregulin, that eventually results in activation of the EGFR/HER2-AKT axis that causes resistance to TKI treatment [270].

Also the ErbB family member HER3 is involved in EGFR-TKI resistance in *EGFR*-mutated tumors [271]. For example, aberrant MET due to *MET* amplification when coupled with HER3 can lead to PI3K/AKT/mTOR signaling in tumors during EGFR-TKI therapy [172]. Moreover, the HER3 ligand Heregulin induces HER2/HER3 coupling and signaling for cancer cell survival independently from the EGFR [272].

Further, rare chromosomal rearrangements have been described in 4–7% of patients, mainly progressing to second-line osimertinib [244], including RET (RET-ERC1, CCDC6-RET and NCOA4-RET), BRAF (AGK-BRAF, ESYT2-BRAF, PCBP2-BRAF and BAIAP2L1-BRAF), NTRK (TPM3-NTRK1), ROS1 (GOPC-ROS1) and FGFR (FGFR3-TACC3) [244,268,273,274].

Additional alterations in other oncogenes have also been found to drive EGFR TKI resistance. RAS mutations, including KRAS and NRAS, have been described as mechanisms of resistance across different TKIs generations, at variable frequencies ranging from 1% to 40% [269,275].

BRAF mutations (mainly the classic V600E) were reported in 3% of patients progressing to first- or second-line osimertinib [268,269].

PIK3CA activating mutations or amplifications are described in 3–5% of patients progressing after first/second-gen TKIs [173] and in 5–12% of patients after progression to third-generation TKIs [268,269]. Analogously, activation of FGFR (Fibroblast Growth Factor Receptor), one of

the few mechanisms identified in the limited amount of data describing adaptation to afatinib treatment [276], and also present in first- and third-gen TKI-resistant tumors [277], operates by favoring transformation toward mesenchymal phenotypes [278]. Likewise, the RTK IGF1R (Insulin-like Growth Factor 1 Receptor) allows mesenchymal trans-differentiation via MAPK and AKT pathways [279]. In the same way, overexpression of the RTK AXL (*AneXeLekto*, uncontrolled), supports EMT-associated resistance to osimertinib, and emergence of tolerant cells via AKT and MAPK [280]. Interestingly, generation of AXL^+ cells in erlotinib-resistant cells is contingent on methylation of a specific CpG island within the promoter of MEST (MEsoderm Specific Transcript), a gene that contains miR-335 in its second intron [281], defining a potentially novel mechanism coupling epigenetics to ontogeny of resistant cells.

Unsurprisingly, expression of IGF (Insulin-like Growth Factor) and Src/FAK, partake in the EGFR-bypass network, by converging on AKT and MAPK to sustain EMT induction [282,283].

6.3. Histologic transformation

SCLC transformation is a phenotypic evidence of acquired resistance to all EGFR-TKIs, that was never identified in patients with *EGFR-wt* chemo-resistant NSCLCs [240]. The original *EGFR* mutations are preserved within the SCLC specimen, providing evidence of their shared clonal origin [284,285]. Tumors that later transform into SCLC carry loss of both *RB1* and *TP53*, similarly to classical SCLC alterations [285]. Therefore, one hypothesis poses that following EGFR-TKI exposure, resistant cells accumulate genetic alterations, such as loss of *RB1* and *TP53*, that allow differentiation along the SCLC lineage, that does not require active EGFR signaling. Moreover, other genetic signature may help identify the SCLC transformation, including alterations in the PI3K pathway as common features characterizing SCLCs genomic signature [286]. Therefore, this mechanism entails molecular by-passing of EGFR through lineage-adaptation.

Rare transformations into squamous cell lung cancer also occur upon treatment with all currently-available TKIs, whose genomics is still unraveled [256]. In reality, another histologic switch occurs in EGFR-TKI-resistant tumors, i.e., the EMT [240,287,288], which is fired up by almost all the molecular events we previously annotated as off-target mechanisms. Since EMT frequently co-occurs with by-pass activation, it is challenging to determine the exact contribution that EMT per se has on therapeutic failure [289].

6.4. Metabolic rewiring

Last, metabolic rewiring is another feature adopted by *EGFRm* cancer cells to defend against EGFR-TKIs. We demonstrated that Cav1 and the glucose transporter GLUT3 have significantly stronger physical interactions in EGFR-TKI resistant than sensitive NSCLC cells, and that Cav1 mediates glucose uptake via GLUT3 only in resistant cells [290]. Cav1 expression was inhibited by using the FDA-approved anti-cholesterol drug Atorvastatin, thus disrupting its oncogenic interaction with GLUT3. Importantly, Atorvastatin-exposed *EGFRm* transgenic mice (*T790M/L858R*) displayed decreased tumor mass [290], demonstrating Atorvastatin can be repurposed to impair a glucose uptake mechanism distinctly found in TKI-resistant NSCLCs. Similarly, Osimertinib-tolerant NSCLCs display abnormal Krebs cycle activity, that could be restored by blocking miR-147b, thus providing a new strategy to prevent drug-tolerance-mediated tumor relapse [291].

Also alterations in lipid metabolism are under investigation, as EGFR on lipid rafts is indicative of resistance, and inhibition of lipid raft formations sensitizes $EGFR^+$ cells to TKI treatments [235, 292–294].

During development of resistance, palmitoylation of EGFR affects its structure, causing abnormal trafficking and localization, such as EGFR translocation to the nucleus or the mitochondria. Fatty Acid Synthase (FASN) is important in the synthesis of palmitate, and its overexpression is associated with poor prognosis, and drug resistance [295,296]. We identified a novel EGFR/FASN signaling axis only occurring in NSCLCs with acquired EGFR TKI-resistance, and showed that palmitoylated nEGFR takes part in acquired resistance [297]. Of relevance, pharmacological inhibition of FASN with Orlistat (an FDA-approved anti-obesity drug) was able to block EGFR palmitoylation, induce EGFR ubiquitination, abrogate EGFRm signaling in TKI-resistant cells and reduce in vivo tumor burden in EGFRm (*T790M/L858R*) transgenic mice [297].

Palmitoylated EGFR enters the nucleus and influences gene regulation patterns to facilitate survival, although what it regulates is still undefined. Most probably, concurrently to TKI inhibiting EGFR signaling, EGFR-addicted cells, under selective pressure, counter-attack by stabilizing its oncogenic signaling via EGFR palmitoylation. In a way, this is another subtlety to induce acquired on-target resistance by coercing fatty acids to strengthen EGFR signaling. Conversely, when palmitoylated EGFR enters the mitochondria, it regulates their dynamics by promoting their fusion [298] to sustain ATP production and sustain cell survival, thus causing a shift of metabolic dependency on mitochondria, which explains why their targeting can re-sensitize resistant cells to TKIs [299].

Overall, the fatty acid metabolism pathway is emerging as candidate target for *EGFRm* NSCLCs that develop resistance to anti-EGFR therapy.

Interestingly, glucose metabolism provides acetyl-CoA for *de novo* fatty acid synthesis, and a recent study demonstrated that suppression of FASN in NSCLC cell lines reduces the activities of glucose metabolism and the AKT/ERK pathway [300]. Recently, it was also demonstrated that Metformin alleviates inflammation through inhibiting endogenous fatty acid synthesis and Akt palmitoylation in macrophages [301], providing evidence that Metformin could act on FASN to ameliorate macrophages-mediated inflammatory diseases.

There is still a lot about EGFR-TKI-resistance that we do not know, with up to 60% patients harboring non-identified genetic and non-genetic resistant mechanisms when treated with new third generation TKIs as first line treatment [261]. Non-coding RNAs may play a part in it. miR-21, which is over-expressed in virtually all cancers [302], hence the alarm-miR appellative [91], is implicated in EGFR-TKI-resistance [303] by downregulating PTEN and PDCD4, and concomitantly activating PI3K/Akt [304]. Similarly, miR-210 is released in high amounts by osimertinib-resistant cells with EMT features [305]. Conversely, several tumor suppressor miRs have been described, such as miR-483-3p that enhances gefitinib sensitivity via reversing EMT;[306] miR-138–5p that reverse gefitinib resistance by negatively regulating the G protein-coupled receptor 124;[307] let-7 and miR-17 that regulate gefitinib resistance by targeting MYC and CDKN1A;[308] miR-130a that restores sensitivity by targeting c-MET-mediated signaling;[309] miR-146b-5p that overcomes resistance by regulating the IRAK1/NFkB signaling pathway;[310] and miR-34a that augments sensitivity to erlotinib, and whose (and miR-133b's) overexpression is capable of regulating EGFR itself, given also miR-133b's ability to directly bind EGFR 3' UTR, thus interrupting EGFR-mediated signaling [311,312].

Recently, also lncRNA has been described as associated with resistance to EGFR-TKIs, with small nucleolar RNA host gene-14 (SNHG14) being able to confer resistance to gefitinib by sponging miR-206-3p [313].

Nowadays, the RNA therapy road is still paved with several challenges that need to be addressed (mainly identification of potential off target effects and improved delivery methods) before their effective translation into future therapeutic applications can take place [91]. However, there is considerable interest in designing miR drugs for pulmonary diseases [91], that will translate into future weapons for our war against NSCLC.

7. Real time follow up of tumor landscapes: the rise of the liquid biopsy

Under-genotyping is still too frequent, also among patients with advanced/recurrent NSCLC. In addition, our incomplete ability to follow-up the molecular dynamics leading up to resistance build-up, is a matter of concern. Currently, tumor biopsies represent the major source of material for biomarker testing. However, sequential invasive biopsies for longitudinal assessment are uncomfortable/risky; cytological specimens can be pauci-cellular; and, most importantly, a single-spot biopsy is unlikely to capture the complexity of the entire genomic scenario. Ideally, repeated liquid biopsies performed at different time-points should be performed, thus overcoming the above hurdles. However, *hic stantibus*, this is a chimera we are still chasing.

Plasma sampling, containing circulating tumor DNA (ctDNA), tumor cells (CTCs) and extracellular vesicles, shed from either the original tumor or its metastases, provides a precious representative screenshot of the total tumor mutation burden (TMB). Cancer-associated genetic alterations, such as point mutations, deletions, copy number variations, and methylation patterns, can be detected in ctDNA [314,315]. Yet, shedding of ctDNA differs among tumors [316] and ~ 15–20% of patients carry non-shedding tumors, most likely due to lack of tumor vascularization, tumor load, or low proliferation rates [317]. In addition, lack of specific biomarkers can still hamper its use in routine diagnostics of recurrent patients. As an example, while evidence of *TP53* and *RB1* loss on ctDNA could trigger tissue re-biopsy to assess for SCLC transformation [318], the molecular landscape in patients with SCC transformation is more complex and, at present, cannot be suggested by ctDNA analysis alone [256].

Historically, serial ctDNA sampling proved capable of revealing both activating *EGFR* mutations and the emergence of T790M upon gefitinib treatment [315], demonstrating its potential to guide clinical decisions. In addition, several tumor-escape mechanisms after first-line osimertinib were characterized by ctDNA analyses during FLAURA phase III trial [183,319], validating ctDNA as a rich source to scan for actionable pathways. Furthermore, the clearance of *EGFR* mutations in ctDNA from NSCLCs upon TKI treatment positively correlated with response rate, PFS and OS, suggesting ctDNA-monitoring could help predict clinical outcomes [320]. In 2016, the FDA approved it as the first liquid biopsy test to analyze the presence of specific *EGFR* mutations (*Ex19Del* or *exon 21* substitution mutations). Nonetheless, several studies found variable concordance rates (57–90%) between the mutation status estimated from plasma and tumor DNA samples [321–325]. Correlation of ctDNA and tissue will be analyzed in currently-active multicenter studies, such as MELROSE [326] or ELIOS (NCT03239340), whose primary objective is indeed performing tumor genetic profiling via serial monthly plasma ctDNAs as well as on renewed tissue biopsies upon progression to osimertinib.

Current guidelines encourage the use of NGS technologies and liquid biopsy, which are usually capable of detecting multiple mutations included in large panels, even if the proportion of tumor cells in the specimen is as low as 1–5% [323,327].

Advantageously, given the increasing use of ctDNA as a monitoring method for disease recurrence, it is likely that alterations will be detected in asymptomatic patients, requiring to start quantifying the length of latency between detection by ctDNA and frank progression [328].

NSCLC served as proof-of-concept that also CTCs could potentially be used in liquid biopsy protocols, as exemplified by i) a study of *EGFRm* NSCLCs in which activating *EGFR* mutations (detected through traditional tumor biopsy) were confirmed in 19/20 patients using a microfluidic CTC-enrichment platform followed by a PCR-based assay;[248] or ii) an *EGFR* mutation analysis in patients treated with EGFR-TKIs in which *T790M* was detected in CTCs from 9/14 patients (64%) who had clinical progression [248]. Recent genomic analyses of single CTCs have been reported in patients with lung cancer [329], that enabled evaluation of CTCs heterogeneity by often disclosing presence of co-existing mutations within a single cell.

EV-derived mRNA can also be adopted and their mutational analyses showed improved sensitivity and better correlation with patients' clinical outcomes over ctDNA-based approaches [330,331]. In addition, since the RNA contained within EVs is well-preserved, this is an ideal specimen to detect gene rearrangements and expression. However, the majority of circulating EVs are not of tumor origin, therefore new platforms capable of capturing tumor-derived EVs from blood must be implemented. Anti-EpCAM-grafted silicon nanowire arrays were recently engineered to mimic the distinctive structures of intestinal microvilli, dramatically increasing surface area and enhancing tumor-derived EV capture, that showed diagnostic applicability [332].

Liquid biopsy is able to capture spatial inter- or intra-tumor heterogeneity, however, coupling this technique with different other techniques, including imaging will provide a more comprehensive picture, if validated in the future. Mass spectrometry-imaging showed that *EGFRm* adenocarcinomas display higher ions intensity of their phospholipids than *EGFR-wt* specimens, consistent with *EGFRm* NSCLCs having a unique lipidomic metabotype in their pleural effusions [333]. Methods based on isotopically-labeled EGFR-TKIs probes were tested for in vivo molecular imaging of EGFR spatial distribution in NSCLC xenografts [334,335]. Once the intrinsic limitations of such methods (mainly a low-throughput methodology requiring large amounts of tissue and complex pre-treatment protocols) will be overcome, they will certainly be adopted in clinical lung cancer diagnostics to image spatial heterogeneity. In addition, therapeutic antibodies were tested in phase I trials and proved safe for imaging applications (Cetuximab-IRDye800CW and Panitumumab-IRDye800CW) [336].

Moreover, liquid biopsy may help understanding the molecular response to pharmacologic treatment and provide information on dynamics of clonal heterogeneity. A number of studies evaluated the correlation between treatment outcome and the amount of somatic mutations in liquid biopsies of NSCLC $EGFR^{+}$ patients treated with TKIs with very positive results [328,337–339]. Therefore, the role of the liquid biopsy to longitudinally monitor patients during treatment and to detect the minimal residual disease is under investigation and validation. Moreover, novel tools integrating different techniques combined together through artificial intelligence algorithms are in development, and, in this scenario, novel minimally invasive techniques such as liquid biopsy and radiomics may be successfully integrated [340–343].

We are slowly chipping the way but such efforts, aimed at developing non-invasive screening tools, will eventually improve therapy resistance management and shorten the current delays in initial lung cancer diagnosis, which contributes to delayed treatments and poor prognoses.

8. Work-in-progress on future EGFR-directed strategies

The single oncogenic driver model is failing to satisfactorily portray the clinical complexity of NSCLC. Therefore, we are at the verge of a new era in which multifaceted therapeutic portfolios must be conceived to tackle the totality of the genomic alterations adopted by NSCLC to grow and thrive.

Innovative trials, such as ORCHARD, that contain a biomarker-directed allocation platform to assign targeted combination treatments to patients that failed first-line Osimertinib treatment, will make great stride to design better integrated future strategies.

Future research will need to establish the best avenues to treat the wide range of molecularly-diverse and divergent (from the original tumor) diseases that arise under drug treatment by i) identifying the most effective combination partners for combination therapy, ii) solving the ongoing dilemma of whether an upfront combination strategy prevents or postpones emergence of resistance with higher efficiency than a sequential approach, as well as iii) continuing developing novel therapies capable of simultaneously hitting multiple resistance-conveying targets.

In the meantime, we have various next-line options, that we will summarize below.

8.1. Logical combinations

Multiple studies, including FLAURA2 are currently assessing Osimertinib with or without platinum-based chemotherapy (PBC). Exploratory analysis demonstrated improved OS of ABCP (atezolizumab, bevacizumab, carboplatin, and paclitaxel) strategies, in patients who failed initial EGFR-TKI treatment [344]. A recent phase III trial found that addition of pemetrexed-carboplatin to gefitinib significantly prolonged median PFS in presence of mutations in exons 18,19, and 21, as compared to gefitinib alone (16 months vs 8 months) [345]. Future clinical studies should assess the efficacy of pemetrexed-carboplatin in combination with afatinib for treatment of patients harboring rare mutations.

Various trials combining different EGFR TKIs exist, such as NCT03122717 testing osimertinib and gefitinib [346]. Strategies attempting simultaneous targeting of by-pass mechanisms are flourishing, as well. Combining osimertinib with crizotinib (a multikinase inhibitor with potent activity against MET) seems effective in treating Osimertinib-resistant patients harboring MET amplification [347]. Other MET inhibitors (savolitinib and tepotinib) are currently tested in clinical trials (SAVANNAH, NCT05009836, NCT02864992). Given the importance of MAPK/ERK pathway to which a substantial fraction of NSCLC is so profoundly addicted, also combinations of osimertinib with MEK (Ras/Raf/MAPK) inhibitors are being pursued. Following promising preclinical data on GEMMs (L858R/T790M) [348], osimertinib plus selumetinib is currently investigated in the clinical trial NCT03392246 [349]. In addition, clinical evidence on the effectiveness of combining osimertinib with drugs targeting other downstream pathways, by adopting a BRAF inhibitor [350] or the multi-kinase inhibitor cabozantinib [351] are starting to emerge. A case report showed impressive radiological and ctDNA response in a patient who developed *BRAF*V600E, by combining osimertinib, dabrafenib (BRAF inhibitor), and trametinib (MEK inhibitor) [352]. Similarly, another case report found that a patient with secondary MET mutations upon crizotinib treatment, clinically benefitted from combinatorial therapy of osimertinib and cabozantinib [353]. *RET* fusion is another well described mechanism of osimertinib resistance and treating patients with cabozantinib, pralsetinib (RET inhibitor) or selpercatinib (RET inhibitor) combined with EGFR inhibition, resulted in significant response [273,354].

As *addendum*, we would like to note that while EGFR-TKIs mainly act on *EGFRm*, accumulating evidence has demonstrated that *EGFR-wt* is critical in the pathogenesis and progression of NSCLC, and elevated *EGFR-wt* expression not only correlates with acquired resistance to third-gen EGFR-TKIs but also participates in the maintenance of mutant *KRAS*-driven NSCLCs [355,356], highlighting the need to identify targeted therapeutics also against abnormal amounts of *EGFR-wt*.

8.2. Targeting the Cancer Stem Cell compartment

Another area of intense preclinical investigation deals with targeting CSC that are major players in therapy resistance. Increased expression Musashi-2 (MSI2) is implicated in EGFR-TKIs acquired resistance [357], and its depletion increases sensitivity to gefitinib and osimertinib in drug-resistant cells, suggesting that combined targeting of MSI2 with EGFR-TKIs may help overcome/prevent recurrence. Similarly, upregulation of the Hedgehog (Hh) signaling contributes to EGFR-TKI-resistance, and combinations of an Hh-signaling inhibitor with EGRF-TKIs displayed a marked synergistic anti-tumor effect in NSCLC cells. Hh tightly regulates EMT through its downstream targets Snail, ZEB1, and TWIST2 [358] and by directly regulating ABCG2, a stem cell marker and major multidrug resistance pump [359,360]. The Hh inhibitor SANT-1 reverted EMT and reduced CSC abundance [361]. EMT per se is described as a CSC-generating process [362] and both EMT and CSCs are involved in acquired resistance to EGFR-TKIs [363–365]. ZEB1, a crucial EMT inducer directly suppresses transcription of miR-200c, that is itself involved in modulating BMI1, an established modulator of CSCs [366,367]. ZEB1 confers EMT-mediated resistance to gefitinib, and NSCLC patients with acquired resistance to EGFR-TKI show high BMI1 expression [368], suggestive of the ZEB1-BMI1 axis acting in clinical resistance settings. Consistently, our data suggest that BMI1 upregulation might be a novel bypass-mechanism to osimertinib resistance in H1975 (L858R/T790M) cells (EL, GM, DGT, Azhar Ali, personal communication). BMI1 is the key regulatory component of the epigenetic Polycomb Repressive Complex-1 (PRC1). We recently demonstrated, at the single nucleus level, that the association of BMI1 to PRC1 is needed for its E3-ligase activity in NSCLC cells [369]. The epigenetic role of BMI1 in NSCLC is particularly intriguing since its overexpression drives stem-like properties associated with induction of EMT, resulting in poor prognosis [370]. Yet, BMI1 is indispensable for the regulation of self-renewal in hematopoietic and leukemic stem cells, as well as for regulating cancer initiating cells, metastasis, invasion and therapy resistance within various cancer types [371]. We previously identified BMI1 as a critical druggable target in NSCLC, whose inhibition in tumorigenic *C/EBPα* null NSCLCs impairs their tumor-propagating ability [372]. Similarly, we observed that *C/EBPα* null hematopoietic stem cells have increased expression of Bmi1 and enhanced competitive repopulating activity [373]. Importantly, by adopting a novel drug (PTC596) capable of negatively impacting BMI1 activity, that recently entered Phase1b (NCT02404480), we decreased in vivo tumor growth in *KRAS*-driven murine models of NSCLC [374].

Since we suspect that BMI1 upregulation might bypass osimertinib resistance, we speculate that PTC596 might be considered for future combined targeting strategies.

Several other actionable pathways converge on stemness, such as the Notch-EGFR cross-talk that promotes drug resistance, EMT, and disease progression in various carcinomas [375]. Notch3 associates with β-catenin, resulting in increased β-catenin stability, which is critical for drug stem-like persistent cells [376,377]. Consistently, inhibition of the β-catenin signal enhances sensitivity to EGFR-TKIs and contributes to suppressing stem cell-like properties that relates to EGFR-TKI resistance [378].

8.3. Targeting novel vulnerabilities

Development of therapeutic approaches for SCLC transformation are underway. Besides the option of extending the time-to-treatment failure of *EGFR/TP53/RB1* triple-mutant NSCLCs by combining upfront EGFR-TKIs with conventional SCLC therapy (platinum/etoposide), recent studies implicate that similar upfront combinations with modulators of EZH2 (Enhancer of Zeste 2), may be beneficial to use [379]. Aurora kinases (AURKA or AURKB) have also been suggested as potential therapeutics, and an interventional clinical trial of osimertinib with the AURKA inhibitor alisertib is currently ongoing (NCT04085315). Another approach utilizes BCL-2 family inhibitors [284]. Finally, targeting cell cycle vulnerabilities created by *RB1* loss, that result in sensitivity to checkpoint kinase 1 (CHK1) and polo-like kinase 1 (PLK1) inhibitors, might yield synthetic lethality combinations [380].

Preclinical studies demonstrated that up-regulated EGFR increases VEGF expression, and elevated VEGF, in turn, contributes to emergence of resistance to EGFR TKIs [381], creating a catch-22 situation. A cohort study addressing efficacy of gefitinib or afatinib in combination with bevacizumab (anti-Vascular Endothelial Growth Factor, VEGF mAb) prolonged PFS in EGFRm NSCLCs [382]. Similarly, combination of erlotinib plus bevacizumab or ramucirumab (anti-VEGFR2 mAb) yielded remarkable PFS benefits, regrettably accompanied by high adverse events. Therefore, several trials are now addressing the dual EGFR-VEGF inhibition hypothesis by testing osimertinib with bevacizumab (NCT02803203, NCT04974879, NCT04181060) or Ramucirumab

(NCT03909334, NCT02789345). *Ad interim* findings from the phase II WJOG917L study, presented at the ESMO Congress 2021, indicated that only patients with history of smoking or an exon 20 deletion seem to derive benefit from the osimertinib and bevacizumab combination [383, 384]. In addition, the combination of the novel multitarget-TKI anlotinib (targets VEGFR, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptors (PDGFR), and c-kit) with PBC is being evaluated in the phase II ALTER-L031 (NCT04136535) trial in patients with disease progression to osimertinib. Furthermore, the association of anlotinib with pemetrexed and toripalimab (anti--programmed cell death protein, PD1 mAb) inhibitor, is being assessed in T790M^{+} patients after osimertinib failure (NCT04316351).

8.4. The immune aspects

Immunotherapy has recently become integrated into the treatment of NSCLC patients, providing long lasting remissions in previously intractable diseases. Its effectiveness depends on the visibility of the tumor to the immune system, i.e. the presence of neo-antigens and the capacity of the tumor to present them. Immune checkpoint inhibitors (ICIs) were introduced in the clinics in 2015 given their ability to block inhibitory pathways that physiologically control the immune response [385]. The major targetable players are the cytotoxic T-lymphocyte-associated-4 (CTLA4) and the PD1 receptors expressed on T cells, that by interacting with PD1 and PD2 ligands (PDL1 or PDL2), expressed on both cancer and immune cells, inhibit immune surveillance.

ICIs have shown remarkable efficacy in *EGFR-wt* NSCLC;[386] however in the *EGFRm* realm we still need to weather the storm, as only patients carrying rare EGFR mutations appear to display some beneficial effect [387,388]. The interplay between EGFR and the immune milieu in NSCLC is still an open debate. Patients with EGFR-mutant NSCLC show poor response to anti-PD1/PDL1 treatment, however, the mechanisms involved are still not clear [389–391]. Several explanations have been proposed to explain lack of therapeutic efficacy in the majority of *EGFRm* cases, including low TMB; an immunosuppressive TME characterized by over-production of negative modulators of immune cells impeding T cell infiltration and cytotoxicity, recruitment of suppressive tumor-associated macrophages (TAMs) and regulatory T cells (Tregs); down-regulation of both class I and II antigens of the major histocompatibility complex; secretion of immunoregulatory exosomes, induction of tumor-promoting inflammatory cytokine, secretion of inhibitory cytokines and metabolites, including an immune-metabolic dysfunction leading to over-production of the powerful immunosuppressive nucleoside adenosine; as well as variable and dynamic PD1/PDL1 expression levels [392–398]. Initial results indeed showed that EGFR-mutant NSCLC display low PDL1 expression, low CD8^{+} tumor-infiltrating lymphocytes (TILs), and low TMB, thus leading to weak immunogenicity [393]. However, the role of PDL1 expression to predict the response to immunotherapy in EGFR mutant NSCLC is controversial [394,399]. On the other side, how EGFR TKIs may interact with the immune microenvironment is under investigation and it is reported that EGFR-TKIs are able to modify both PDL1 expression [393] and TMB [400].

EGFR can upregulate PDL1 on tumor cells; however, while EGFR-TKIs seem to initially reduce PDL1 expression, in some patients PDL1 levels increase following treatment and are associated with primary resistance [393,401]. EGFR-TKIs are associated with a significant increase in PDL1, especially in T790M^{-} patients [402]. However, unsatisfactory results with PD1 inhibitors were also obtained in *EGFRm* patients with higher PDL1 levels, possibly as a result of immunosuppressive factors, including increased amounts of Tregs and CD73 expression on tumor cells [402]. PDL1 expression on TILs and TAMs [403], may also be a confounding element in data interpretation. Therefore, future data on how EGFR TKIs are able to modulate tumor microenvironment may also help identify EGFR mutant patients likely to respond to immunotherapy in subsequent lines.

In addition, mechanistic studies on PD1 have been largely focused on its role on T cells, whereas, PD1 is also expressed on tumor cells [404]. Induction of PDL1 on tumor cells is regulated by two pathways: one driven by IFN-γ and another controlled by constitutive oncogenic signaling [396]. In this respect, we observed that EGFRm cells resistant to Osimertinib upregulate the oncogene BMI1, and PDL1. Although a causal link has not been formally established yet, if verified, these data may imply that anti-BMI1 treatments could help manage Osimertinib resistance by acting on transformed epithelial cells, that not only overexpress BMI1 but also PDL1.

Interestingly, also a new perspective of non-immunological functions of PDL1 in regulating cancer-intrinsic activities including mesenchymal transition, glucose and lipid metabolism, as well as stemness, has been suggested [405]. PDL1 can regulate expression of BMI1 in breast CSCs; [406,407] promotes expression of stemness-associated genes (Oct4, ABCG2, ALDH1, and Bmi1) in colorectal CSCs [408]; and promotes migration and invasion by impinging on the TGF-β/Smad pathway in EGFRm NSCLCs resistant to Gefitinib [409]. Therefore, this PDL1-BMI1 correlation may well exemplify the age-old chicken or egg dilemma. PDL1 gene is also a direct target of miR-200 family in NSCLC cells [410], and both miR-200c and miR-141, two members of the miR-200 family, are repressed by BMI1 [411]. These data trigger the hypothesis that the BMI1 increase we observed in Osimertinib resistant cells may lead to PDL1 overexpression, by directly inhibiting PDL1 inhibitors (such as miR-200 family members).

Noteworthy, the tumor suppressor p53 upregulates miR-34 family members [412], and miR-34a acts itself as tumor suppressor miR by repressing PDL1 and > 30 oncogenes [413]. In addition, mir-200c can target BMI1 in bladder cancer [414]. Thus, the complex interplay between miRs, oncogenes and PDL1 must be further investigated to be able to comprehend their roles in immunosuppression.

PDL1 is also positively associated to cancer glucose metabolism in NSCLC [415] and skillfully increases cancer lipid uptake in gastric adenocarcinomas that compete with tissue-resident memory T cells for metabolites, thus causing evasion of anti-tumor immunity [416]. Collectively, PDL1 plays a dual role by shutting down T cell immunity as well as by turning on pro-tumor programs linked to CSCs and invasiveness [405].

Last, we want to touch on few other immune-based emerging therapies for *EGFRm* NSCLCs. One promising avenue to treat malignancies, i.e., the synthetic chimeric antigen receptor (CAR)-redirected T cell therapy, has achieved dramatic successes in hematological diseases, although few studies regarding solid tumors, particularly NSCLC, are starting to emerge. In murine models, modified CAR-T cells exhibited regression of human lung cancer xenografts [417]. A phase I study of EGFR-targeted CAR-T cells was conducted, which demonstrated objective response in 2/11 and disease control in 7/11 patients, though survival data was immature at the time of publication [418]. In addition to numerous ongoing EGFR CAR-T trials in China, pediatric evaluation of a second-gen agent targeting both CD137 (a co-stimulatory molecule regulating immune response) and EGFR is underway in the United States (NCT03618381).

An additional EGFR-targeting approach undergoing investigation in pancreatic cancer, entails collecting autologous lymphocytes and expanding them in culture in presence of OKT3 (anti-CD3 mAb) and cetuximab, to generate bispecific antibody-armed T cells (BATs), for subsequent infusion. In a small trial, innate immune response activation and better-than-expected responses to subsequent chemotherapy were achieved [419]. A second line phase Ib study is underway to confirm these findings (NCT04137536).

Another therapeutic strategy which is being assessed in a small exploratory trial (n = 30 patients with advanced *EGFRm* NSCLC) involves combining gefitinib with allogeneic CD8^{+}CD56^{+} natural killer T (NKT) cells, a unique subset of lymphocytes that present characteristics of T and NK cells, and exert cytotoxicity on tumor cells [420]. Such cells could effectively prime patients' alloimmune T cells and reverse TME-induced immune suppression, without causing graft-vs-host

disease.

Finally, vaccination against EGF might enhance the efficacy of EGFR TKIs and delay the emergence of resistance. In preclinical studies, anti-EGF vaccine inhibited EGF-induced cell proliferation and downstream signaling in *EGFR*-mutated cells [421]. Sera from patients treated with the vaccine enhanced the activity of the TKIs evaluated, and delayed the emergence of resistance clones (EPICAL trial, NCT03623750).

9. Summary and conclusions

The anti-EGFR era coached us along the way, as we went from identifying the EGFR oncogene and its variants, to proving their oncogenic potential by adopting clinically relevant preclinical models, that also expedited comprehension of the molecular mechanisms driving tumorigenesis.

A plethora of clinical trials were run, to prove high response rates of the newer TKIs. Given therapy resistance, more potent inhibitors are constantly required, that ideally should address both systemic disease and CNS penetration, while also being less toxic oral monotherapies that become the newest evidence-based backbones for future use in clinical settings. In the meantime, osimertinib-based combination therapies will probably be part of the clinical trial portfolio for the next decade or so. Osimertinib has indeed set the paradigm for adjusting targeted therapy based on the mechanisms of resistance identified. Such complex resistance-machinery results in major intra-tumor diversity that ends up exacerbating pharmacological management. Multiple (translated and un-translated) resistance mechanisms can even arise simultaneously within the same patient, requiring dynamic tumor-tailored approaches. Single-cell technologies are needed to meticulously analyze such evolution of tumor heterogeneity in the form of TKI-sensitive cells mixed with resistant cells, or even detect events occurring in the TME, which is the most tumor-intermingled "organ" [91] that plays significant roles in drug-resistance. However, no single study has yet demonstrated that the resistance mechanisms identified so far do indeed occur within the same cells containing the founder EGFR mutation, nor has even addressed whether such resistance mechanisms originate within defined epithelial subsets of transformed cells. High-resolution transcriptomics is expected to advance our understanding about how transcripts perform at the single-cell molecular level in contexts as diverse as naïve tumors, or during drug treatment and development of resistance. The epithelial component is still understudied at the single cell level. Recently, adopting a *Kras*-driven NSCLC model, we reported a prototype-study describing how drug treatment affects in vivo distribution of transformed epithelial subpopulations [374]. Briefly, we identified a unique epithelial cluster, only present in tumors from *Kras* mutant mice, which was conserved in *KRAS* mutant human adenocarcinomas, and was targetable through PTC596. Besides emphasizing how GEMMs act as powerful preclinical model to mimic human disease, we proved how solid tumors can be interrogated to assess single-cell adaptation to drug treatment [374]. Similar studies, performed on *EGFRm* transgenic models, paired to clinical specimens, could identify vulnerable subpopulations, describe resistance mechanisms, and concomitantly test therapeutic options. Single-cell technologies are not immediately available in the clinical practice. However, we anticipate a forthcoming merging of histology with high technology, resulting in spatially resolved transcriptomics to fine-tune our understanding the dynamic topography of the complex molecular events building up patients' tumor recurrence.

Emerging new technologies such as CRISPR/Cas9-mediated genome editing protocols, by specifically correcting mutated alleles [422] could even eliminate the endless process of drug-induced new resistance mutations. To break ice on EGFR mutations, precise excision of *L858R* in H1975-xenografted tumors was achieved, which resulted in rapid tumor regression [423], indicating mutant allele-specific editing can be a promising method of cancer treatment to ablate or correct EGFR mutations. Ideally, after detection of EGFR mutations in cancer biopsies, mutant genes could be repaired or destroyed with virus-delivered CRISPR/Cas systems. Careful management of potential off-target effects, and efficient delivery will be necessary. However, with future improvements of CRISPR/Cas technology, combining this molecular approach with traditional surgery, radiation, and/or chemo/TKI treatment would have the potential to significantly improve the survival of patients with *EGFRm* NSCLC.

Overall, multiple interdisciplinary contributions including i) identification of single cell specific genetic and epigenetic alterations, ii) discovery of diagnostic and prognostic biomarkers, iii) implementation of efficient and specific diagnostic tools, iv) design of genomic editing protocols, v) planning of well-designed therapeutic strategies that take into consideration the best sequential options to delay/prevent development of recurrence, and vi) design of efficient delivery methods to target cytotoxic payloads or prime an immune-mediated response, will all converge as a well-orchestrated multifaceted approach to address the presently incurable nature of the disease. The EGFR saga is to be continued and we are awaiting for the next sequel of advancements that, despite being designed under the EGFR aegis, will certainly benefit the entire oncology field.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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