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REVIEW

Implementing anti-epidermal growth factor receptor (EGFR) therapy in metastatic colorectal cancer: challenges and future perspectives

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Epidermal growth factor receptor (EGFR) inhibitors are valuable therapeutics in metastatic colorectal cancer (mCRC). Anti-EGFR monoclonal antibodies (MoAbs), such as cetuximab or panitumumab, in combination with chemotherapy are effective treatment options for patients with *RAS* and *BRAF* wild-type mCRC. Nevertheless, several issues are still open concerning the optimal use of anti-EGFR drugs in the continuum of care of mCRC. Novel approaches for increasing the efficacy of anti-EGFR therapies include better molecular selection of EGFR-dependent mCRC, intensification of chemotherapy, combination of anti-EGFR MoAbs and immune checkpoint inhibitors, and reintroduction of EGFR blockade or 'rechallenge' in selected patients who have previously responded to anti-EGFR MoAb therapy. An extensive translational research program was conducted in the Cetuximab After Progression in KRAS wIld-type colorectal cancer patients-Gruppo Oncologico dell' Italia Meridionale (CAPRI-GOIM) study with the aims of determining which subgroups of patients could benefit from the continuous inhibition of EGFR, from evaluating the role of liquid biopsy-based and its concordance with tissue-based molecular testing, and from investigating novel potential mechanisms of resistance to anti-EGFR therapies. In this review, we summarize the translational and clinical findings of the CAPRI-GOIM program in the context of the current knowledge of therapeutic strategies and of ongoing research on more appropriate uses of anti-EGFR therapies in *RAS* and *BRAF* wild-type mCRC patients.

Key words: anti-epidermal growth factor receptor monoclonal antibodies, liquid biopsy, metastatic colorectal cancer, molecular selection, predictive biomarkers, rechallenge therapy

INTRODUCTION

At the end of the twentieth century, the prognosis of metastatic colorectal cancer (mCRC) was relatively poor: the most effective medical option was therapy with 5-fluorouracil with a median overall survival (mOS) ranging from 8 to 12 months.[1] Today, there are several therapeutic options for the continuum of care of patients with mCRC, with at least four active lines of treatment leading to significant improvements in mOS, which now reach approximately 30 months.[2] Molecular tumor characterization, choice of the optimal initial medical treatment, the possibility of surgical intervention for selected patients with metastatic disease, and the appropriate sequence of lines of therapy are the pillars of mCRC clinical management. Among these therapeutic possibilities, the addition of molecular-targeted drugs to chemotherapy has significantly contributed to the improvement in survival.[2]

The epidermal growth factor receptor (EGFR) is a valuable therapeutic target in mCRC. Nevertheless, treatment with anti-EGFR monoclonal antibodies (MoAb), such as cetuximab or panitumumab, is effective only in a subset of patients.[3–6] Activating mutations in hot spot regions of exons 2, 3, and 4 of *KRAS* or of *NRAS* genes, which occur in approximately 55% of mCRC, are the major intrinsic mechanisms of resistance to anti-EGFR MoAbs and are currently used for excluding patients from treatment with these drugs.[7,8] Other molecular mechanisms of intrinsic and/or acquired cancer cell resistance have been suggested in *RAS* wild-type (WT) tumors.[9,10] Therefore, the identification of patients whose tumors are truly dependent upon EGFR activation is an open clinical question for the optimal use of anti-EGFR drugs in mCRC treatment.

To gain insights on the role of anti-EGFR agents in the continuum of care of mCRC patients, we started an academic, non-profit research program in 25 Italian centers within the cooperative network of the Gruppo Oncologico

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dell' Italia Meridionale (GOIM) in 2009. The Cetuximab After Progression in KRAS wIld-type colorectal cancer patients (CAPRI)-GOIM study was designed to investigate the strategy of continuing EGFR inhibition in second-line therapy of mCRC patients after progression from first-line treatment with cetuximab plus chemotherapy. Briefly, mCRC patients with *KRAS* exon 2 WT tumors (at that time the only known biomarker for anti-EGFR therapy patient selection) received FOLFIRI plus cetuximab as first-line treatment and, at disease progression, were randomly assigned to receive either FOLFOX or FOLFOX plus cetuximab.[11–13] An extensive translational research program was conducted in the CAPRI-GOIM study to determine which subgroups of patients could benefit from the continuous blockade of EGFR, to evaluate the role of liquid biopsy-based molecular testing and its concordance with tissue-based molecular testing, as well as to investigate novel potential mechanisms of resistance to anti-EGFR therapies.[14–18] Here, we summarize the major translational and clinical findings of the CAPRI-GOIM program that have provided evidence for a more appropriate use of anti-EGFR therapies in mCRC. These results are discussed in the context of current knowledge and of ongoing research in this field.

CORRELATION BETWEEN THE EFFICACY OF FIRST-LINE TREATMENT WITH FOLFIRI PLUS CETUXIMAB AND TUMOR MOLECULAR CHARACTERIZATION

Three hundred and forty patients with *KRAS* exon 2 WT mCRC received FOLFIRI plus cetuximab as first-line treatment (Table 1).[11] *KRAS* mutations were determined by local laboratories. In the intention-to-treat population, the results were consistent with data from randomized trials (CRYSTAL, OPUS, and FIRE 3) that have evaluated the efficacy of the addition of cetuximab to chemotherapy as first-line treatment of *KRAS* exon 2 WT mCRC patients.[19–21] For 182/340 (53.5%) patients, formalin-fixed paraffin-embedded tissue samples were available for next generation sequencing (NGS) analysis. Tumor samples were analyzed with Ion Torrent technology, using the Colon and Lung Cancer Panel that can identify mutations in 87 hot spot regions of 22 genes (*ALK*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *MET*, *DDR2*, *KRAS*, *PIK3CA*, *BRAF*, *AKT1*, *PTEN*, *NRAS*, *MAP2K1*, *STK11*, *NOTCH1*, *CTNNB1*, *SMAD4*, *FBXW7*, *TP53*). Based on the results of a previous validation study, a 2% sensitivity threshold was set for this panel.[22] Only less than one-third of tumors were WT for all 22 genes tested (58/182; 31.9%). One or more genes were mutated in 124/182 (68.1%) samples. Unexpectedly, *KRAS* exon 2 mutations were identified in 29/182 (15.9%) samples, although they had been classified as *KRAS* exon 2 WT tumors by local laboratory assessment. These findings represented the first report of discordance between centralized and local laboratory analysis of *KRAS* exon 2 mutations in a prospective clinical trial, and highlighted the need for external quality assessment as well as the use of more sensitive techniques, in order to ensure the standardization of *RAS* testing for clinical practice.[23] Mutations in exons 3 or 4 of the *KRAS* gene were identified in 16/182 (8.8%) samples, while *NRAS* exon 2 or 3 mutations were reported in 13/182 (7.1%) cases. *PIK3CA* mutations were found in 24/182 (13.2%) cases, whereas $BRAF^{V600E}$ mutations were identified in 10/182 (5.5%) tumors.[11]

We investigated if mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes could influence the efficacy of FOLFIRI plus cetuximab treatment. The subset of patients with quadruple WT tumors had the greatest benefit from this treatment with median progression-free survival (mPFS) of 11.3 months and overall response rate (ORR) of 64.4%, while mPFS was 7.7 months and ORR 47.4% in patients with a mutation in at least one of these genes (Table 1). These results have been confirmed in subsequent studies of first-line treatment of molecularly selected mCRC with chemotherapy plus anti-EGFR MoAbs, such as the VALENTINO trial that investigated the combination of FOLFOX plus panitumumab.[24]

We conducted a *post hoc* analysis for assessing the feasibility and the efficacy of FOLFIRI plus cetuximab in elderly patients who were enrolled in the CAPRI-GOIM clinical trial, considering that the majority of mCRC cases are diagnosed in the elderly.[25] Phase II and III studies have shown the safety and clinical activity of bevacizumab in combination with fluoropyrimidine-based regimens in elderly patients with mCRC who are unfit for chemotherapy doublets.[26,27] Few data are available for elderly patients who are suitable for anti-EGFR treatment in combination with chemotherapy, however.[28,29] In the CAPRI-GOIM study, FOLFIRI plus cetuximab treatment resulted in similar PFS and ORR among the elderly population, with a similar incidence of all grade adverse events. Nevertheless, in patients $\geq$75 years old, higher rates of grade 3–4 diarrhea and neutropenia were reported.[13]

TUMOR HETEROGENEITY FOR *RAS*, *BRAF*, AND *PI3KCA* MUTATIONS AND EFFECTS ON ANTI-EGFR THERAPIES

Intratumor genetic heterogeneity refers to the concept that in a single tumor, different cancer cell clones, carrying different molecular alterations, coexist.[30] Recently, greater attention has been focused on the issue of tumor heterogeneity, since the presence of cancer cell clones with different mutations could constitute a mechanism of primary and/or secondary resistance to molecular targeted therapies.[31] In the CAPRI-GOIM trial, the relevance of heterogeneity of *KRAS*, *NRAS*, *BRAF*, and *PI3KCA* mutations on the clinical activity of anti-EGFR therapy was evaluated.[14] Tumor heterogeneity was measured by quantitative assessment of mutant allele frequency (MAF) by NGS with normalization for cancer cell content in the tumor tissue. Assuming that somatic mutations generally affect one allele, the heterogeneity score (HS) was calculated by multiplying by two the frequency of mutant alleles in cancer cells. As an example, HS = 100 suggests that all cancer cells are mutated; HS $<$100 indicates that only a fraction of cancer cells are mutated; HS $>$100 implies gene copy number variation, either gain of the mutant allele or loss of

Table 1. Clinical activity of FOLFIRI plus cetuximab as first line treatment of metastatic colorectal cancer (mCRC) in the intention to treat population (ITT) and in the next generation sequencing (NGS) cohort

First line: FOLFIRI + cetuximab	ITT (*N* = 340 patients)	NGS (*N* = 182 patients)	*KRAS*, *NRAS*, *BRAF*, and *PIK3CA* WT (*N* = 102 patients)	*KRAS*, *NRAS*, *BRAF*, or *PIK3CA* mut (*N* = 78 patients)
Complete response (%)	26/340 (7.6%)	12/182 (6.6%)	8/104 (7.7%)	4/78 (5.1%)
Partial response (%)	166/340 (48.8%)	927182 (50.5%)	59/104 (56.7%)	33/78 (42.3%)
Stable disease (%)	115/340 (33.8%)	61/182 (33.5%)	28/104 (26.9%)	33/78 (42.3 %)
Disease progression (%)	33/340 (9.7%)	17/182 (9.3%)	9/104 (8.6%)	8/78 (10.3%)
ORR (%)	192/340 (56.4%)	104/182 (57.1%)	67/104 (64.4%)	37/78 (47.4%)
mPFS, months	9.9	9.8	11.3	7.7

Subgroup analysis on the efficacy of FOLFIRI plus cetuximab in *KRAS*/*NRAS*/*BRAF* and *PIK3CA* wild-type or in *KRAS*/*NRAS*/*BRAF*/*PIK3C* mutant cohorts.
Mut, mutant; mPFS, median progression-free survival; ORR, overall response rate; WT, wild-type.

the WT allele.[14] *KRAS* HS ranged between 12 and 260, as in most mCRC patients the majority of cancer cells had mutant *KRAS*. Similar results were found for *NRAS* mutant cases. In contrast, for *BRAF* or for *PIK3CA* mutant cases, generally only a fraction of cancer cells were mutated.

We investigated whether there was a threshold value for *KRAS* mutations to predict response to therapy and if the HS could represent a feasible biomarker for this purpose. Among 10 patients carrying *KRAS* mutant tumors with HS <33, the ORR to FOLFIRI plus cetuximab therapy was 70%. On the other hand, patients with *KRAS* mutant tumors with HS >33 had a response rate in line with the activity of FOLFIRI alone (45.7%), as expected for mCRC patients with *RAS* mutant tumors. The mPFS was similar for the high *KRAS* and low *KRAS* mutant groups (7.97 versus 8.37 months), however, suggesting that even a low content in *KRAS* mutant cancer cells is sufficient to determine resistance to anti-EGFR therapies. These data are in agreement with the current knowledge on resistance to targeted agents. A low fraction of cancer cells that carry a resistance mutation may not prevent a transient clinical response to a specific drug, but the duration of the response is relatively short for the rapid clonal expansion of the resistant cancer cells, eventually leading to disease progression. Nevertheless, the threshold of *KRAS* MAF that could be used to predict the clinical efficacy of EGFR blockade is still debated. In the CAPRI-GOIM trial, even very low MAFs determined resistance to cetuximab.[14,18] In the CRYSTAL trial, *RAS* mutational status was retrospectively validated with a cut-off of 5%, since *RAS* MAF values <5% did not affect the clinical efficacy of FOLFIRI plus cetuximab.[4] A retrospective study suggested that mCRC patients with *RAS* MAF values <1% who were treated with anti-EGFR therapies had similar clinical outcomes compared with patients with *RAS* WT tumors.[32] A recent large multicenter retrospective analysis that compared standard-of-care *RAS* testing with NGS multigene assessment reported no improvement in the selection of patients for anti-EGFR therapy by lowering the threshold in tissue samples from 5% to 1% MAF.[33] As expected, in this study anti-EGFR treatment was significantly better in mCRC patients with *KRAS*/*NRAS*/*BRAF*/*PIK3CA* WT tumors.[33]

Of note, in the CAPRI-GOIM study we found in 7/10 cases of low *KRAS* mutational load the presence of additional mutations in *PIK3CA*, *TP53*, *BRAF*, *ERBB2*, *FGFR3*, and/or *FBXW7* genes, which could equally contribute to anti-EGFR cancer cell resistance. These findings highlight the existence of a subgroup of mCRC with a mixed genotype, which is characterized by different potentially driver mutations that affect the EGFR pathway. The complexity of tumor mutations and of cancer cell heterogeneity suggests that biomarker-selected combinations of different molecular targeted drugs will probably be necessary to effectively control cancer growth and, thus, to determine the relevant clinical benefit in mCRC.

CETUXIMAB CONTINUATION AFTER FIRST PROGRESSION IN mCRC

In the CAPRI-GOIM trial, 153 patients who obtained a clinical benefit (stable disease, partial or complete response [CR]) from FOLFIRI plus cetuximab as first-line therapy, were randomly assigned to second-line treatment with FOLFOX plus cetuximab (arm A, *n* = 74) or FOLFOX (arm B, *n* = 79) (Table 2) at disease progression.[12] In the intention-to-treat population we found a trend in improvement in PFS and OS. In the molecularly selected population (*KRAS*, *NRAS*, *BRAF*,

Table 2. Clinical outcome of second line treatment with FOLFOX plus cetuximab (experimental arm) and FOLFOX (control arm) in the intention-to-treat population (ITT)

Second line: FOLFOX + cetuximab versus FOLFOX	FOLFOX + cetuximab ITT (*N* = 74 patients)	FOLFOX ITT (*N* = 79 patients)	FOLFOX + cetuximab *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* WT (*N* = 34 patients)	FOLFOX *KRAS*, *NRAS*, *BRAF*, or *PIK3CA* mut (*N* = 32 patients)
ORR (%)	21.6%	12.7%	29.4%	9.4%
mPFS, months	6.4	4.5 (HR: 0.81; *P* = 0.19)	6.9	5.3 (HR: 0.56; *P* = 0.025)
mOS, months	17.6	14 (HR: 0.86; *P* = 0.41)	23.7	19.8 (HR: 0.57; *P* = 0.056)

Subgroup analysis on the efficacy of FOLFOX plus cetuximab in *KRAS*/*NRAS*/*BRAF* and *PIK3CA* or in *KRAS*/*NRAS*/*BRAF*/*PIK3CA* mutant cohorts.
HR, hazard ratio; mOS, median overall survival; mPFS, median progression-free survival; mut, mutant; ORR, overall response rate; WT, wild-type.

and *PIK3CA* WT) representing 76%, we observed a statistically significant improvement in mPFS, with a trend in improvement of mOS of about 4 months and an ORR three times higher for the experimental arm (29.4% versus 9.4%). These data are encouraging when compared with other second-line options; this strategy could be an option for quadruple WT mCRC patients who require tumor shrinkage after first-line therapy.[34–36] The CAPRI-GOIM trial has been the only randomized study to provide evidence that a potentially relevant clinical benefit could be obtained by the continuous blockade of EGFR in a subset of patients with mCRC whose tumors remain dependent on EGFR activation. The results of a retrospective analysis of two non-randomized single-arm parallel studies, in which mCRC patients with *RAS* WT tumors were treated with chemotherapy alone or with chemotherapy plus cetuximab after first-line treatment with cetuximab plus chemotherapy, have been reported.[37] In patients with all *RAS* WT tumors who had early tumor shrinkage in first-line treatment, the continuation of cetuximab after progression in combination with a different chemotherapy backbone resulted in a statistically significant advantage in terms of OS, PFS, and ORR. No benefit was found in patients with *RAS* mutant tumors or in *RAS* WT patients who did not achieve early tumor shrinkage in first-line treatment.[37]

RAS TESTING BY LIQUID BIOPSY AND CORRELATION WITH CLINICAL OUTCOME

The term liquid biopsy refers to an analytical technique that allows tumor molecular profiling through the use of tumor-derived biomarkers in body fluids (including peripheral blood, urine, and cerebral-spinal liquor). In this respect, the search and the analysis of circulating tumor DNA (ctDNA) from cancer cells could represent an important source of clinical information on the prognosis of intensively treated CRC patients, on the evaluation of sensitivity to specific molecular targeted therapies, and on the early detection of mechanism(s) of cancer cell resistance.[38]

In the CAPRI-GOIM study we also evaluated the predictive role of *RAS* testing with liquid biopsy. For 92/340 mCRC patients, both tissue samples for NGS analysis and baseline plasma samples for beads, emulsions, amplification, magnetics testing were available.[15] *RAS* mutations were found in both tissue and plasma samples in 33/92 patients (35.9%). The concordance rate between tissue and plasma was only 78.3%, however. Of note, 10 patients were tissue *RAS* mutant and plasma *RAS* WT, whereas 10 patients were tissue *RAS* WT and plasma *RAS* mutant. There were no major differences in mPFS, mOS, and ORR of *RAS* WT or *RAS* mutant patients according to either tissue or liquid biopsy *RAS* testing.[15] We found a lower concordance rate between *RAS* analyses on tumor tissues and plasma samples compared with other reports.[39,40] For plasma *RAS* WT but tissue *RAS* mutant cases, possible causes of discordance could be identified in the prevalence of lung and node metastases, which are known to reduce the sensitivity of *RAS* testing in liquid biopsy.[41,42] Moreover, in seven patients the primary tumor was surgically removed before drawing blood, and this could have reduced tumor burden and the levels of ctDNA and, therefore, could have affected liquid biopsy sensitivity.[43] To investigate the discordant cases, further analysis was performed with digital droplet PCR (ddPCR), a highly sensitive analytic technique. Interestingly, ddPCR detected *RAS* mutations only in 2/10 patients who were plasma *RAS* WT and tissue *RAS* mutant. In contrast, ddPCR detected the presence of *RAS* mutations at low allelic frequencies ranging between 0.15% and 1.15%, which were below the 2% threshold of NGS that was used in the CAPRI-GOIM study in all 10 patients who were tissue *RAS* WT but *RAS* mutant by liquid biopsy. These results suggest the presence of subclonal *RAS* mutations in these patients. Remarkably, mCRC patients with *RAS* WT tumor on tissue and *RAS* mutant tumor on liquid biopsy had a similar PFS and OS compared with patients with *RAS* mutant tumors on tissue.[15] Despite the limits of a *post hoc* analysis that was conducted on a relatively small patient group, these data highlight the capability of liquid biopsy to detect spatial and temporal tumor heterogeneity. Therefore, liquid biopsy testing for ctDNA could be an effective tool in the diagnosis of *RAS* mutations and could represent a valid instrument for monitoring the emergence of cancer resistance to anti-EGFR therapies.

NOVEL MECHANISMS OF RESISTANCE TO ANTI-EGFR DRUGS

The benefit of cetuximab- or panitumumab-based treatments for patients with mCRC could be limited by primary or acquired mechanisms of resistance. *KRAS* or *NRAS* mutations are predictive biomarkers for intrinsic (or innate) and secondary (or acquired) resistance to anti-EGFR therapy. Other gene alterations, however, might be involved in determining resistance to these drugs (Figure 1). Several mechanisms of acquired or secondary cancer resistance have been identified, including activation of angiogenesis, novel development of *RAS* mutations and/or EGFR mutations, gene amplification, or mutations in other tyrosine kinase receptors (RTK).[9,10,44–46] We have previously shown in preclinical models of human CRC that acquired resistance to anti-EGFR drugs could be associated with increased levels of vascular endothelial growth factor and that inhibiting angiogenesis could contribute to tumor growth inhibition.[47] Under the selective pressure of EGFR blockade, approximately one-third to one-half of mCRC patients will develop secondary resistance through the emergence of *RAS* mutant cancer cell subclones.[48] Another mechanism of acquired resistance could be the onset of mutations in the EGFR extracellular domain (ECD). Montagut et al.[49] identified the S492R mutation in the ECD of EGFR that prevents the binding of cetuximab to EGFR. Of note, the S492R EGFR ECD mutation has never been detected in mCRC patients before exposure to anti-EGFR MoAb treatment.[50] Human EGFR 2 (*HER2*) gene amplification has been described in approximately 3% to 5% of *RAS* WT tumors as a primary and/or secondary mechanism of resistance to anti-EGFR therapy.[51,52] In this respect, HER2

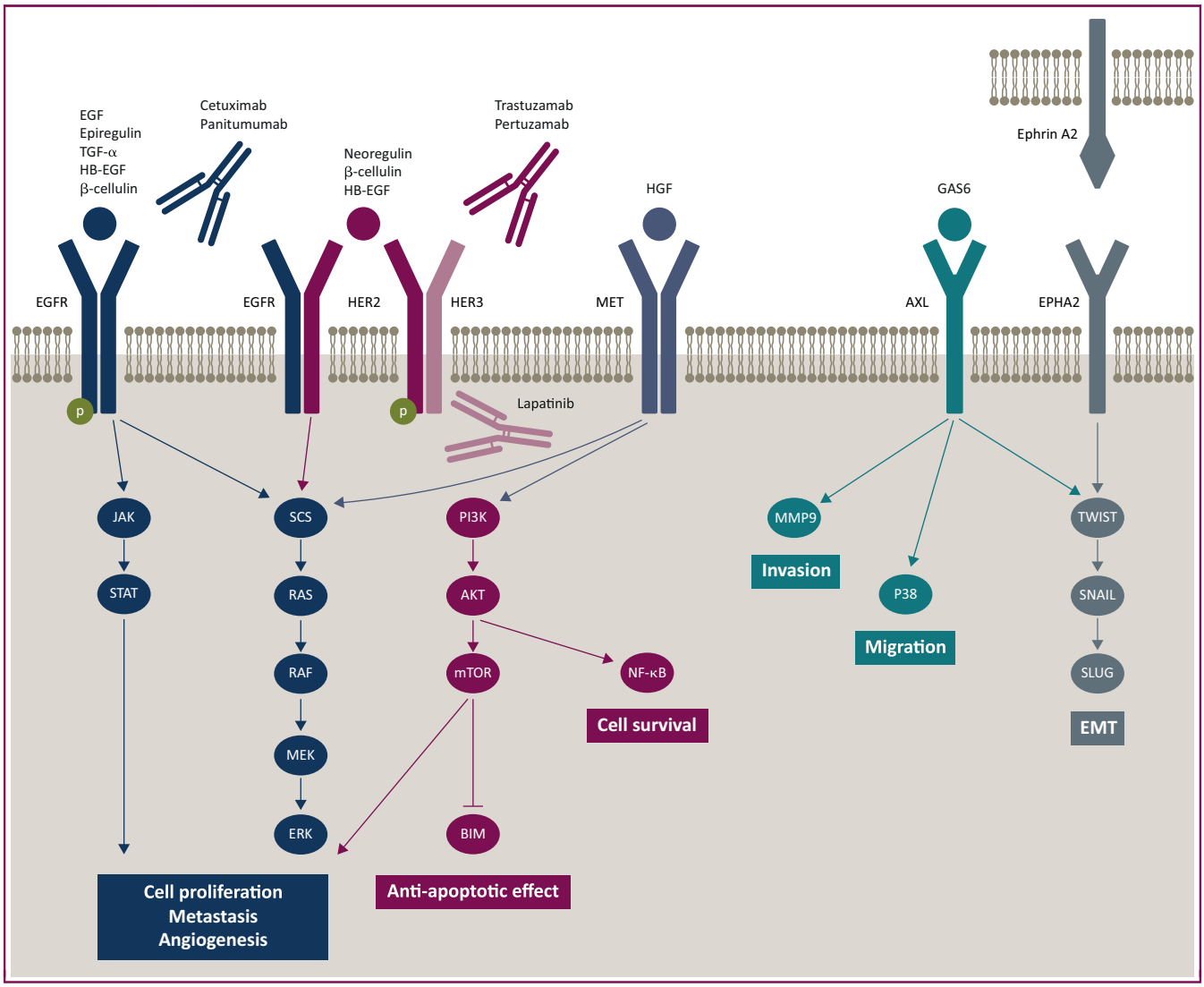

Figure 1. Epidermal growth factor receptor (EGFR) signaling pathway and potential mechanism of resistance to cetuximab and panitumumab.
AXL, tyrosine-protein kinase receptor UFO; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EPHA2, erythropoietin-producing hepatocellular A2 receptor; GAS6, growth arrest-specific 6; HB-EGF, heparin-binding EGF-like growth factor; HER2, human epidermal growth factor receptor 2; HER3, human epidermal growth factor receptor 3; HGF, hepatocyte growth factor; MET, tyrosine-protein kinase Met; TGF-α, transforming growth factor alpha.

inhibition is a promising strategy in HER2 amplified mCRC.[53–57] *MET* gene amplification has been found as a subclonal alteration in a small group of patients. In these patients, during anti-EGFR treatment, *MET* amplified cancer cells undergo a positive selection and become the dominant cancer cell population.[58,59]

To identify novel potential biomarkers of resistance to cetuximab treatment, we performed a comprehensive molecular profiling of 21 quadruple WT tumors from mCRC patients enrolled in the CAPRI-GOIM study to investigate if the presence of any gene alteration(s) could be correlated with a poorer clinical outcome.[18] One patient with a short PFS had a tumor with the c.169A>G mutation in the *MAP2K1* gene that codes for the MEK1 protein. This variant results in the inactivation of the MEK1 negative regulatory domain and determines a gain of function of MEK1 protein.[60] Two patients with short mPFS had tumors with mutations in *NF1*, a gene that encodes for an inhibitory regulator of *RAS* signaling pathway.[61] In particular, we found an insertion (c.638_639insA; p.Asn214Lys fs*2) in one tumor sample and an single nucleotide variation (c.5101A>T; p.Lys1701Ter) in the other tumor sample. These mutations result in the loss of function of *NF1* and could cause the constitutive activation of *RAS* signaling. To our knowledge, these *NF1* mutations have never been reported as negative biomarkers for anti-EGFR drugs in a prospective clinical trial in mCRC. Three missense mutations were found in the *FBXW7* gene. The prognostic role of these gene alterations is still controversial. Recently, *FBXW7* variants in a tumor sample from a patient who was refractory to chemotherapy plus cetuximab have been reported.[62,63] In the CAPRI-GOIM study, one patient with this variant had CR, with a PFS of 18 months; on the contrary the other two patients did not respond to FOLFIRI plus cetuximab treatment. For these two patients, c.1798G>A (p.Asp600Asn) and c.1513C>T single nucleotide variation (p.Arg505Cys) *FBXW7* mutations were found. In the

patient who had a significant clinical benefit from FOLFIRI plus cetuximab treatment, the *FBXW7* mutation coexisted with *GAS6* gene amplification. In this respect, elevated *GAS6* expression has been correlated with a good prognosis in CRC.[64] Collectively, these results indicate that mechanisms of anti-EGFR drug resistance are extremely complex and, in some cases, cannot be only explained by the presence of a single gene alteration.

We also investigated the role of other growth factor receptor-driven signaling pathways that could be involved in either primary or secondary resistance to anti-EGFR MoAbs. Erythropoietin-producing hepatocellular (EPH) A2 receptor is a member of the EPH RTK family.[65] EPHA2 overexpression has been described as a poor prognostic factor in early stage CRC and has been shown as a negative predictive biomarker of response to anti-EGFR therapy.[66,67] We evaluated the expression of EPHA2 by immunochemistry (IHC) in tumor samples from a cohort of 82 patients *RAS* WT in the CAPRI-GOIM study.[16] EPHA2 expression by IHC was detected in 55 of 82 (67%) tumor specimens. To better understand the predictive role of EPHA2, we developed a semi-quantitative immune-histoscore (HSCORE). Patients with high EPHA2 HSCORE had a statistically significant inferior mPFS compared with patients with low EPHA2 HSCORE. We evaluated EPHA2 as a mechanism of resistance to anti-EGFR MoAbs by using human CRC cell lines with primary or acquired resistance to cetuximab.[16,68] The combination of ALW-II-41-27, a small molecule tyrosine kinase inhibitor of EPHA2, with cetuximab was able to restore the sensitivity to anti-EGFR therapies, resulting in significant antitumor activity.[16] Taken together, these data suggest that EPHA2 is a novel biomarker of resistance to EGFR targeting therapies, and that EPHA2 could be an innovative therapeutic target in order optimize treatment of patients with *RAS* WT mCRC.

AXL is a member of the TAM RTK family.[69] We have previously demonstrated in human CRC cell lines that AXL activation could promote proliferation, migration, and EMT, and that AXL blockade may exert sustained antitumor activity and induce resistance to anti-EGFR therapy.[70–72] We retrospectively analyzed, by IHC, 102 *RAS* WT tumor samples of patients with mCRC (of whom 68 patients were included in the CAPRI-GOIM study) who received chemotherapy plus cetuximab as first-line treatment.[16] AXL-positive patients (9%) were refractory to chemotherapy plus cetuximab, with a statistically significant worse mPFS compared with AXL-negative patients (4.3 versus 12.1 months; $P = 0.001$) and with a lower OS (20.1 versus 30.2 months). With the limitation of a retrospective analysis on a small cohort of patients, these results might suggest a role of AXL as a predictive biomarker of lack of response to cetuximab in *RAS* WT mCRC.[16]

NOVEL APPROACHES FOR OPTIMIZING THE EFFICACY OF ANTI-EGFR THERAPIES

FOLFOX or FOLFIRI doublets in combination with cetuximab or panitumumab are first-line treatment options for *RAS* and *BRAF* WT mCRC patients, according to European Society for Medical Oncology clinical practice guidelines.[2,73] Nevertheless, several issues are still open for the optimal use of anti-EGFR drugs in the continuum of care of mCRC.

A first question is how to improve the activity and efficacy of anti-EGFR drugs in combination with chemotherapy. The intensification of first-line treatment using triple-drug chemotherapy regimens in combination with EGFR blockade is a potential strategy to intensify the efficacy of EGFR inhibitors in mCRC.[74] The MACBETH study has demonstrated that cetuximab could be added to modified FOLFOXIRI as first-line therapy for fit *RAS* and *BRAF* WT mCRC patients with high ORR and a manageable safety profile.[75] Furthermore, the final results of the VOLFI study, a randomized phase II trial evaluating the addition of panitumumab to FOLFOXIRI as initial treatment of *RAS* WT mCRC patients, have been recently reported.[76] The study met its primary end point, which was ORR. ORR was 87.3% in the experimental arm compared with 60.6% in the FOLFOXIRI control arm ($P = 0.0041$). An increased rate of surgical resection of liver metastases was also obtained. Nevertheless, despite a significant increase in responses, mPFS was similar between the two arms, without statistically significant improvements in mOS for the experimental arm (probably due to the increased number of censored cases for surgical resection of liver metastatic disease).[76] A critical issue for the combination of anti-EGFR agents and triplet chemotherapy is side-effects, including gastrointestinal toxicity, that could limit the feasibility of these regimens. Nevertheless, *RAS* WT mCRC patients with a good performance status and who require intense and rapid tumor shrinkage might benefit from a combination of FOLFOXIRI plus anti-EGFR MoAbs. In this respect, TRIPLETE, a multicenter randomized phase III trial, that is comparing FOLFOXIRI plus panitumumab versus FOLFOX6 plus panitumumab as initial therapy for fit patients with unresectable *RAS* and *BRAF* WT mCRC, is currently ongoing.[77]

A potential intensification of first-line therapies with anti-EGFR drugs could be the combination of EGFR inhibitors and immune checkpoint inhibitors. Immunotherapy is generally of limited efficacy in mCRC.[78] Whereas in deficient mismatch repair or in high microsatellite instable mCRC there is clinical evidence of immune checkpoint inhibitors efficacy, approximately 90% to 95% of mCRC patients who have a proficient mismatch repair or a microsatellite stable tumor do not benefit from immunotherapy.[78] Nevertheless, experimental preclinical data suggest that blocking EGFR with cetuximab in cancer cells and in the tumor microenvironment could modulate immune infiltration and therefore could activate the antitumor activity of the immune system.[78,79] On the other hand, in a preclinical model of CRC, the expression of EGFR on myeloid cells in the tumor microenvironment contributed to tumor development and progression through the production of immune-modulatory cytokines, such as interleukin 6.[80] Furthermore, cetuximab could promote opsonization and phagocytosis of human colon cancer cells by dendritic cells through the stimulation of tumor antigen presentation to T cells and, thus,

cetuximab could potentially activate an immune response.[81] Moreover, cetuximab may induce natural killer cell-mediated antibody-dependent cellular cytotoxicity.[82] On this basis, trials are currently investigating the combination of cetuximab with immune checkpoint inhibitors in different cancers, such as head and neck squamous cell carcinoma and non-small-cell lung cancer. In mCRC, the AVETUX phase II study enrolled 43 patients with *RAS* and *BRAF* WT tumors. First-line treatment consisted of avelumab, an anti-programmed death ligand 1 (PD-L1) MoAb, plus cetuximab in combination with FOLFOX6. An interim analysis of the AVETUX trial that was conducted on the first 20 treated patients has reported a 75% ORR (15/20) with a 95% disease control rate (19/20).[83] A further type of therapy intensification is currently being evaluated in the AVETRIC trial, a single-arm study of FOLFOXIRI in combination with cetuximab and avelumab as initial treatment of *RAS* and *BRAF* WT mCRC patients. After induction therapy with all drugs, maintenance treatment with cetuximab plus avelumab is scheduled until disease progression (EudraCT Number 2019-0041501-24).

Another potential area for improving the efficacy of anti-EGFR agents in the continuum of care of mCRC is the reintroduction of EGFR blockade or 'rechallenge' with EGFR inhibitors in selected patients who have been previously treated with anti-EGFR MoAbs. Rechallenge refers to anti-EGFR drug re-treatment of mCRC patients with *RAS* WT tumors who have had an initial significant clinical benefit from anti-EGFR drugs, such as a major response or prolonged disease stabilization, and who, upon disease progression, received a different medical treatment.[84] The rechallenge concept was first explored in a study that evaluated the potential clinical activity of cetuximab plus irinotecan. Thirty-nine patients with *KRAS* exon 2 WT mCRC, who had a clinical benefit from anti-EGFR drugs in combination with chemotherapy as first-line therapy, at disease progression were treated with a second-line therapy. After further disease progression, these patients received cetuximab plus irinotecan.[85] The reported RR was 53.8% (with 19 partial response [PR] and 2 CR) and the mPFS was 6.6 months.[85] A possible explanation of these results is the dynamic temporal evolution of resistance mechanisms to anti-EGFR drugs. In fact, it has been shown that cancer cell subclones, that are harboring *RAS* or ECD EGFR mutations, evolve and are rapidly selected as the predominant clones during anti-EGFR therapies, thus causing disease progression.[86–88] These mutant cancer cells decay after the interruption of EGFR inhibitor treatment, however, with a cumulative half-life of approximately 4 months.[89] In line with this hypothesis, the results of CRICKET, a small single-arm proof-of-concept prospective study of rechallenge with cetuximab and irinotecan, have recently been published.[90] Twenty-seven patients with *RAS* and *BRAF* WT mCRC, who had at least a PR and PFS of 6 months or more after first-line cetuximab plus chemotherapy, were enrolled. Interestingly, a *post hoc* analysis revealed that all patients who achieved a PR had *RAS* WT tumors at the baseline liquid biopsy testing for ctDNA before cetuximab plus irinotecan rechallenge.[90] Several trials of anti-EGFR rechallenge therapies are currently on-going (Table 3).

Our research group is currently conducting two studies that are evaluating the activity of two different rechallenge strategies. The CAVE mCRC-GOIM trial is a multicenter, single-arm phase II study, which is investigating third-line treatment with cetuximab in combination with the anti-PD-L1 MoAb avelumab in 75 *RAS* and *BRAF* WT mCRC patients who have obtained a partial or a CR with an anti-EGFR MoAb-based chemotherapy in first line. Overall survival is the primary end point (EudraCT Number 2017-004392-32). VELO is a multicenter randomized phase II trial which is investigating panitumumab plus trifluridine-tipiracil versus trifluridine-tipiracil as third-line therapy in 112 patients with *RAS* WT mCRC, who received first-line chemotherapy plus an anti-EGFR drug and who had a clinical benefit from initial treatment (complete or PR). The primary end point is PFS (EudraCT Number 2018-001600-12).

Finally, the results of the CAPRI-GOIM study suggest that a potentially relevant clinical benefit could be obtained by continuing EGFR blockade after first-line disease progression in a subset of molecularly selected patients with mCRC whose tumors are highly dependent on EGFR signaling. These results provide evidence that, despite the onset of secondary mechanisms of resistance that could be responsible for disease progression in a large group of patients, a subset of patients has tumors that remain dependent on EGFR activation and, therefore, could be effectively treated with a different chemotherapy regimen while continuing EGFR blockade. Liquid biopsy now allows a non-invasive dynamic evaluation of the complex molecular heterogeneity of mCRC. Serial assessments of ctDNA during treatment with anti-EGFR drugs allow the evaluation of cancer molecular evolution and the identification of the onset of resistance mechanisms. In this regard, our cooperative research group is currently organizing a proof-of-concept prospective clinical study of sequential treatments of mCRC patients with *RAS* and *BRAF* WT tumors (CAPRI 2-GOIM trial). Treatment choices will be defined by liquid biopsy ctDNA testing. As illustrated in Figure 2, two sequence strategies will be evaluated, with the aim of optimizing anti-EGFR therapies according to cancer molecular evolution. A continuum treatment with cetuximab in first line, in second line, and, eventually, in third line (with chemotherapy regimen changes: FOLFIRI, FOLFOX, and irinotecan, respectively) will be done in patients whose tumors remain *RAS* WT by liquid biopsy assessment. On the contrary, if, after first-line progression, a *RAS* mutation is detected, second line will consist of FOLFOX plus bevacizumab. In this case, if at the time of second-line disease progression *RAS* WT is found at liquid biopsy testing, patients will be treated in third line with irinotecan plus cetuximab according to the anti-EGFR rechallenge strategy. In the case of persistence of *RAS* mutations, a standard-of-care third-line treatment (trifluridine/tipiracil or regorafenib) will be provided.

Table 3. Ongoing clinical trial investigating different 'rechallenge' strategies for *RAS* wild-type (WT) metastatic colorectal cancer (mCRC)

Study name	Recruitment status	Treatment	Study population	End point
CAVE mCRC GOIM (EudraCT: 2017- 004392-32)	Active, recruiting	Cetuximab + avelumab	Pretreated patients (>2 lines) with *RAS* WT mCRC who obtained a PR/CR in first line with anti-EGFR treatment and received at least a second line of treatment (last administration EGFR >4 months)	Primary end point: OS Secondary end points: PFS, ORR
VELO (EudraCT: 2018-001600-12)	Active, recruiting	Panitumumab + TAS102 versus TAS102	Third-line treatment in patients with *RAS* WT mCRC who obtained a PR/CR in first line to an anti-EGFR treatment and received a second line of treatment (last administration EGFR >4 months)	Primary end point: PFS Secondary end points: OS, ORR
CHRONOS (NCT03227926)	Active, recruiting	Panitumumab	Third-line treatment in patients with *RAS* WT mCRC who obtained a PR/CR in first line to an anti-EGFR treatment and received a second line of treatment; *RAS* extended mutational load measured at RML; a >50% drop in *RAS* extended mutational load between BML and RML	Primary end point: ORR Secondary end point: PFS, OS
FIRE 4 (NCT02934529)	Active, recruiting	Cetuximab + irinotecan versus standard of care III line	Third-line treatment in patients with *RAS* WT mCRC who were treated with FOLFIRI + cetuximab as first-line therapy and obtained a PR/CR (PFS >6 months) and received a second line of treatment with FOLFOX plus bevacizumab	Primary end point: OS Secondary end point: PFS, ORR, molecular biomarker
A-REPEAT (NCT03311750)	Active, recruiting	Cetuximab + irinotecan/FOLFIRI/FOLFOX	Third-line treatment in patients with *RAS* WT mCRC who obtained a PR/CR in first line to an anti-EGFR treatment and received a second line of treatment (last administration EGFR >2 months)	Primary end point: ORR Secondary end point: ORR by *RAS* status, PFS, OS
REGAIN (NCT02316496)	Completed	Cetuximab + irinotecan	Third-line treatment in patients with *RAS* WT mCRC who were treated with FOLFIRI + cetuximab as first-line therapy and obtained a PR/CR and received a second line of treatment with FOLFOX plus bevacizumab (last administration EGFR >6 weeks)	Primary end point: ORR Secondary end points: DCR, DOR, PFS, OS
NCT03524820	Active, recruiting	Cetuximab	Third-line treatment in patients with *RAS* WT mCRC who obtained a PR/CR in first line to an anti-EGFR treatment and received a second line of treatment (last administration EGFR >3months)	Primary end point: ORR Secondary end point: liquid biopsy biomarker analysis
NCT03087071 (cohort 3)	Active, recruiting	Panitumumab	Patients with *RAS* WT mCRC who received cetuximab-based therapy and had a clinical benefit (PR, CR, PFS >4 months) and progressed to 5-FU, irinotecan, and oxaliplatin with a liquid biopsy negative for EGFR ECD mutation and *RAS*, *BRAF* WT on ctDNA	Primary end point: ORR Secondary end point: PFS, OS

BML, rechallenge mutational load; CR, complete response; ctDNA, circulating DNA; DCR, disease control rate; DOR, duration of response; ECD, extracellular domain; EGFR, epidermal growth factor receptor; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PR, partial response; RML, baseline mutational load.

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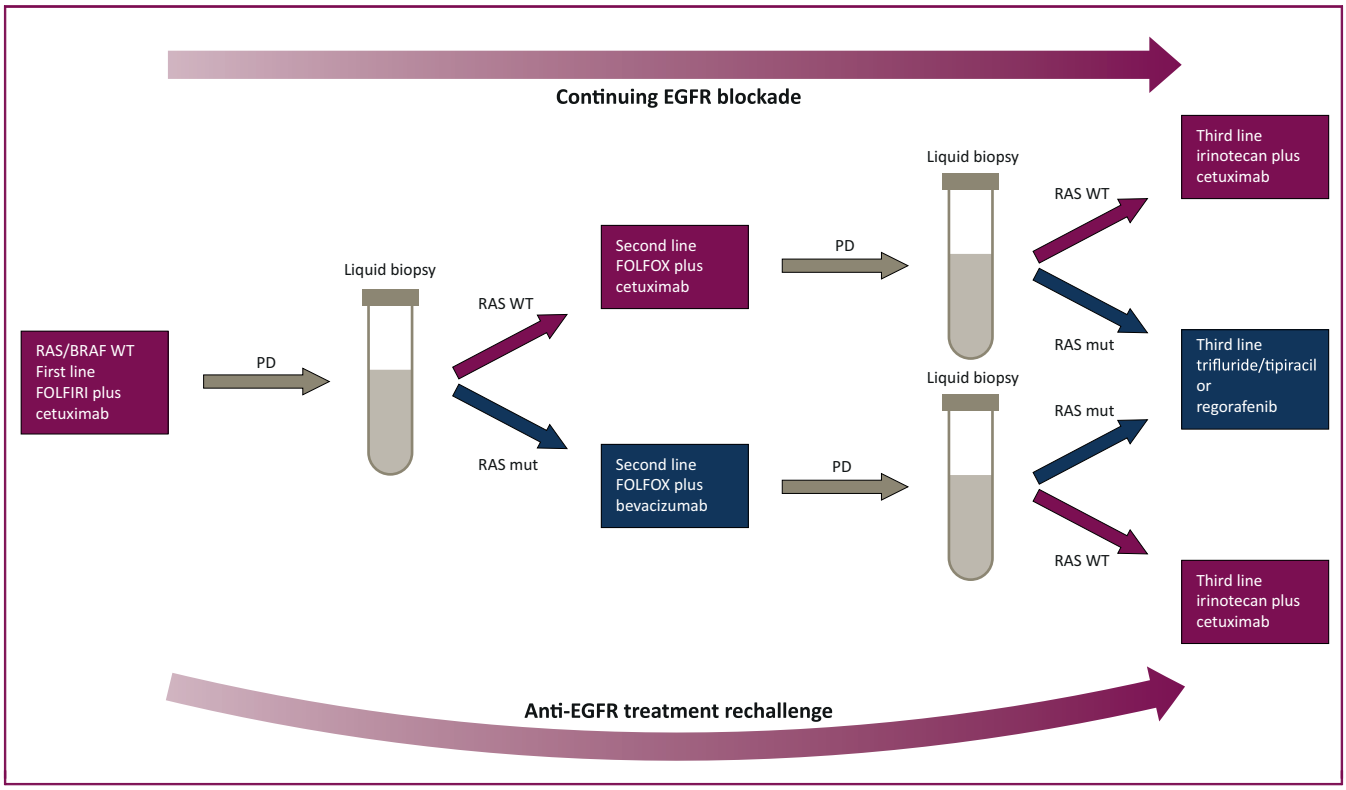

Figure 2. Schematic overview of the Cetuximab After Progression in KRAS wIld-type colorectal cancer patients-Gruppo Oncologico dell' Italia Meridionale (CAPRI 2-GOIM) trial. A proof-of-concept prospective clinical study of sequential treatments of metastatic colorectal cancer (mCRC) patients with *RAS* and *BRAF* wild-type (WT) tumors.

EGFR, epidermal growth factor receptor; PD, progressive disease; RAS mut, *RAS* mutant tumors.

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E.M.: Advisory Board role for Amgen, Astra-Zeneca, Bayer, Merck KgA, Roche, Servier, Sanofi. D.C.: travel grant from Sanofi. T.T.: Amgen, Bayer, Merck KgA, Roche, Sanofi. N.N.: Astra-Zeneca, Bayer, Illumina, Incyte, MSD, Merck KgA, J&J, Quiagen, Roche, Thermofisher. E.M.: Advisory Amgen, Astra-Zeneca, Bayer, Merck KgA, Roche, Servier, Sanofi. T.L.: Roche, Servier, Sanofi. F.D.V.: Amgen, Cellgene, Lilly, Roche.

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