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STATEMENT OF SIGNIFICANCE:

Precision cancer medicine relies on the possibility to match, in daily medical practice, detailed genomic profiles of a patient's disease, with a portfolio of drugs targeted against tumor-specific alterations. Clinical blockade of oncogenes is effective but only transiently; an approach to monitor clonal evolution in patients and develop therapies that also 'evolve' over time may result in improved therapeutic control and survival outcomes.

ABSTRACT

Recent technological advances in the field of molecular diagnostics (including blood-based tumor genotyping) allow the measurement of clonal evolution in patients with cancer, thus adding a "new dimension" to precision medicine: time. The translation of this new knowledge into clinical benefit implies rethinking therapeutic strategies. In essence, it means considering as a target not "only" individual oncogenes but also the evolving nature of human tumors. Here we analyze the limitations of targeted therapies and propose approaches for treatment within an evolutionary framework.

INTRODUCTION

A major issue in the treatment of patients suffering from cancer is the development of resistance to therapies. This ability of cancer to adapt to pharmacological pressures can be described in terms of "tumor evolution", and stems from its intrinsic diversity, or "heterogeneity". Tumor heterogeneity refers to the coexistence of cellular populations bearing different genetic or epi-genetic alterations within the same lesion, or in different lesions of the same patient. Tumor evolution depicts changes in tumor heterogeneity along the temporal axis, and describes the dynamics by which, under environmental pressure,

sub-populations of cancer cells bearing “selective advantages” emerge at the expense of others. This process appears to be particularly marked when cancer undergoes sudden selective pressures imposed by medical treatment.

Recent advances in the longitudinal detection and quantification of tumor specific mutations in blood, through liquid biopsy, have allowed the definition of patterns of clonal evolution as measurable characteristic of a patient’s cancer. Therapy approaches have so far been based on the characterization of tumors in a “two- dimensional way” i.e. by means of *in situ* tissue analyses (depicting disease as the sum of molecular alterations of a particular lesion at a definite time-point); now longitudinal tracking of cancer mutations offers unprecedented opportunities to attempt to modulate tumor evolution for therapeutic purposes.

This review will discuss the relevance of measuring tumor evolution as a read-out for response to therapy and the possibility to exploit evolution itself to harness cancer. We will first present the technical approaches which support the “measurement” of tumor evolution; we will then discuss the role of tumor evolution in the development of resistance to therapy and propose different strategies to exploit the evolving nature of tumors for the benefit of cancer-patients.

TECHNIQUES FOR MONITORING TUMOR EVOLUTION

Multi-region sequencing

The comparison of synchronous samples derived, in a single patient, from multiple regions of one or more neoplastic lesions led to the notion that solid tumors are genetically heterogeneous, which has been demonstrated across different cancer-types through sequencing of spatially separated samples (1–5).

Translating Darwin’s evolutionary principles to cancer pathogenesis, tumor heterogeneity has been interpreted as result of both the acquisition of (epi)-genetic variability, fostered by genetic instability, and the selection of distinct subpopulations driven by external pressures, microenvironmental conditions, as well as “mere” geographical factors (neutral evolution(6)). Consequently, information issued from multi-region biopsies could effectively be used to reconstruct the evolutionary dynamics (or “history”) of a tumor, graphically rendered as “tumor phylogenetic trees”, where trunk or clonal alterations, which are present in all tumor cells, represent ancestral events, while heterogeneous genetic alterations constitute the branches(7) (Box 1). However static punctual assessment of multiple but limited tissue samples is not sufficient to fully describe (spatial) tumor heterogeneity, nor to appropriately describe the profound dynamics of non-homeostatic biological systems such as tumors.

Liquid biopsies

As clonal evolution is defined by changes of tumor heterogeneity over both space *and* time (temporal heterogeneity), its analysis requires the ability to track tumor specific genetic alterations in real-time. Multi-region sequencing proves that single biopsies from geographically localized tumor areas cannot recapitulate the complexity of spatial heterogeneity (1–5), Moreover, morbidity related to the surgical procedure strongly constrains repeated longitudinal sampling, especially in patients with metastatic disease undergoing therapy, thus limiting reliance on tissue biopsies for the measurement of clonal evolution.

Recently the increase in sensitivity of DNA sequencing techniques has allowed genetic characterization of tumors from the analysis of circulating tumor DNA (ctDNA) isolated from plasma, and other biological fluids (liquid biopsy)(8). Analysis of ctDNA is based on the identification of tumor specific alterations, which accounts for its high specificity and sensitivity(9–11) and detection rates comparable to those of tissue biopsies(12–14). Moreover, the half-life of cfDNA being about two hours(8), changes in the allelic frequencies of genetic alterations can be monitored in real-time. Both clonal and subclonal alterations can be detected by liquid biopsy: “phylogenetic ctDNA tracking” was effectively performed in early stage non-small cell lung cancer (NSCLC) patients who underwent multi-region biopsy sampling and had a selected panel of single nucleotide variants representative of trunk and branch mutations longitudinally monitored through liquid biopsy (15–17). This shows that information obtained at the time of surgery from multi-region biopsy analysis (on spatial heterogeneity) can be, to a certain extent, translated to ctDNA analysis.

Liquid biopsy allows the tracking of the evolution of different cell sub-clones (see also Box1), and this was proven to be particularly effective in the follow-up of patients treated with targeted therapy in the metastatic setting. In this setting the increase in the relative frequency (allelic fraction) of alterations mediating resistance to specific targeted-agents had been used to measure the rise (evolution) of refractory tumor sub-populations (branches). In cetuximab and panitumumab-treated CRCs for example, blood-based detection of increasing levels of *KRAS* mutated variants in plasma allowed the identification of resistant sub-clones before relapse was evident by imaging diagnostics (12,13); in the same setting, multiple *KRAS* alterations and concomitant KRAS and NRAS mutations (polyclonal drug resistance) were identified in several studies (9,18–20). Similar observations have been reported for patients with both breast (21) and lung cancer (22). Exome sequencing can be applied to ctDNA to systematically dissect clonal evolution as suggested by the study of Murtaza and colleagues, who investigated genetic markers of resistance in a long-term follow up of patients with breast, ovarian and lung cancers undergoing different lines of treatment (23). Whole genome sequencing analysis of ctDNA from the blood of colorectal and breast cancer patients allowed detection of chromosome copy number and structure alterations (24); Shoda and colleagues monitored in plasma the dynamics of *HER2* amplification in a gastric cancer patient treated with trastuzumab (25), and Liang et al. detected, through ctDNA analysis, the coexistence of EGFR

mutation and *EML4-ALK* gene translocation in a patient with metastatic NSCLC who had relapsed following first and second-line EGFR-inhibition, supporting the effective treatment with two lines of ALK inhibitors(26).

Differences in the amount of ctDNA are related to tumor histological type, location and stage. Most patients with advanced stage ovarian and liver cancers as well as metastatic cancers of the pancreas, bladder, colon, lung, stomach, breast present with measurable ctDNA compared to a minority of patients with medulloblastomas, gliomas and metastatic cancers of the kidney, prostate or thyroid(9). The proportion of patients with detectable levels of ctDNA is high in advanced disease but significantly reduces in early stages(9). However minimal residual disease monitoring through ctDNA in patients with colon (27,28) breast (29) and lung cancers (17) has been reported. Diffusion of free tumor DNA is also limited by human anatomy and transit through blood-brain barrier is limited: a recent study emphasised that in primary tumors of the brain and brain metastasis the cerebrospinal fluid is a more informative source of ctDNA than plasma (30); similarly cfDNA collected from thoracic and peritoneal effusions might be highly informative: Krimmel and colleagues successfully identified TP53 mutations in cell free DNA from peritoneal fluid from patients with high grade serous ovarian tumors(31). Bronchoalveolar lavage and pleural fluids were successfully used to detect EGFR mutations in advanced non-small lung cancer (32). ctDNA has also been isolated from saliva and urine (33,34). Therefore, analysis from multiple sources could be useful in dissecting inter-lesion heterogeneity. Indeed while multi-region tissue sampling allows the dissection of spatial heterogeneity this is impossible with liquid biopsy alone: for example ctDNA releasing mutant cells could either be dominant in one or few lesions or consist of smaller populations intermixed within all metastatic sites; in both cases the resulting circulating mutant allelic fractions could be similar. Besides, factors like size of the lesions, necrosis, vascularisation may also play a role on the relative amount of mutant DNA released in the circulation.

Effective monitoring of tumor evolution would thus also require the clinician to careful select tissue samples to be biopsied (with the specific aim to depict tumor heterogeneity), chose timepoints for circulating DNA detection and integrate the molecular scenario with information derived from “standard” techniques, such as imaging diagnostics and protein markers. Comprehensive studies encompassing multiple diagnostic techniques to monitor tumor evolution during treatment such as TRACERx recently provided evidence of feasibility of this approach (16,17).

TUMOR EVOLUTION IN RESPONSE TO THERAPY

Analysis of post-treatment samples sheds light on how, despite undeniable proof of clinical efficacy of targeted therapies(35–37), with few exceptions(35,38), the emergence of acquired drug resistance inevitably limits the gains achieved in overall survival with such treatments (39–45). Similarly, markers of increased tumor heterogeneity (the substrate for evolution) have been associated with worse outcome beyond targeted therapy, for example in head and neck(46), non-small cell lung cancer (NSCLC)(4), ovarian cancer(47) and CLL(48). Evidence of widespread primary and emerging acquired resistance to

immunotherapy(49–52) suggests that at least some tumors are capable of adapting to a therapeutically unleashed immune response. Thus evolutionary adaptation (to therapy) appears as a "hallmark of cancer" and the possibility to understand and quantify this hallmark highlight the need (and opportunities) of devising cancer therapies aimed at overcoming disease recurrence.

Pre-existing secondary resistance to therapy: the paradigm of kinase inhibitors

The possibility of systematically identifying, with high resolution sequencing techniques, genetic markers (alterations) of targeted-drug resistance in relapsed tumors and pre-treatment samples has revealed that small populations of genetically resistant cell sub-clones often already pre-exist treatment, supporting the idea that clonal selection of pre-existent populations is the main mechanism for acquired resistance to targeted therapy(53,54).

For example, in lung adenocarcinoma bearing activating exon 19 deletions or L858R mutations, the emergence of EGFR mutation T790M is the most common mechanism of resistance to EGFR inhibitors erlotinib and gefitinib (55) and the identification of the T790M allele in pre-treatment samples has been associated with shorter progression-free survival(56). Similarly, amplification of the *MET* oncogene is also detected in 22% of lung specimens developing resistance to EGFR kinase inhibitors(57) and was found in patients and cell lines prior to drug exposure(58). The co-existance, in these tumors, of different phylogenetic branches characterized by diverse genetic profiles explains how the dynamic balance between different sub-clones allows tumors to escape even from administration of next generation inhibitors designed to specifically target resistant cells. Indeed in tumors treated with osimertinib, one of the third generation EGFR inhibitors capable of overcoming T790M-mediated resistance (59,60), not only the EGFR C797S resistance mutation emerges among T790M positive clones, but also the increase of the tumor fraction positive for the EGFR activating alterations but lacking T790M mutation has been witnessed (61). Similarly resistance to third generation inhibitor rociletinib may not only be mediated by EGFR (L798I, C797S) mutations, but also by alterations of MET, PIK3CA, ERRB2, and KRAS(22), and by the negative selection of T790M mutant sublcones(62).

Analogous observations were made in colorectal adenocarcinoma (CRC) treated with anti-EGFR with the antibodies cetuximab and panitumumab. In this setting RAS pathway mutations and mutations in the extracellular (ECD) domain of the EGFR are predominant resistance mechanisms(12,13,19,20,63). These mutations often coexist in the same tumor (12,18,19), where different cell clones can harbor distinct KRAS, NRAS and BRAF alterations(13,18,19,64). Moreover, Siravegna et al. showed, through liquid biopsy, that upon drug withdrawal, the allelic frequencies of mutated *KRAS* decline in the blood of CRC-patients resistant to anti-EGFR agents(18).

De novo acquired secondary resistance to therapy

Mathematical modeling of CRC tumor growth in patients supports the notion that the

complex patterns of polyclonal resistance often witnessed in clinical practice are unlikely to originate only, or mainly, *de novo,* within the short time-frame of pharmacological treatment (12). However while the presence of RAS mutated clones in CRC resistant to anti-EGFR antibodies is detected prior to treatment in patients and cell lines(13) the same is not observed for EGFR ECD mutations (19,20,63), suggesting that these variants might originate primarily upon treatment. Indeed in patient-derived lung cancer cells treated with gefitinib Hata and colleagues described both the emergence of early-resistant sub-clones, derived from pre-existing T790M mutated cells, and the detection of late-emerging resistant populations (65); the latter showed appearance *de novo* of T790M mutation in drug-tolerant, persister cells (66) in which resistance exists at the epigenetic level. Interestingly these cells appear to be less sensitive to third generation EGFR inhibitors (65). Moreover, Ramirez and colleagues demonstrated that multiple resistance mechanisms could emerge from a single drug-tolerant clone of PC9 cells sensitive to erlotinib (67) and drug sensitivity of drug-tolerant PC9 cells is restored by IGF-1R inhibition (66). EGFR ECD mutations were shown by Van Emburgh and colleagues to emerge later in cfDNA when compared with RAS mutations and to be associated with longer progression-free survival in patients with metastatic CRC(68); this observation is consistent with a two step progression model in *de novo* acquired resistance. Thus liquid biopsy could possibly be used to identify patients in whom a therapy directed against persister cells might eradicate the *reservoirs* of drug resistance .

Several reports also highlight that non genetic mechanisms of resistance are involved in response to targeted therapy and might play an important role in clonal evolution. For example, increased secretion of TGFbeta and amphiregulin by CRC cells resistant to cetuximab was shown to sustain neighboring sensitive cells (69). A study of 67 secondary resistant melanomas treated with MAPK inhibitors revealed that 39% of cases were not accounted for by any validated mutational mechanism (70) suggesting non-genomic adaptive resistance. In T cell acute lymphoblastic leukemia, gamma secretase-resistant persister cells were found to be dependent on chromatin regulator BRD4 overexpression, and BRD4 inhibition re-sensitized cells to therapy (71). Acquired resistance to antiPD1 checkpoint inhibitor in NSCLC had been correlated with upregulation of alternative immune checkpoints (50), showing that adaptive epigenetic evolution mediates therapeutic resistance in several settings**.** Indeed liquid biopsy might allow effective integration of epigenetic markers by determination of methylation profiles from ctDNA and possibly through the characterization of tumor derived exosomes (72,73). Transcriptional analysis of circulating tumor cells is also informative as shown by the identification of non-canonical Wnt signaling pathway activation in androgen-resistant prostate cancer patients (74).

TARGETING CANCER EVOLUTION

If clonal evolution eventually drives resistance to therapy, the possibility to measure it through tissue and liquid biopsy might be pivotal in guiding the identification of the most effective additional lines of treatment (Figure1). Here, we discuss the rationale,

applicability and possible limits of strategies having as an endpoint the modulation of a tumor's evolution which are schematically represented in Figure2.

Modulating genomic instability

Tumor evolution is fueled by (epi-)genetic alterations leading to reduced genomic stability. Examples range from familial and sporadic colorectal cancers with loss of function of mismatch repair proteins, BRCA1 and BRCA2 deficient breast and ovarian cancers with deficiency in homologous recombination repair, to ultramutated tumors characterized by impaired proofreading activity of polymerase epsilon and delta (75). Recently over-activation of APOBEC family proteins has been suggested to increase mutational rate across half of human cancers (76) and to represent a common cause of subclonal diversification in NSCLC(17).

Indeed the actionability of molecular alterations in genes controlling genome stability has only partially been tested. A well known example is the use of a synthetic lethal approach to selectively kill homologous recombination deficient cells, as demonstrated by the activity of PARP inhibitors in BRCA deficient and BRCA-like tumors (77). Interestingly this paradigm suggests that increasing genomic instability (i.e. by targeting a complementary pathway of DNA repair) over the threshold of tolerability might lead to a breakdown in genomic integrity, and consequently to cell death.

Moreover tumors bearing mismatch deficiency show extremely high response rates to immune checkpoint inhibitors and exceptionally long lasting responses, thus correlating levels of mutational burden with therapeutic efficacy (78). In this regard, increase in the levels of genetic instability could be exploited therapeutically, as suggested by the induction of MSI phenotypes reported in patients treated with alkylating agents such as temozolomide (79), who might further benefit from immune checkpoint blockade. On the other hand restraining genomic instability for therapeutic purposes might slow down tumor progression. However, with the exception of p53 loss in preclinical models (80), dependency of tumor cells on specific "mutagenic" alterations has yet to be proven and the efficacy of APOBEC inhibition awaits further validations. It is reasonable to think that such an approach might best impact on patients' prognosis in particular in the preventive/adjuvant setting when disease burden and tumour heterogeneity are low.

Targeting clonal mutations.

Since the (epi)genetic heterogeneity of tumor subclones favors evolution under the selective pressure of anti-cancer drugs it would be intuitive to think that administration of drugs targeting truncal alterations (present in all cells) could better increase the odds of durable control of disease

In this regard, recent work by Pearson et al. has shown that patients with gastric cancer

who responded to the FGFR inhibitor AZD4547 harbored tumours with high-level clonal FGFR amplifications. In contrast, tumours that did not respond harbored sub-clonal or low-level amplification(81). In a study of 120 breast cancer patients undergoing treatment with PI3K/AKT/mTOR inhibitors, tumors with clonal PIK3CA mutations showed a trend for better response which was however not statistically significant (82); indeed the high frequency of sub-clonal alterations of PI3K-mTOR across different tumors, as reported in a TCGA-based study by McGranahan et al. (83), suggests that this could at least partially account for the modest results held by PI3K inhibitors in patients with solid malignancies(84).

Accordingly knowledge of the clonal status of actionable drug targets(83) in individual cancers could help the design and implementation of therapies aimed at lowering the odds of acquired resistance. However, direct targeting of clonal alterations is not always feasible: this is the case of tumor suppressors like APC. Restoration of APC results in induction of apoptosis(85) in colorectal cancer cell lines and tumor regression in preclinical models(86). Unfortunately, pharmacological restoration of APC activity has not yet been achieved. Analogously, restoration of TP53, which is significantly enriched in clonal mutations across different tumor types (17,83), led to tumor regression in autochthonous mouse sarcomas and lymphomas(80); unfortunately the actionability of p53 with targeted agents remains challenging (87).

Moreover aiming at a single truncal oncogenic variant might be insufficient to produce long term benefit This had been witnessed in the context of metastatic melanoma, where mutated *BRAF* is a *bona fide* trunk driver, but therapy with vemurafenib only provides a 2 months increase in overall survival compared to dacarbazine (88). In patients with acquired resistance to BRAF inhibition multiple molecular lesions in MAPK as well as PI3K pathways are commonly detected in the same tumor or among multiple tumors from the same patient(89). Similarly in Ph+ acute lymphoblastic leukemia (ALL), in which *BCR-ABL* translocation is a trunk alteration(90), high rates of relapse to imatinib are observed despite a high initial response rate(91).

Combinatorial approaches

Empirical associations of multiple effective drugs largely support the effectiveness of chemotherapeutic regimens in both hematologic and in solid malignancies (92,93).
Conceptually the same paradigm might be applied to targeted drugs such as inhibitors of oncogenic signaling pathways. Drug association is further sustained by mathematical modeling of acquired resistance; for example studies on pancreatic, colorectal and melanoma patients suggests that, in metastatic cancer, monotherapy with targeted agents cannot eradicate the disease, even in the presence of limited tumor burden (range $8{,}5x10^{8}$-$1{,}2x10^{11}$ cells), while dual combination therapy offers hope of success only for low tumor burden and in the absence of cross-resistance mutations(94). Therefore three or higher order combination therapy might be needed to obtain tumor eradication even with agents targeting truncal alterations; analogously inhibition of distinct pathways would also be required to avoid cross-resistance.

Targeting trunk mutations with immunotherapy

As previously discussed affecting multiple clonal alterations with targeted agents is limited by druggability and toxicity issues. A strategy to overcome these limitations involves targeting clonal neoantigens, or dominant branched antigens that were selected through evolutionary bottlenecks such as surgery or systemic therapy, through personalised vaccines or adoptive cell therapy (95). The possibility to target multiple neoantigens through these approaches would significantly reduce the odds of resistance. The latter has been shown to be associated with loss of expression of neoantigens (either by genetic or epigenetic mechanism) in two melanoma patients who underwent T-cell adoptive infusion (96). Moreover, in Acute Lymphoblastic Leukemia patients who responded to CART-19 infusion mechanisms of resistance implicated acquired mutations but also alternative splicing of immuogenic epitopes (97). In this context liquid biopsies could be particularly effective in tracking dynamics of the targeted neoantigens, and, coupled with TCR sequencing from blood, in predicting the odds of relapse (98) However, immune-evasion from T-cells aimed at clonal neoantigens could for example arise through clonal selection of tumor cells bearing mutations or loss of HLA (99,100); the latter was recently reported in a metastatic CRC patient who relapsed after adoptive T-cell transfer (101); alterations of IFNgamma pathway effectors could also impair a targeted T cell mediated immune response (102). This suggests that immunotherapy alone might not be sufficient to eradicate a tumor, and integration with other forms of therapy coupled with diagnostic monitoring of tumor evolution might be needed to maximize efficacy.

Preventive combination therapy

The observation that resistant cell clones often pre-exist (though undetectable) the start of treatment supports the idea that early administration of combinatorial treatments stands higher chances of eradicating such clones when their number is very low, before acquired resistance is overtly diagnosed. *Ab initio* combination therapies are particularly effective in preventing resistance in other pathological contexts, such as infectious diseases(103). In this setting drug combinations have proven effective against fast evolving pathogenic agents such as HIV (103). In oncology however, the narrower therapeutic window between tumor cells and host poses limits to the number of agents which can be simultaneously combined. Liquid biopsy and multi-region sequencing could effectively guide evolution-based combination regimens aiming initially at reducing the odds of resistance and further exploiting escape mechanism to maintain tumor growth control when resistance develops.

Targeted-drugs association *ab initio* could aim at simultaneous targeting the bulk tumor (with a drug active on the trunk) and the expected secondary resistance mechanism, thus providing a significant advantage in survival compared to administration at relapse(94).

Acquired resistance mediated by the emergence of secondary mutations of the drug-target has often been witnessed with imatinib, dasatinib and nilotinib in CLL and with different generations of EGFR inhibitors in lung cancer, and suggests that reactivation of the inhibited pathway is a biologically favored mechanism (104,105). Similarly, 14 different metastatic lesions from a breast cancer patient bearing an activating PIK3CA mutation

who relapsed under therapy with PI3Kα inhibitor BYL719 bore different PTEN genetic alterations, resulting in convergent loss of PTEN expression which was reverted by simultaneous PI3K p110β blockade (106). Indeed the observation that often, upon inhibition of an oncogenic driver, a relevant number of escape mechanisms converge on that same pathway suggests that at least in certain tumors preventive combination therapy providing vertical inhibition of a trunk target and its downstream effectors might reduce the probability of relapse (i.e. delay it). Moreover, synchronous targeting of downstream players of drug resistance would not just represent a preventive action but could also result in increased cytotoxic effects on the bulk of the tumor and thus in deeper reduction in tumor burden. This could limit also *reservoirs* of *de novo* resistances.

In CRCs, for example, secondary resistance to anti-EGFR antibody therapy, which interfere with signaling through the MAPK cascade, is often mediated by reactivation of the pathway through additional gain of function alterations in RAS, MEK and MET(54,107). Following these observations, Misale et al. demonstrated that combinatorial treatment of EGFR-sensitive colorectal cancer models with vertical inhibition of EGFR and MEK (which is a downstream effector of MAPK pathway) prevents the occurrence of resistance(108) and a clinical trial adopting this approach in EGFR sensitive CRC is ongoing to test the hypothesis (EudraCT 2014-002460-33). Similarly combined EGFR/MEK inhibition was reported to prevent emergence of resistance in EGFR-mutated lung cancer models(109). Crystal et al. described the establishment of a platform of patient-derived models of acquired resistance for the identification of effective targeted-drug combinations: cell lines were derived from lung cancer patients and made resistant to single agent inhibition of a primary driver oncogene and screened for agents capable of overcoming resistance. Selected compounds were tested in mice. Notably combination treatments *ab initio* showed increased response compared to combinations at resistance (110).

A limit to this approach is the variability of resistance mechanisms witnessed in patients: the combination of BRAF and MEK inhibitors results in increased survival in melanoma patients(111) (while sequential therapy showed no such benefit(112)), nevertheless tumor relapse is observed(113). Indeed in metastatic melanoma which lost sensitivity to MAPK inhibitors not only alterations of *BRAF*, *NRAS*, *KRAS*, *MEK1* or *MAP2K1* are correlated to relapse but also activation of divergent escape pathways as suggested by the evidence of gain-of-function events in *PIK3CA*, *AKT1*, *AKT3* and loss-of-function events in *PIK3R2*, *DUSP4*, *CDKN2A*, *PTEN*, and possibly non genomic alterations like MET over-expression, B-Catenin and YAP1 deregulation (70). Recently combinatorial agents capable of preventing divergent by-pass escape mechanisms were tested in the form of antibody mixtures. For example, the EGFR antibody mixture Sym 004 was shown to overcome cetuximab resistance mediated by EGFR extracellular domain mutations in CRC (114) and Pan-HER, targeting EGFR, HER2, and HER3 was shown to act synergistically on tumor cells possibly preventing bystander resistance due to compensatory activation of EGFR family receptors and increased production of ligands (115,116).

Of note in this setting, the levels of heterogeneity might be systematically underestimated in preclinical models, where the reduced number of cells (compared to a patient with

metastatic disease), implies a parallel reduction in heterogeneity. Thus comprehensive integration of data from multi-region and liquid biopsies in large cohorts of patients should guide towards the definition of effective preventive combinations, with two or more drugs, and lead to the identification of more favorable clinical conditions, where higher efficacy could be achieved (i.e. after debulking surgery).

Adaptive therapy

Cell-specific fitness in the presence of therapy could also be exploited to harness tumor evolution. Resistance may come at a fitness cost and sub-clones showing a growth advantage under (targeted) therapy could lose their fitness advantage in the absence of the selective pressure. In CRC patients who became resistant to cetuximab or panitumumab and showed emergence of a KRAS mutated cell population the interruption of therapy or the treatment with a different class of compounds (i.e. a VEGF inhibitor) correlated with a reduction of KRAS mutant allelic fraction in plasma, as assessed with liquid biopsy(18,117). In a patient-derived xenograft model of melanoma displaying resistance to BRAF inhibition, intermittent dosing of vemurafenib led to long-term control of tumor growth(118), unlike continuous treatment(118), in line with the observation of responses upon re-challenge in melanoma patients with acquired resistance to BRAF-inhibitors(119,120).

Mathematical analysis of tumor evolution further supports these observations. Gatenby and colleagues modeled clonal dynamics in the presence or absence of treatment, showing that while high dose regimens lead to rapid expansion of resistant populations (competitive release)(121), modulation of therapy (adaptive therapy)(122) allows to control tumor growth. This is achieved by keeping a balance between drug sensitive tumor cells, which proliferate better in the absence of drug, and resistant cells, which prove fitter only in the presence of the drug itself, as exemplified in ovarian and breast cancer xenograft models(122–124).

Competition between tumor clones could be therefore exploited for therapeutic purposes. In this setting measurement of clonal evolution through liquid biopsy could guide “precise administration” of drug-holidays and re-challenge (Figure 3). However the pre-existence of resistant clones prior to therapy suggests that, albeit at different rates, both drug-resistant and drug-sensitive populations are susceptible to proliferate in a drug-free environment, thus supporting the introduction of sequential treatment strategies at molecular relapse. With this perspective the arising polyclonal multigenic mechanisms of resistance could be turned by liquid biopsy into a therapeutic opportunity for adaptive therapy, allowing the possibility to fine-tune intra-tumor clonal competition and enforce tumor growth control by alternating two (“evolutionary double bind”(125)) or more drugs specific for different tumor branches (and resistance mechanisms).

A complementary strategy to high dose alternating regimens implies the administration of reduced doses of therapy (the amount needed to achieve control of tumor growth rather

than the maximum tolerated dose) on a continuous schedule (metronomic therapy). In chemotherapy-treated breast cancer xenografts metronomic therapy allowed better control of tumor growth than full dose therapy (123). In this model intermittent doses (i.e. drug holidays) failed to control tumor growth(123). Indeed low doses maintenance regimens have for long been evaluated in clinical practice(126), however evidence showing the control of tumor evolution through metronomic approaches in patients is lacking. In this setting measuring tumor evolution could offer additional criteria to assess the efficacy of complex regimens and possibly to model more effective sequences of induction and maintenance therapy for solid tumors. The ethical and clinical challenges of adopting novel clinical trial paradigms implementing evolutionary modeling, superseding current approaches of treatment until progression of disease, should not be underestimated.

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LEVERAGING BIOMARKERS AND REAL-TIME ASSESSMENT OF TUMOR EVOLUTION

How then should medical intervention be guided? (Precision) cancer medicine has relied so far on predictive biomarkers to help the choice of the most effective therapeutic regimens. The impact of at least partially forecasting tumor evolution (that is to be able to predict the tumor's next moves) is suggested by evidence of parallel evolution in cancer(127). The ways tumors evolve could be relatively limited even across (epi)genetically different tumors. As already discussed a definite selective pressure could result in the de-regulation of a common pathway. In this regard, in the same patient, genetically distinct sub-clones often harbor genetic alterations targeting the same gene, or pathway through parallel evolution(3,128–130).

Exhaustive follow-up studies coupling multi-region biopsies with ctDNA analysis and analysis of tumor heterogeneity through autopsy analysis will define to what extent tumor evolution might be predictable (16,17), and hopefully these studies will provide a comprehensive understanding of the "evolutionary rule-book" of cancers, by distinguishing driver events that are always clonal from those that are often or rarely clonal, by estimating frequencies and dynamics of driver alterations across molecular subtypes, and possibly by revealing how molecular profiles associate with evolution patterns under the pressure of anti-cancer therapy.

Meanwhile, clinical and preclinical studies testing the efficacy of drug combinations targeting simultaneously the bulk tumor (therefore having a *bona fide* ubiquitous target) and resistance mechanisms will deliver valuable information to identify associations to administer *ab initio*. For example in a cetuximab-resistant CRC patient described by Russo et al. the combination of anti-EGFR panitumumab and MEK inhibitor trametinib was effective on a *MEK* mutated metastatic lesion (but not on a *KRAS* mutated clone)(64). Another study coupling functional analysis and tissue genotyping revealed that a *MET* amplification conferred resistance to the combination of panitumumab and vemurafenib in

a *BRAF* mutated CRC. In the same patient a combination of vemurafenib with the dual ALK-MET inhibitor crizotinib was capable, even if temporarily, to overcome resistance(131); interestingly the choice was supported by pharmacologic analysis on a BRAF-mutant cell line made resistant to BRAF-inhibition and showing emerging MET amplification. Similarly, in the study described by Crystal et al. sequencing of patient samples alone was not sufficient for the prediction of effective combinations suggesting that patient derived "avatar" could also be exploited to functionally define patient-specific drug associations(110).

Further preclinical insight into specific patterns of evolution could point out ways to steer tumor evolution towards more favorable or more targetable molecular backgrounds: for example T790M mutations in lung cancer, which renders resistant clones sensitive to third generation inhibitors, or EGFR ECD mutations in CRC which are effectively targetable with oligoclonal antibody MM-151(20,114). Steering tumor evolution could be possibly achieved by targeting specific phenotypes correlated with the "unwanted" genotypes. As an example the recently reported increased dependency of KRAS mutated clones on mitochondrial metabolism(132), and on increased uptake of dehydroascorbate (133) could be exploited in order to decrease the odds of emergence of KRAS-mediated resistance. Longitudinal analysis with liquid biopsy could then enable in patients real-time monitoring for the emergence of the desired phenotype.

It is important to underscore that several technical issues such as the ability to query spatial and temporal heterogeneity presently limits our capability to foresee tumor evolution. Moreover every tumor is unique, even when only genetic alterations are considered (134), and stochastic events might constitute an intrinsic barrier to the prediction of specific mechanism of drug resistance(135). The clinical implementation of liquid biopsies could provide real-time assessment of tumor evolution, thus allowing physician to undertake appropriate therapeutic measures and chose the best strategy to harness the evolution of a patient's tumor. The assessment of tumor heterogeneity through multi-region biopsies should not only be regarded as a tool for research, and the necessity to sample multiple lesions should be considered with a therapeutic purpose. Indeed multiple parameters, such as specific markers of susceptibility/resistance to targeted therapy, as well as proxies of response to immunotherapy could simultaneously be evaluated and possibly be hold as endpoints for therapeutic success, alongside standard imaging-based parameters.

FUTURE DIRECTIONS

The study of tumor evolution through multi-region sequencing and liquid biopsy has shed new light in our understanding of the neoplastic process and of the mechanisms by which tumors escape to therapy. Progress in these areas will likely be fostered by technological advances and decrease in the costs of sequencing: clinical application of multi-region biopsy can be supported by single cell analysis(136), which could provide high resolution readout of tumor heterogeneity even with limited sampled material. Reduced sequencing

costs and increased accessibility to standardized platforms will further foster implementation of liquid biopsies in clinical practice. Moreover, the evidence of epigenetic drivers of targeted therapy resistance(70) as well as the need for the evaluation of the tumor (micro)environemnt for the follow-up of response to other class of therapeutics (i.e. immunotherapy) calls for development of new ways to exploit circulating tumor DNA. Transcriptional analysis of tumor RNA retrieved from exosomes, or from circulating tumor cells, could widen our ability to identify and target non-genetic drivers of tumor evolution, and further studies are needed to assess the value of such approaches. Genetic analysis of T cell receptors is being exploited in the attempt to trace the "evolution" of T Lymphocytes in response to tumor evolution itself (98,137,138). Thus, recognition of patterns of tumor progression through multi region-biopsy and liquid biopsies might provide new therapeutic strategies tailored to cancer evolution and tumour-microenvironmental background in individual patient. Designing of clinical trials comparing liquid biopsy-driven therapeutic decisions vs. standard algorithms will be pivotal to promote progress in this area.

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FIGURE LEGENDS

Figure 1. Diagnostic approaches to measure the impact of cancer therapies on clonal evolution. Tumors are molecularly heterogeneous. Multi-region biopsies provide a snapshot of this heterogeneity, allowing the reconstruction of a tumor "phylogenetic tree" and the identification of ubiquitous, shared or private alterations. Liquid biopsy allows, through ctDNA analysis, real-time monitoring of changes in tumor heterogeneity under the selective pressure of anticancer-treatments. Analysis of the allelic frequencies of subclonal alterations provides a measure of growth dynamics of the different cell populations within a tumor, while quantification of trunk alterations allow normalizing for tumor burden. Circulating Tumor cells could integrate biological information obtained by ctDNA sequencing and circulating immune cells could help describe the "evolution" of tumor-responsive immune microenvironment.

Figure 2. Strategies to target clonal evolution. Measurements of clonal evolution through liquid biopsy (and multi-region biopsy) allows to "select" and integrate appropriate strategies to harness tumor evolution. The identification of targetable trunk alterations diminishes the odds of escape of clonal "branches" lacking the targeted alteration. "Preventive" combination therapy might allow exterminating resistant cells before the appearance of further resistance mechanisms. Adaptive strategies like the alternating administration of "drug holidays" and of treatments targeting different "branches" of the tumor, coupled with liquid biopsy monitoring

of subclonal growth ratios, could foster clonal competition and keep overall tumor growth under control. Immune response could be guided against evolution, either by selection and infusion of Tumor lymphocytes specific for trunk alterations, and thus capable to withstand evolution, or by triggering evolution in order to foster the immune response, by increasing the number of neoantigens.

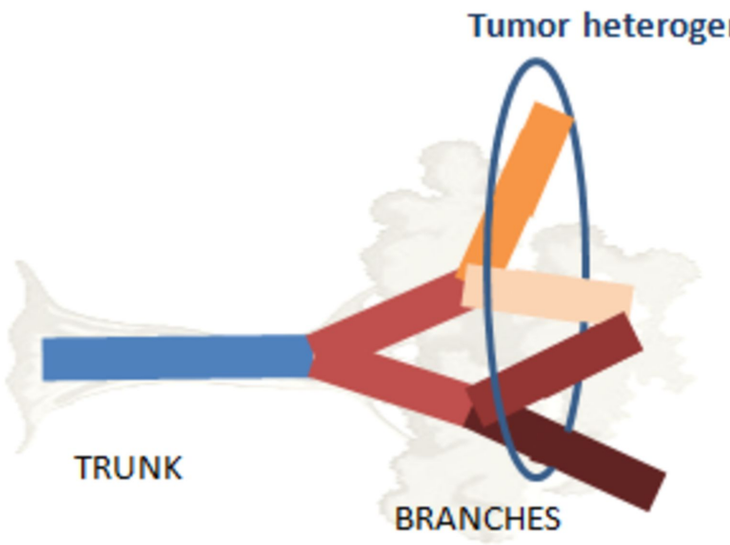
Figure 3. Liquid biopsy as a tool to guide adaptive treatment approaches. In CRCs that respond to treatment with anti EGFR antibodies, KRAS mutations often emerge as a mechanism of acquired resistance. Notably, KRAS mutations decline in ctDNA when EGFR blockade is suspended(18). Monitoring the evolution of KRAS mutant alleles in circulating tumor DNA can be used to design additional lines of therapies aimed at rechallenging patients that initially respond and then relapse to EGFR blockade.

BOX 1. Cancer evolutionary phylogenetic trees and liquid biopsy

Multi-region biopsy consists in parallel analysis of tissue derived from different regions of a single neoplastic mass, and from distinct metastatic lesions from the same patient (1). By assessing their pattern of occurrence, in the different samples, clonality of individual alterations is extrapolated. Clonal alterations, present in all samples analysed (blue) likely represent "ancestral " events , occurred early in tumorigenesis, and are therefore represented as the phylogenetic "trunk" of the tumor (2), while heterogeneous (sub-clonal) events (shades of brown) have likely occurred later and therefore represent the "branches" of the phylogenetic tree. Sub-clonal alterations are the ground for tumor heterogeneity, adaptability to therapy and cancer evolution. Liquid biopsy allows longitudinal assessment of the growth dynamics of different sub-clones , by cross-comparison of the relative frequencies of mutated sub-clonal alleles and normalization on (putative) trunk alterations (3).

1. Several regions of the tumor masses are sequenced in parallel

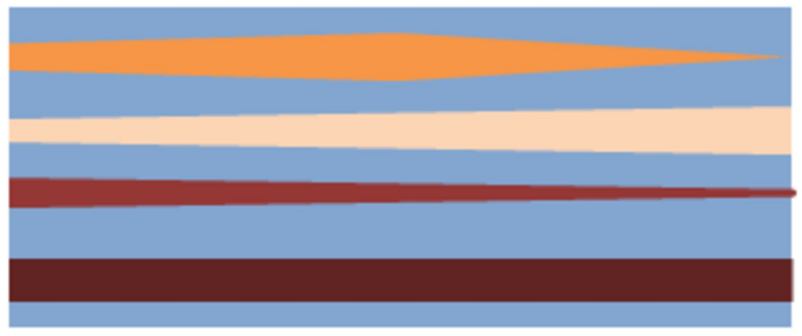
2. A "phylogenetic-tree" representing the "past" evolution of the tumor is reconstructed.



Clonal alterations, presenti n all tumor cells

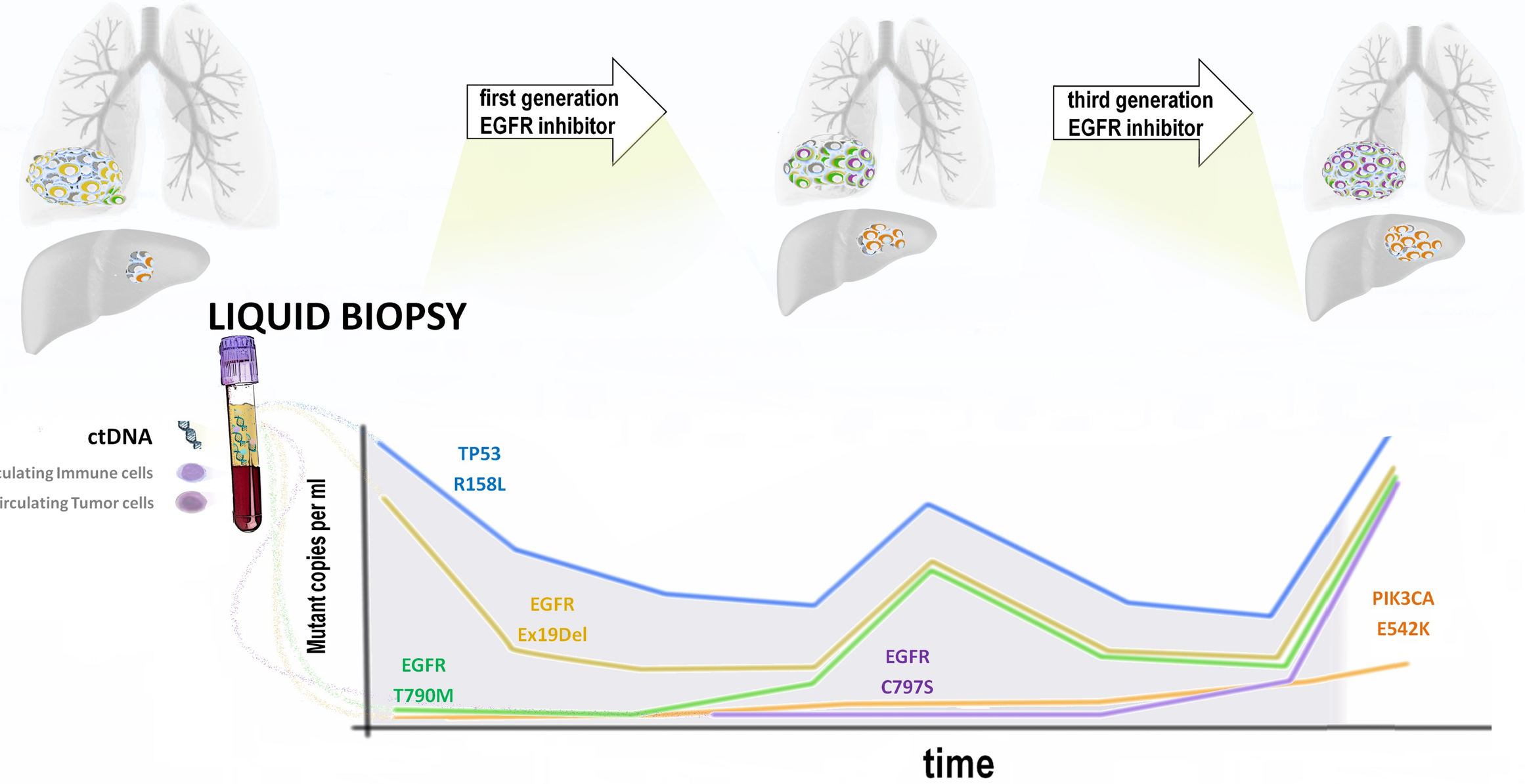
Sub-clonal alterations, ground for tumor heterogeneity

3. Liquid biopsy allows real-time mesurement of the growth dynamics of the different branches



Past tumor evolution

Future tumor evolution


first generation
EGFR inhibitor
third generation
EGFR inhibitor
LIQUID BIOPSY
ctDNA
Circulating Immune cells
Circulating Tumor cells
Mutant copies per ml
TP53
R158L
EGFR
Ex19Del
EGFR
T790M
EGFR
C797S
PIK3CA
E542K
time
MULTI-REGION
BIOPSY
Private
Shared
Ubiquitous
Trunk Branches
EGFR
T790M
PIK3CA
E542K
EGFR
Ex19Del
TP53
R158L
EGFR
T790M
EGFR
C797S
PIK3CA
E542K
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Ex19Del
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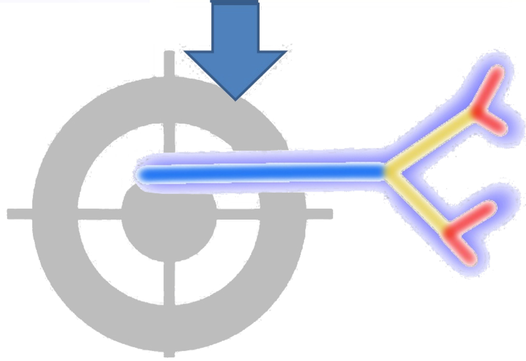
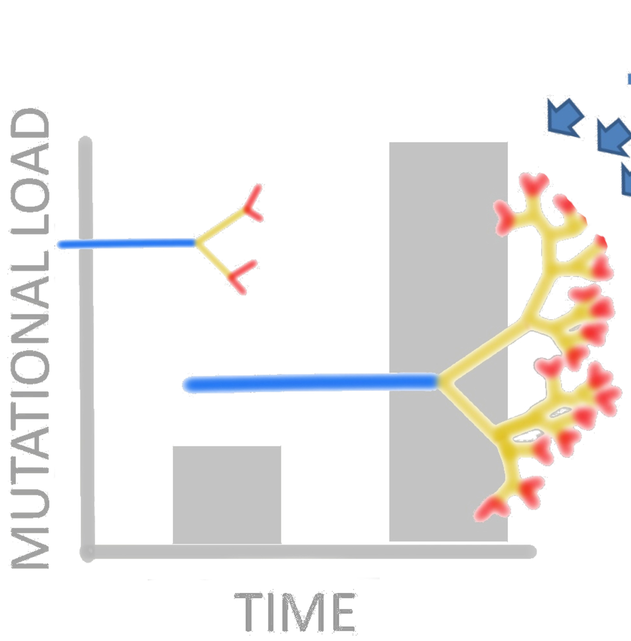
R158L
Tumor
heterogeneity
Tumor evolution

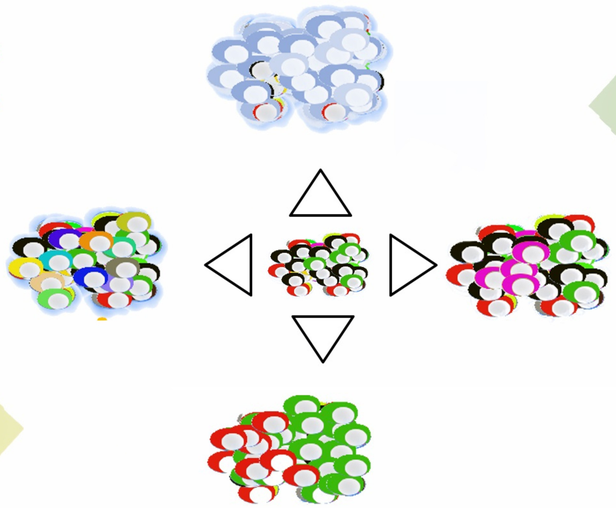


Targeting trunk mutations



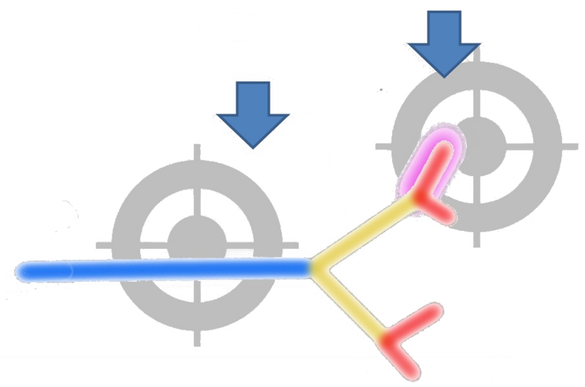
Hijacking evolution

Immunotherapy

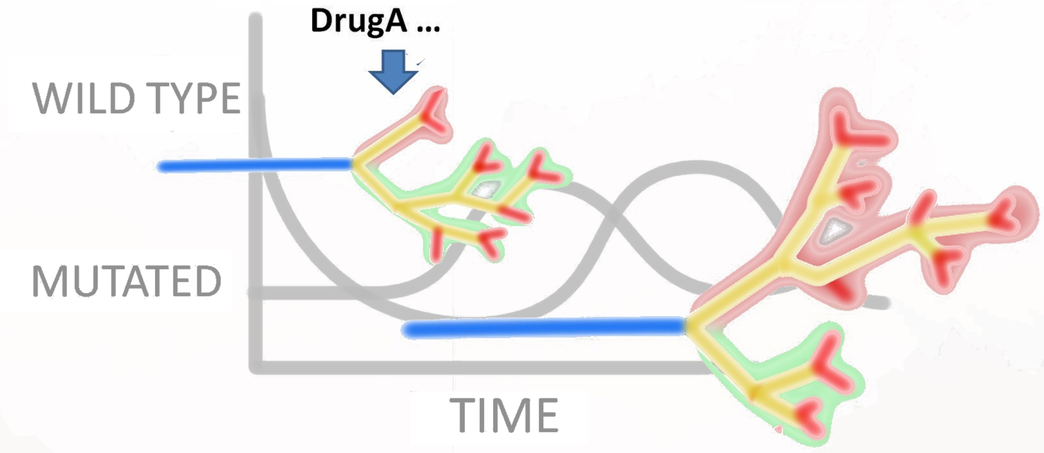


Preventive combination therapy

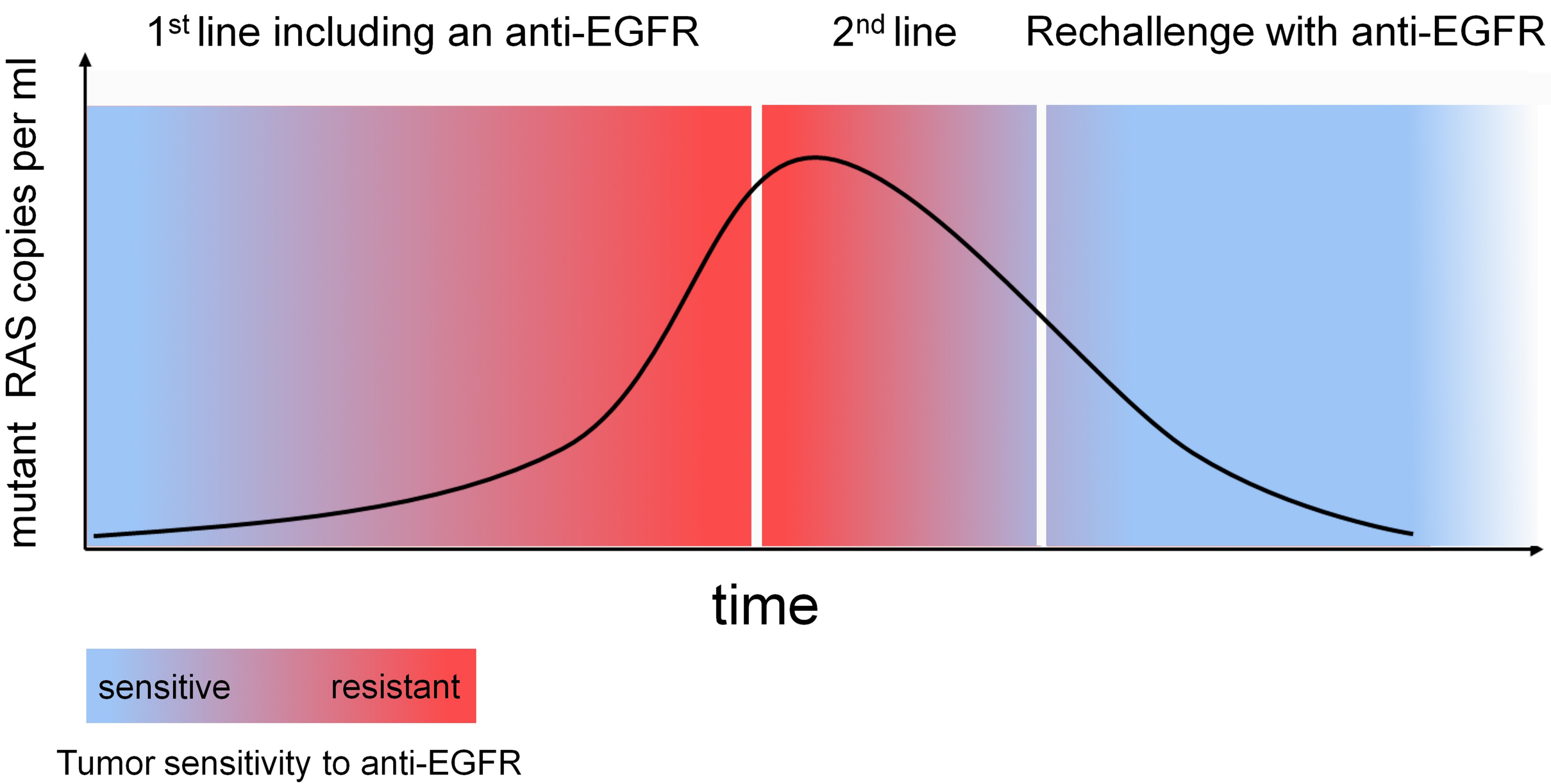
DrugA + DrugB



Adaptive therapy



...DrugB


1st line including an anti-EGFR
2nd line
Rechallenge with anti-EGFR
mutant RAS copies per ml
time
sensitive
resistant
Tumor sensitivity to anti-EGFR