Prognostic and predictive value of RAS gene mutations in colorectal cancer : moving beyond KRAS exon 2

Prognostic and Predictive Value of *RAS* Gene Mutations in Colorectal Cancer: Moving Beyond *KRAS* Exon 2

Running title: Prognostic and Predictive Value of New *RAS* Mutations in Colorectal Cancer

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Abstract

The advent of anti-EGFR therapy resulted in a significant progress in the treatment of metastatic colorectal cancer patients. However, many patients do not respond to this therapy or develop acquired resistance within a few months after the start of treatment. Since 2008, anti-EGFR therapy is restricted to *KRAS* wild-type patients as it has been shown that *KRAS* exon 2-mutated patients do not respond to this therapy. Still, up to 60% of *KRAS* exon 2 wild-type patients show primary resistance to this treatment. Recently, several studies investigating the predictive and prognostic role of *RAS* mutations other than in *KRAS* exon 2 demonstrated that patients with these mutations are not responding to therapy. However, the role of these mutations has long been questioned as The National Comprehensive Cancer Network Guidelines in Oncology and the European Medicines Agency indications had already been changed in order to restrict anti-EGFR therapy to all *RAS* wild-type colorectal cancer patients, while the Food and Drug Administration guidelines remained unchanged. Recently, the Food and Drug Administration guidelines have also been changed, which implies the importance of *RAS* mutations beyond *KRAS* exon 2 in colorectal cancer. In this review, we will discuss the most important studies regarding the predictive and prognostic role of *RAS* mutations other than in *KRAS* exon 2 in order to demonstrate the importance of these *RAS* mutations in patients with metastatic colorectal cancer treated with anti-EGFR therapy.

Key Points

RAS mutations, other than *KRAS* exon 2, are also responsible for resistance to anti-EGFR therapy in patients with metastatic colorectal cancer.

Mutation-analysis on *KRAS* and *NRAS* exon 2 (codon 12 and 13), 3 (codon 59 and 61), and 4 (codon 117 and 146) on tumor tissue of mCRC patients is advised before starting anti-EGFR therapy with detection platforms that are sensitive enough to detect mutations at an allele frequency threshold of ≤5%.

A lot of patients will benefit from extended *RAS* testing as they will no longer be exposed to unnecessary toxicities and costs.

1. Introduction

Colorectal cancer (CRC) is a widespread type of cancer, characterized by high morbidity and mortality. It is the second most commonly diagnosed cancer in females following breast cancer and the third in

males, following lung and prostate cancer. In 2008, 608.700 CRC patients died and 1,2 million new CRC patients were diagnosed worldwide [1].

CRC patients can be treated effectively or can even be cured by complete surgical resection of the primary tumor and the local lymph nodes when the tumor is detected in its early stages. However, surgery has limited efficacy when the tumor has spread to other organs. The 5-year survival rate of patients with CRC detected and treated in its early stages is 90% [2, 3]. This rate drops to 10% in patients with metastatic colorectal cancer (mCRC). At diagnosis, approximately 35% of patients have metastatic disease and during the course of disease, 20% to 50% of stage II or III patients develop metastases [4]. A subset of mCRC patients, with metastases limited to the liver and the lungs, can be cured with surgery, preceded and/or followed by chemotherapy. However, in the vast majority of mCRC patients, surgery is not curative [5].

Targeted therapies, such as cetuximab and panitumumab, have been developed for treatment of mCRC patients. Cetuximab and panitumumab are both monoclonal antibodies directed against the epidermal growth factor receptor (EGFR). Activation of the EGFR pathway in cancer cells has been linked to increased cell proliferation, angiogenesis, metastasis, and decreased apoptosis [6]. Inhibition of this pathway by anti-EGFR therapy has shown survival improvements of mCRC patients in several clinical trials [7-10].

Unfortunately, cetuximab and panitumumab are only effective in approximately 10% to 20% of chemoresistant CRC patients [11-14]. Only a fraction of CRC patients respond to anti-EGFR therapy and almost all responders become resistant after a few months of treatment [12, 13, 15]. In addition, this therapy is costly and associated with potential harmful side effects, such as skin toxicity, neutropenia, fatigue, nausea, vomiting, diarrhea, anorexia, constipation, and hypomagnesia [15, 16]. Therefore, there is a growing need for biomarkers that are able to identify patients who will respond to anti-EGFR therapy. A lot of research has already been performed on this theme in order to improve patient selection. A better selection can avoid unnecessary toxicities and costs in patients that will not respond. In addition, the survival and quality of life of these patients might increase, as other and potentially more effective therapies can be started earlier.

Since 2008, anti-EGFR therapy has been restricted to *KRAS* exon 2 wild-type (WT) patients, as it was shown that *KRAS* exon 2-mutated patients do not respond to this therapy [7-10, 17]. However, up to 60% of these *KRAS* exon 2 WT patients are still resistant to anti-EGFR therapy [18, 19].

Recently, interesting results on anti-EGFR therapy in first-line setting as well as other lines of treatment were published. In these studies, it has been shown that patients with mutations in *KRAS*, other than

exon 2, and *NRAS*, the so-called new *RAS* mutations, did not respond to anti-EGFR therapy [20, 21]. Therefore, the European guidelines and the National Comprehensive Cancer Network Guidelines in Oncology (NCCN) for both cetuximab and panitumumab have been revised to recommend that CRC patients with any *KRAS* or *NRAS* mutation should not be treated with eiter cetuximab or panitumumab [11, 22, 23]. Recently, the US Food and Drug Administration guidelines (FDA guidelines) have been adapted as well which means that all important agencies agree on this theme. In this review, we will summarize and discuss the most important studies that have investigated the significance of new *RAS* mutations in order to understand the real predictive and prognostic value of *RAS* mutations, beyond *KRAS* exon 2.

2. EGFR pathway

The epidermal growth factor receptor is a member of the EGFR family, a group of receptor tyrosine kinases, that mediate cell proliferation, survival, migration, and differentiation [24]. In addition to EGFR, the EGFR family has three other members: ERBB2 (formerly HER2/neu), ERBB3 (formerly HER3), and ERBB4. These receptor tyrosine kinases are transmembrane glycoproteins that exert their enzymatic activity in the cytoplasm. The receptors are inactive as single molecules but form activated homo- or heterodimers when a ligand binds to the extracellular ligand-binding domain of the receptor. In this way, the receptors can translate extracellular signals into intracellular activity [6, 25]. EGFR is frequently overexpressed and activated in colorectal tumors, and therefore a possible target in CRC treatment [16, 26, 27].

EGFR is activated by binding a ligand, such as epidermal growth factor (EGF), transforming growth factor-α, amphiregulin, and epiregulin to its extracellular domain [6]. EGFR exerts its activity via two main pathways, the RAS/RAF/MAPK (mitogen-activated protein kinase) pathway and the (phosphatidylinositol-3-kinase) PI3K/AKT pathway (Figure 1) [28, 29].

In the RAS/RAF/MAPK pathway, receptor dimerization due to ligand-binding leads to the activation of RAS [30]. The RAS family is encoded by three genes: *HRAS*, *NRAS,* and *KRAS* [31]. The RAS proteins are small guanine nucleotide-binding proteins (GTPases) that act as intracellular signal transducers. These proteins transduce extracellular signals to the cytosol and the nucleus leading to the activation of different transcription factors [3, 32]. RAS proteins show a spontaneous dephosphorylation activity and can be present in two different states, the active guanosine triphosphate (GTP)-bound state and the inactive guanosine diphosphate (GDP)-bound state. Switching between both states is supported by regulatory proteins, such as RAS guanine nucleotide exchange factors (GEFs) and RAS GTPase-activating proteins (GAPs), as the intrinsic GTPase-activity of RAS is very slow. The function of GEFs is the activation

of RAS by releasing GDP out of the GDP-RAS complex. This release creates a binding opportunity for GTP, which is present in high concentration in the cytoplasm. The inactivation of RAS on the other hand is supported by GAPs, which stimulate the dephosphorylation of GTP [3, 33].

Active GTP-bound RAS recruits the serine-threonine protein kinase BRAF to the cell membrane which results in the activation of BRAF [30]. Afterwards, BRAF activates mitogen-activated protein kinase kinase (MAP2K or MEK), which induces the activation of MAPK. Finally, MAPK activates transcription factors that are involved in cell proliferation, survival, growth, angiogenesis, and motility [3].

In the PI3K/AKT pathway, ligand-binding to EGFR leads to the activation of PIK3CA, which phosphorylates phosphatidylinositol-4-5-biphospate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). Next, PIP3 activates AKT resulting in the phosphorylation of downstream protein effectors, including mTOR. The pathway is negatively regulated by PTEN, which dephosphorylates PIP3 to PIP2 [34, 35].

3. Current guidelines regarding the use of anti-EGFR therapy

Cetuximab, a mouse chimeric monoclonal antibody and panitumumab, a fully human monoclonal antibody are both directed against the EGFR pathway. These antibodies target the extracellular ligand-binding domain of the receptor, which prevents ligand-binding and receptor dimerization. This results in the inhibition of ligand-induced cell survival, cell growth, cell proliferation, and angiogenesis (Figure 1) [3].

Both monoclonal antibodies have been reported to be effective as single agents and in combination with chemotherapy for mCRC treatment, as shown in different clinical trials by improved progression-free survival (PFS), response rate (RR) or quality of life [12-14, 36]. In 2004 and 2006 respectively, cetuximab and panitumumab were approved for the treatment of mCRC patients [37]. However, it was clear that more research on predictive markers was needed, as many patients did not respond to anti-EGFR therapy.

In 2006, Lièvre et al. were the first to report that CRC patients carrying a mutation in *KRAS* exon 2 showed resistance to anti-EGFR therapy [8]. Since then, rapidly accumulating publications of clinical trials provided compelling evidence that patients with *KRAS* mutations in codon 12 or 13 of exon 2 are resistant to cetuximab and panitumumab [7, 9, 10, 13, 17, 38-45].

Codon 12 and 13 encode two adjacent glycine residues that are located in the proximity of the catalytic site of KRAS. Mutations in this area cause a reduction in the intrinsic GTPase activity, impeding the normal inactivation of KRAS [46]. These mutations result in constitutively activated KRAS and consequently in a constitutive activation of the RAS/RAF/MAPK pathway, even in the presence of cetuximab or panitumumab, or in the absence of a ligand that binds to EGFR [10, 15].

In 2009, the American Society of Clinical Oncology (ASCO) released provisional guidelines recommending restriction of anti-EGFR therapy to patients with *KRAS* exon 2 WT tumors. It was advised to analyze the mutation status of *KRAS* codon 12 and 13 in all candidate patients for anti-EGFR therapy. If a mutation was detected in one of these codons, patients should not receive anti-EGFR therapy [18]. Subsequently, the FDA indications for anti-EGFR therapy were also changed reflecting the same guidelines [47]. During the last five years, there has been some debate about the predictive value of the *KRAS* G13D mutation, as some authors have reported that patients with this mutation might respond to anti-EGFR therapy. However, there are still some doubts about the real predictive value of this mutation (see section 4. Predictive value of the *KRAS* G13D mutation in patients receiving anti-EGFR therapy). Therefore, the guidelines have not been altered and anti-EGFR therapy is still restricted to patients that have no mutations in *KRAS* codon 12 and 13.

Unfortunately, not all *KRAS* exon 2 WT patients respond to anti-EGFR therapy. A lot of research has been performed in order to study the effect of mutations in genes encoding other proteins involved in the EGFR pathway, including *NRAS*, *BRAF*, *PTEN*, and *PIK3CA* [21, 48-50]. Recently, consistent results have been published on the mutation status of *NRAS* and *KRAS* (beyond exon 2). The conclusion of these new data holds that patients with *NRAS* and rare *KRAS* mutations do not respond to anti-EGFR therapy [20, 21]. Due to these findings, the NCCN Guidelines for CRC have been adapted. Currently, the NCCN Guidelines recommend that all mCRC patients should have either primary or metastatic tumor tissue tested for *KRAS* and *NRAS* mutations [11]. Patients harboring any *RAS* mutation cannot be treated with anti-EGFR therapy. In addition, the European Medicines Agency (EMA) and FDA indications for anti-EGFR therapy have been updated to restrict the therapy to all *RAS* WT patients [22, 23, 51, 52]. In the following part, the most important studies, assessing the role of new *RAS* mutations in mCRC patients treated with anti-EGFR therapy will be summarized. In addition, the predictive value of the *KRAS* G13D mutation will be discussed, as there is still some debate about the role of this mutation.

4. Predictive value of the *KRAS* G13D mutation in patients receiving anti-EGFR therapy

Although the mutation-analysis in *KRAS* exon 2 (codon 12 and 13) is performed before starting anti-EGFR therapy today, there is still some discussion about the effect of the glycine to aspartate mutation at codon 13 (G13D) in *KRAS* on clinical outcomes in patients treated with anti-EGFR therapy. Contrasting results have been published on this topic.

Two retrospective analyses have suggested that patients harboring the *KRAS* G13D mutation benefit from anti-EGFR monoclonal antibody therapy in chemotherapy-refractory settings and in first-line

combination therapy with irinotecan or oxaliplatin [53, 54]. De Roock et al. (2010) compared the outcomes of patients with the *KRAS* G13D mutation to those of patients with other *KRAS* mutations among patients with chemotherapy-refractory mCRC treated with cetuximab. Thirty-two patients harbored the *KRAS* G13D mutation. These patients had longer PFS (4.0 vs. 1.9 months, p=0.004) and overall survival (OS) (7.6 vs. 5.7 months, p=0.005) than patients with other *KRAS* mutations, but no significant difference in RR (6.3% vs. 1.6%, p=0.19) was seen. In addition, there was no significant difference in PFS (4.0 vs. 4.2 months, p=0.66) and OS (7.6 vs. 10.1 months, p=0.79) between *KRAS* G13D-mutated and *KRAS* WT patients, but there was a significant difference in RR (6.3% vs. 26.4%, p=0.02). The authors believe that patients with *KRAS* G13D-mutated tumors respond to cetuximab therapy, but with a lower RR than *KRAS* WT patients. In addition, De Roock et al. performed an *in vitro* and *in vivo* mouse model analysis that showed that *KRAS* G13D-mutated colorectal cancer cells were sensitive to cetuximab, similar to *KRAS* WT cells, while *KRAS* G12V-mutated colorectal cancer cells were insensitive. It was concluded that the prolonged PFS and OS of *KRAS* G13D-mutated patients compared to patients with other *KRAS* mutations was due to a delay in progression but not to a real reduction in tumor burden. A possible explanation is that the proliferation of tumor cells is inhibited (cytostatic effect) on EGFR inhibition instead of undergoing apoptosis (cytotoxic effect). However, it should be taken into account that the response rate of *KRAS* G13D-mutated patients to cetuximab monotherapy was very low. None of these patients responded to monotherapy. This might indicate that the responses that were seen in the cetuximab plus chemotherapy group may reflect responsiveness to chemotherapy rather than to cetuximab [53].

Tejpar et al. (2012) investigated the association between the *KRAS* mutation status (WT, G13D, G12V, or other mutations) and PFS, OS, and response in pooled data from the CRYSTAL (Cetuximab Combined With Irinotecan in First-line Therapy for Metastatic Colorectal Cancer) and OPUS (Oxaliplatin and Cetuximab in First-line Treatment of Metastatic Colorectal Cancer) studies. Out of 1378 eligible patients, they found 533 patients (39%) with mutations in *KRAS*. G13D, G12V, and other mutations were found in 83 (16%), 125 (23%), and 325 (61%) patients, respectively. By comparing patients with the *KRAS* G13D mutation to patients harboring all other *KRAS* mutations (including G12V), significant variations were found in tumor response (p=0.005) and PFS (p=0.046). Among all *KRAS* G13D-mutated patients, the addition of cetuximab to chemotherapy compared to chemotherapy alone resulted in a significantly improved PFS (7.4 vs. 6.0 months, p=0.039) and RR (40.5% vs. 22.0%, p=0.042). However, no improvement in OS was seen (15.4 vs. 14.7 months, p=0.68). Contrary to *KRAS* G13D-mutated patients, patients with other *RAS* mutations did not benefit from the addition of cetuximab. Furthermore, the

KRAS G13D-mutated patients that received only chemotherapy had worse RR (22.0% vs. 43.2%, p=0.032) than patients harboring other *RAS* mutations. Tejpar et al. concluded that the addition of cetuximab was beneficial for patients with the *KRAS* G13D mutation in first-line treatment with chemotherapy. The observed positive treatment effect for these patients was caused by the combination of the poor prognosis observed when those patients received only chemotherapy, and the improved outcome under treatment with cetuximab [54]. Other studies performed by Benvenuti et al. (2007) and Molinari et al. (2011) reported partial response in 1 out of 6 and 2 out of 11 *KRAS* G13D-mutated patients, respectively [17, 55].

Next to these clinical trials, some *in vivo* studies have shown similar results. Preclinical studies have suggested that individual *KRAS* codon 12 or 13 alleles show quantitative and/or qualitative differences in transforming capacity and other biologic phenotypes. Specifically, in comparison to *KRAS* codon 13 mutations, *KRAS* codon 12 mutations seem to have greater *in vitro* transforming ability [56-58].

Furthermore, Alamo et al. (2014) recently showed by injecting recombinant clones of the SW48 CRC cell line expressing the *KRAS* G12V mutation or the *KRAS* G13D mutation in mice cecum that *KRAS* G12V mutations have a higher metastatic potential than *KRAS* G13D mutations [59].

Contrary to these results, Peeters et al. (2013) found that patients with mutations in *KRAS* codon 12 or 13 do not benefit from panitumumab therapy. A retrospective analysis of three randomized phase III studies was performed in order to assess the prognostic and predictive impact of these mutations on survival outcomes in 1053 mCRC patients. None of the individual mutant *KRAS* alleles were consistently associated with panitumumab treatment effect on PFS or OS outcomes, across the three studies. However, the collective group of mutant *KRAS* alleles was a negative predictive factor for both PFS and OS in therapies containing panitumumab. The authors concluded that patients with *KRAS* codon 12 or 13 mutations are unlikely to benefit from panitumumab treatment [41].

Furthermore, Schirripa et al. (2014) conducted a prospective trial in order to confirm the aforementioned findings of De Roock et al. and Tejpar et al. and in order to evaluate the clinical relevance of cetuximab in *KRAS* G13D-mutated patients. Therefore, 12 *KRAS* G13D-mutated mCRC patients treated with cetuximab monotherapy were enrolled. If only three or less of these patients would have been progression-free at four months after treatment start, the hypothesis that *KRAS* G13D-mutated patients experience benefit from cetuximab would have been rejected. At four months after treatment start, it was found that three patients (25%) showed disease stabilization and that no RECIST responses were observed. The authors concluded that there was no clinically relevant benefit with

cetuximab monotherapy in *KRAS* G13D-mutated mCRC patients and that these patients should not be treated with cetuximab [60].

In addition, a recent retrospective analysis of 110 patients treated with cetuximab, was performed by Gajate et al. (2012). They reported that patients with the *KRAS* G13D mutation did not benefit from cetuximab treatment. In these patients, a trend towards lower OS was detected compared to *KRAS* WT patients or patients with other *KRAS* mutations [61].

In conclusion, there is still no consensus about the predictive value of the *KRAS* G13D mutation. As long as there is no clear evidence of tumor response in *KRAS* G13D-mutated patients, the guidelines will remain unchanged.

5. Predictive value of new *RAS* mutations in patients receiving anti-EGFR therapy

During the last decade, a lot of research has been performed on the predictive potential of the *RAS* mutation status in mCRC patients treated with anti-EGFR therapy. We report the most important studies according to the monoclonal antibody that has been used in the different clinical trials.

The frequency of all new *RAS* mutations per study are reported in table 1. In addition, the response rates in the different studies and the survival outcomes are shown in table 2 and 3, respectively. In general, all studies support a negative predictive role of new *RAS* mutations in patients treated with anti-EGFR therapy.

5.1 Cetuximab-based therapy

Loupakis et al. (2009) were one of the first to study *KRAS* mutations outside exon 2. They investigated the role of mutations in *KRAS* codon 61 (exon 3) and 146 (exon 4) regarding resistance to cetuximab plus irinotecan in a cohort of patients with no mutations in *KRAS* codon 12 and 13. Seven patients (8%) with a mutation in *KRAS* codon 61 and 1 patient (1%) with a mutation in *KRAS* codon 146 were identified. None of these patients showed response to therapy while 22 of 68 WT *KRAS* patients did respond (p=0.096). In addition, patients with mutations in codon 61 and 146 had a significantly shorter PFS than *KRAS* WT patients (3.8 vs. 5.1 months, p=0.028). However, no significant difference in OS was reported (9.7 vs. 14.7 months, p=0.390), which may be explained by the low amount of mutated cases [62].

De Roock et al. (2010) studied the effect of mutations in *KRAS*, *NRAS*, *BRAF,* and *PIK3CA* on the efficacy of cetuximab in patients with chemotherapy-refractory mCRC treated with chemotherapy plus cetuximab. *KRAS* mutations were detected in 40% of patients (299/747), including 36.3% in exon 2 (codon 12/13), 2.1% in codon 61, and 2.0% in codon 146. They also detected one patient with a *KRAS* mutation in codon 59. Double *KRAS* mutations were detected in four tumors (G12V+G12S, A146T+Q61L,

and twice G12V+A146T). *KRAS*-mutated patients had a significantly lower RR than *KRAS* WT patients (6.7% (17/253) vs. 35.8% (126/352), p<0.0001). In addition, they had a shorter median PFS (12 vs. 24 weeks, p<0.0001) and OS (32 vs. 50 weeks, p<0.0001). Among the evaluable *KRAS*-mutated patients, 13 patients had a mutation in codon 61. Compared to *KRAS* WT patients, a significantly lower RR (p=0.0055) was seen in *KRAS* codon 61-mutated patients, as none of them responded to therapy. In contrast to these findings, there was no significant difference in RR between patients with mutations in *KRAS* codon 146 and *KRAS* WT patients (p=0.34), as 2 out of 11 patients (18.2%) with *KRAS* codon 146 mutations did respond to therapy. Although the number of *KRAS*-mutated patients was low, these results indicate that patients with *KRAS* codon 61 mutations do not respond to anti-EGFR therapy, while patients with codon 146 mutations might still respond. In addition, De Roock et al. detected *NRAS* mutations in 2.6% of *KRAS* WT patients (17/644). Most of them occurred in codon 61, rather than in codon 12 or 13. These patients had a significantly lower RR compared to *NRAS* WT patients (7.7% (1/13) vs. 38.1% (110/289), p=0.013) and a trend towards shorter median PFS (14 vs. 26 weeks, p=0.055) and OS (38 vs. 50 weeks, p=0.051). These results show the inefficacy of cetuximab in *NRAS*-mutated mCRC patients. Since the number of *NRAS*-mutated patients is low, the effect on survival is not clear [48].

Heinemann et al. (2014) recently published the results of the re-analysis of the FIRE-3 study, a randomized, open-label, phase III trial. This study was originally designed in order to compare the objective response in *KRAS* exon 2 WT mCRC patients treated with FOLFIRI plus cetuximab vs. FOLFIRI plus bevacizumab. In this subgroup of patients, there was no significant difference in objective RR (62.0% vs. 58.0%, p=0.18) and median PFS (10.0 vs. 10.3 months, p=0.55) in both treatment arms. However, the median OS was significantly longer in the FOLFIRI plus cetuximab arm vs. the FOLFIRI plus bevacizumab arm (28.7 vs. 25 months, p=0.017). For the re-analysis, the tumor mutation status of *KRAS* exon 3 (codon 61), exon 4 (codon 146) and *NRAS* exon 2 (codon 12 and 13), exon 3 (codon 59 and 61), and exon 4 (codon 117 and 146) was assessed using pyrosequencing in 407 evaluable patients. New *RAS* mutations were detected in 65 patients (16%). Similar results were obtained for the objective response (65% vs. 60%, p=0.32) and PFS (10.4 vs. 10.2 months, p=0.54) when comparing the cetuximab arm to the bevacizumab arm in patients with all *RAS* WT tumors. However, there was a marked advantage in OS for patients treated with FOLFIRI plus cetuximab (33.1 vs. 25.6 months, p=0.011). Several possible explanations exist for the fact that these patients experience an OS benefit without a difference in PFS or RR. First, some bias can be present in response assessments as there was no independent radiological review of the response data. Further, response to therapy might not be captured adequately by RECIST criteria when using different strategies of targeted therapy. Last, although the number of patients that

received second-line therapy was similar in each treatment group, and although the number of patients that crossed over to the alternative anti-VEGF or anti-EGFR therapy was similar, the sequence of targeted agents for patients in the treatment groups was in many cases reversed. This can lead to changes in tumor biology during first-line therapy, which might be related to the difference in OS between both treatment groups. In experimental models, the upregulation of VEGF in association with resistance to cetuximab has been reported [63-65]. Such phenotypic changes could benefit second-line anti-VEGF treatment after first-line cetuximab therapy. In the subgroup of patients that were WT for *KRAS* exon 2 but mutant for other *RAS* mutations, PFS was significantly lower in the cetuximab arm vs. the bevacizumab arm (6.1 vs. 12.2 months, p=0.004), but the difference in RR (38% vs. 58%, p=0.14) and OS (16.4 vs. 20.6 months, p=0.57) did not reach statistical significance. This study confirms that excluding all *RAS*-mutated patients for anti-EGFR therapy will lead to a population that is more likely to benefit from cetuximab and that the addition of cetuximab to *RAS*-mutated patients might have detrimental effects [63].

Recently, a re-analysis of the OPUS study was performed. In this study, mCRC patients were randomized to oxaliplatin, fluorouracil (FU), and leucovorin (FOLFOX4) treatment with or without cetuximab for first-line treatment. It was found that patients with mutations in *KRAS* exon 2 had no benefit from the addition of cetuximab, while the addition of cetuximab significantly improved PFS (8.3 vs. 7.2 months, p=0.0064) and RR (57% vs. 34%, p=0.0027) in *KRAS* exon 2 WT patients. Results for OS were improved but did not reach statistical significance (22.8 vs. 18.5 months, p=0.39) [66]. Now, beads, emulsion, amplification, and magnetics technology (BEAMing) was used in order to screen for mutations in *KRAS* exon 3 and 4, and *NRAS* exon 2, 3, and 4 in patients with *KRAS* exon 2 WT tumors. New *RAS* mutations were found in 26% of patients (31/118). The addition of cetuximab to FOLFOX4 significantly improved RR (57.9% vs. 28.6%, p=0.008) and PFS (12.0 vs. 5.8 months, p=0.062) in all *RAS* WT patients, but there was no significant difference in OS (19.8 vs. 17.8 months, p=0.8). The lack of OS benefit in this group might be explained by the small sample size. Furthermore, the OPUS study is a phase II study where efficacy is a primary endpoint instead of OS. As the number of patients in the group of new *RAS*-mutated patients was low, treatment effect could not be assessed. However, no benefit was seen from the addition of cetuximab to FOLFOX4 in RR (37.0% vs. 50.7%, p=0.087) and PFS (5.6 vs. 7.8 months, p=0.031) in patients with any mutation in *RAS*. There was even a trend for worse outcome in the cetuximab arm (OS: 13.5 vs. 17.8 months, p=0.157) [67].

Van Cutsem et al. (2015) showed updated results of the phase III CRYSTAL trial. This study originally showed that patients with *KRAS* exon 2 WT tumors benefit from the addition of cetuximab to FOLFIRI in

first-line treatment, evidenced by significantly improved PFS (9.9 vs. 8.4 months, p=0.0012), OS (23.5 vs. 20.0 months, p=0.0093), and RR (57.3% vs. 39.7%, p<0.001) [38, 68]. Recently, the *KRAS* exon 2 WT patients were re-analyzed using BEAMing in order to find out whether these patients have mutations in *KRAS* exon 3 (codon 59 and 61) and exon 4 (codon 117 and 146), and in *NRAS* exon 2 (codon 12 and 13), exon 3 (codon 59 and 61), and exon 4 (codon 117 and 146). New *RAS* mutations were detected in 15% of patients (63/430). In the subgroup of all *RAS* WT patients, a significant improvement in RR (66.3% vs. 38.6%, p<0.001), PFS (11.4 vs. 8.4 months, p<0.001), and OS (28.4 vs. 20.2 months, p=0.0024) was seen when cetuximab was added to FOLFIRI compared to FOLFIRI alone. Contrary to these results, no difference between both treatment arms was seen in RR (34.4% vs. 35.5%, p=0.97), PFS (7.2 vs. 6.9 months, p=0.56), and OS (18.2 vs. 20.7 months, p=0.50) in the group of patients with new *RAS* mutations [69, 70].

In sum, many studies indicate that patients with new *RAS* mutations do not benefit from cetuximab, while all *RAS* WT patients are susceptible to cetuximab, evidenced by improved response and survival outcomes. Furthermore, the addition of cetuximab to *RAS*-mutated patients might even be detrimental and consequently should be avoided.

5.2 Panitumumab-based therapy

In 2013, Peeters et al. analyzed 320 samples for mutations in nine genes (*KRAS* (codon 61), *NRAS* (codon 12, 13, and 61), *BRAF*, *PIK3CA*, *PTEN*, *TP53*, *EGFR*, *AKT1*, and *CTNNB1*) using massively parallel multigene sequencing in a randomized phase III study of mCRC in order to evaluate whether these mutations predicted response to panitumumab monotherapy. They reported that 1 out of 6 patients with mutations in *KRAS* codon 61 showed partial response. In addition, *NRAS* mutations were detected in 5% of patients (n=14), 3 of them had mutations in both *KRAS* and *NRAS*. None of the *NRAS*-mutated patients responded to panitumumab, while 17% of *NRAS* WT patients did respond to therapy. Furthermore, among *KRAS* and *NRAS* WT patients (n=138), treatment with panitumumab compared to best supportive care was associated with improved PFS (p<0.001), while panitumumab treatment was no longer associated with improved PFS (p=0.379) in *KRAS* WT and mutant *NRAS* patients (n=11) [21].

Douillard et al. (2013) performed a prospective-retrospective analysis of the treatment effect of *RAS* (*KRAS* and *NRAS*) and *BRAF* mutations on PFS and OS in a randomized phase III study of panitumumab plus FOLFOX4 compared to FOLFOX4 alone in patients with previously untreated mCRC. Therefore, *KRAS* exon 2 WT patients of the PRIME trial (Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy) were re-analyzed. These patients were screened for mutations in *KRAS* exon 3 (codon 61) and exon 4 (codon 117 and 146), *NRAS* exon 2

(codon 12 and 13), exon 3 (codon 61), and exon 4 (codon 117 and 146), and *BRAF* exon 15 (codon 600). Among the 620 patients that were originally categorized as not having mutations in *KRAS* exon 2, 108 patients (17%) harbored new *RAS* mutations. There were 24 patients with mutations in *KRAS* exon 3, 36 *KRAS* exon 4-mutated patients, 22 *NRAS* exon 2-mutated patients, and 26 *NRAS* exon 3-mutated patients. No mutations were found in *NRAS* exon 4. Comparing the panitumumab arm to FOLFOX4 alone in this group of 108 new *RAS*-mutated patients, there seemed to be a negative treatment effect of panitumumab plus FOLFOX4 on PFS (7.3 vs. 8.0 months, p=0.33) and OS (17.1 vs. 17.8 months, p=0.12) but these results did not reach statistical significance. However, in the group of patients with any mutation in *RAS*, the survival outcomes were significantly worse in the panitumumab arm than in the FOLFOX4 arm (PFS: 7.3 vs. 8.7 months, p=0.008; OS: 15.5 vs 18.7 months, p=0.04), clearly showing a detrimental effect of adding panitumumab to first-line FOLFOX4 in patients with mutations in *RAS*. These results were comparable to those in the subgroup of *KRAS* exon 2-mutated patients (PFS: 7.3 vs. 8.8 months, p=0.02; OS: 15.5 vs. 19.2 months, p=0.16). Contrary to these findings, among the *RAS* WT patients (n=512), a significant improvement in both PFS (10.1 vs. 7.9 months, p=0.004) and OS (25.8 vs. 20.2 months, p=0.009) was detected in the panitumumab plus FOLFOX4 arm compared to FOLFOX4 alone. This finding confirms the positive effect of panitumumab in *RAS* WT patients. The authors concluded that all tested *RAS* mutations were negative predictive factors for treating mCRC patients with anti-EGFR therapy, as patients with mutant *RAS* tumors did not benefit from panitumumab treatment. Moreover, the addition of panitumumab to *RAS*-mutated patients was even detrimental [20]. Recently, the final results of PFS and OS from PRIME were published, 30 months after the last patient was enrolled. These results were similar to those described above [71].

André et al. (2013) performed a single-arm multicenter, phase II study in order to evaluate the efficacy and safety of the combination of irinotecan and panitumumab in *KRAS* WT mCRC patients that were heavily pretreated. In addition, this study explored other potential predictive genetic alterations. Therefore, *KRAS* exon 2 WT patients were further screened for mutations in *KRAS* codon 59, 61, 117, and 146, in *NRAS* codon 12, 13, and 61, and in *BRAF* codon 600 using direct sequencing. Among 60 patients, 6 patients had a *KRAS* mutation in codon 12. This finding was surprising, as only patients with a *KRAS* codon 12 and 13 WT tumor were included in the study based on local molecular determination of the mutational status performed in routine diagnosis. Central analysis using an allelic discrimination strategy based on TaqMan mutation-specific probes for *KRAS* screening revealed discrepancies between laboratories. These discrepancies might be due to other testing methodologies or differences in expertise. Beside these *KRAS* codon 12 mutated patients, 4 patients had rare *KRAS* mutations (1 in codon

59 and 3 in codon 61), 5 patients had *NRAS* mutations (1 in codon 12, 1 in codon 13, and 3 in codon 61), and 4 patients had *BRAF* mutations. None of them responded to therapy. Among the original group of *KRAS* exon 2 WT patients, the overall RR was 29.2%, PFS was 5.5 months, and OS was 9.7 months. All parameters seemed to be improved in the subgroup of patients without mutations in *KRAS*, *NRAS*, and *BRAF*, as the RR was 46.3%, PFS was 8.7 months, and OS was 15.8 months. Contrary to these results, a drop in all parameters was seen in the subgroup of mutated patients. RR was 0%, PFS was 1.9 months, and OS was 4.6 months. These results confirm that *RAS*-mutated patients do not respond to anti-EGFR therapy. Moreover, excluding all *RAS*-mutated patients will lead to a population that is more likely to benefit from anti-EGFR therapy [72].

Patterson et al. (2013) published some additional results of the randomized, phase III mCRC study (20020408). In this monotherapy study, the addition of panitumumab was compared to best supportive care. It had been shown that patients with mutations in *KRAS* exon 3 and *NRAS* exon 2 and 3 did not benefit from the addition of panitumumab [21]. These results were expanded in order to study the effect of *KRAS* and *NRAS* mutations in exon 4. Of a total amount of 243 *KRAS* exon 2 WT patients, 9 and 2 patients harbored mutations in *KRAS* and *NRAS* exon 4, respectively. They also discovered one patient with mutations in both *KRAS* and *NRAS* exon 4. Among 95 *KRAS*/*NRAS*-mutated patients in the panitumumab arm, there was 1 mutant *KRAS* exon 4 patient that showed partial response. The overall RR of patients with mutations in *KRAS* or *NRAS* was 1% in the panitumumab arm, while the overall RR was 15% in patients with *KRAS* and *NRAS* WT tumors. In the best supportive care arm, no responses were shown. The authors concluded that patients with mutations in exon 4 of both *KRAS* and *NRAS* did not benefit from panitumumab therapy. Furthermore, patients with mutations in *RAS* exon 4 need to be excluded before starting anti-EGFR therapy despite the fact that these mutations are rare [73].

Schwartzberg et al. (2014) performed an extended *RAS* mutation-analysis on patients of the PEAK study (Panitumumab Efficacy in Combination With mFOLFOX6 against Bevacizumab Plus mFOLFOX6 in mCRC Subjects With Wild-Type *KRAS* tumors). The PEAK study is a phase II, open-label randomized study originally designed to estimate the effect of panitumumab in combination with modified FU, leucovorin, and oxaliplatin (mFOLFOX6) relative to bevacizumab plus mFOLFOX6 as first-line therapy in patients with *KRAS* exon 2 WT mCRC. In the *KRAS* exon 2 WT group, PFS was similar in both arms (10.9 months in the panitumumab arm vs. 10.1 months in the bevacizumab arm, p=0.353), while OS was significantly longer in the panitumumab arm compared to the bevacizumab arm (34.2 vs. 24.3 months, p=0.009). A secondary objective of this study was to assess PFS and OS in patients with *RAS* WT mCRC. Therefore an extended *RAS* analysis of exon 2 (codon 12 and 13), exon 3 (codon 59 and 61), and exon 4 (codon 117

and 146) in both *KRAS* and *NRAS* was performed using real-time quantitative PCR in the central laboratory or other validated assays in local laboratories. New *RAS* mutations were detected in 51 of 221 patients (23%). *KRAS* exon 3 and 4 mutations were detected in 9 and 17 patients and *NRAS* exon 2, 3, and 4 mutations were found in 12, 13 and 0 patients, respectively. In the all *RAS* WT group, PFS was improved in the panitumumab arm compared to the bevacizumab arm (13.0 vs. 9.5 months, p=0.029) and there was a trend for an improvement in OS (41.3 vs. 28.9 months, p=0.058). In patients with *KRAS* exon 2 WT tumors that did have other *RAS* mutations, PFS seemed to be worse in the panitumumab arm compared to the bevacizumab arm, although these results were not statistically different (7.8 vs. 8.9 months, p=0.318). OS, on the other hand, was improved in the panitumumab arm (27.0 vs. 16.6 months, p=0.020). This surprising result for OS can be explained by the relatively high percentage of patients in the panitumumab arm that received subsequent chemotherapy (83%) and anti-VEGF therapy (53%). In sum, this study confirms that only patients with *RAS* WT tumors benefit from anti-EGFR therapy and that these patients have more benefit from anti-EGFR therapy than anti-VEGF therapy in combination with mFOLFOX6 [74].

Peeters et al. (2014) reported new results on the phase III study 20050181. This study was originally designed in order to assess the effect on PFS and OS of panitumumab plus FOLFIRI vs. FOLFIRI alone in second-line treatment of mCRC patients. A significant improvement in PFS (6.7 vs. 4.9 months, p=0.023) and a trend towards improved OS (14.5 vs. 12.5 months, p=0.37) were detected in the panitumumab arm in *KRAS* exon 2 WT patients [7, 75]. Recently, a re-analysis was performed on the group of *KRAS* exon 2 WT patients. Mutations in *KRAS* exon 3 and 4 and in *NRAS* exon 2, 3, and 4 were investigated by bidirectional Sanger sequencing. New *RAS* mutations were found in 18% of the *KRAS* exon 2 WT patients (107/597). In the all *RAS* WT group, better PFS results (6.4 vs. 4.6 months, p=0.007) and a trend towards improved OS (16.2 vs. 13.9 months, p=0.08) were found in the panitumumab plus FOLFIRI arm vs. FOLFIRI alone. No benefit could be detected from the addition of panitumumab to FOLFIRI in *RAS*-mutated patients for PFS (4.8 vs. 4.0 months, p=0.14) and OS (11.8 vs. 11.1 months, p=0.34). The authors found that similar to patients with mutations in *KRAS* exon 2, *RAS*-mutated patients are unlikely to benefit from the addition of panitumumab to FOLFIRI [76].

5.3 Cetuximab- or panitumumab-based therapy

Molinari et al. (2011) evaluated retrospectively the objective tumor responses in 111 evaluable mCRC patients that were treated with cetuximab- or panitumumab-based regimens. *KRAS* codon 12, 13, and 61 were analyzed for mutations. *KRAS* exon 2 mutations were found in 43 cases (39%). Most of them occurred in codon 12 (31 cases, 74%) and 11 cases (26%) showed a mutation in codon 13. One patient

showed *KRAS* mutations in both codon 12 and 13. In addition, *KRAS* exon 3 mutations were detected in 4 cases (4%), including Q61H (2 cases), Q61L, and G60D. The G60D-mutated patient and 2 *KRAS* G13D-mutated patients did respond to cetuximab- or panitumumab-based therapy. Three patients were not evaluable for mutations in exon 3 due to a lack of material. The 3 *KRAS* codon 61-mutated patients showed progression of disease, but these mutations occurred concomitantly with other mutations. Therefore, the predictive value of *KRAS* codon 61 mutations could not be determined [55].

A meta-analysis was recently performed by Sorich et al. (2015) to investigate whether new *RAS* mutations are predictive for resistance to anti-EGFR therapy. The analysis was based on eight randomized controlled trials. New *RAS* mutations were detected in 19.9% of *KRAS* exon 2 WT tumors (n=1911). *KRAS* exon 3 mutations were found in 4.3% of patients, *KRAS* exon 4 in 6.7%, *NRAS* exon 2 in 3.8%, *NRAS* exon 3 in 4.8%, and *NRAS* exon 4 in 0.5% of patients. Moreover, the efficacy of anti-EGFR therapy was significantly inferior for tumors in the new *RAS* mutant subgroup compared to tumors in the all *RAS* WT subgroup regarding PFS (p=0.001), OS (p=0.008), and RR (p=0.001). There was no significant difference detected regarding PFS (p=0.88), OS (p=0.35), or RR (p=0.32) when the new *RAS* mutant subgroup was compared to the *KRAS* exon 2 mutant subgroup. In sum, this meta-analysis also confirmed the previous findings that patients with new *RAS* mutations do not benefit from anti-EGFR therapy [77].

Another study was performed by Schirripa et al. (2015) who analyzed mutations in *KRAS* and *NRAS* (codon 12, 13, and 61 in both genes) in 786 mCRC patients. *KRAS* mutations were detected in 393 patients (50%). Among these patients, 308 patients (78%) had a mutation in codon 12, 70 patients (18%) in codon 13, and 16 patients (4%) in codon 61. *NRAS* mutations were detected in 47 out of 321 *KRAS* and *BRAF* WT (15%) patients or in 6% of the total study population. Mutations in *NRAS* codon 12, 13, and 61 were detected in 14 (30%), 6 (13%), and 27 (57%) patients, respectively. A small subgroup of 8 *NRAS*-mutated patients received anti-EGFR therapy and was evaluated for response to treatment. Five of these patients were treated with cetuximab plus irinotecan, 2 patients received cetuximab monotherapy and 1 patient received panitumumab monotherapy. Seven of these patients did not respond to therapy and showed disease progression, while 1 patient showed initial disease stabilization. After eight weeks, this patient also experienced disease progression. This study confirms the negative predictive effect of *NRAS* mutations on anti-EGFR therapy [78].

6. Prognostic value of new *RAS* mutations

Next to the predictive value of new *RAS* mutations, there was also growing interest in their prognostic value. First, the prognostic role of new *RAS* mutations in patients receiving anti-EGFR therapy will be discussed, followed by the prognostic role of new *RAS* mutations in patients receiving other therapies.

6.1 Anti-EGFR therapy

The survival results of the aforementioned studies that are summarized in table 3, suggest that new *RAS* mutations have a negative prognostic effect in mCRC patients treated with anti-EGFR therapy. Among the group of patients treated with anti-EGFR therapy in each study, an increased PFS and OS is seen in the *RAS* WT group compared to the new *RAS*-mutated group, pointing towards a negative prognostic value of *RAS* mutations in patients treated with anti-EGFR therapy [20, 48, 62, 63, 67, 69, 72, 74, 76]. However, Jonker et al. (2008) compared survival results in mCRC patients treated with cetuximab plus best supportive care or best supportive care alone. They reported that the mutation status of *KRAS* had no influence on survival among patients treated with supportive care alone [45]. In the aforementioned studies, cetuximab treatment was never compared to best supportive care. Therefore, it seems that no hard conclusions can be made on the negative prognostic effect of *RAS* mutations. However, it remains clear that *RAS*-mutated patients should not be treated with anti-EGFR therapy as they experience no survival benefit.

6.2 Other therapies

The role of new *RAS* mutations in CRC patients that were not treated with anti-EGFR therapy has also been investigated. Some studies analyzed only the role of *KRAS* codon 61, while in other studies all new *RAS* mutations were analyzed.

Richman et al. (2009) investigated whether *KRAS* mutations were associated with prognosis in advanced CRC. Therefore, they assessed the mutation status of *KRAS* codon 12, 13 and 61 in patients participating in the MRC FOCUS trial (Medical Research Council Fluorouracil, Oxaliplatin and Irinotecan: Use and Sequencing). Patients were randomly assigned to different sequences of chemotherapy, including first-line FU alone, FU/irinotecan, or FU/oxaliplatin. *KRAS* mutations were detected in 288 (40.5%) and 23 (3.2%) patients in exon 2 (codon 12/13) and codon 61, respectively. Although there was no difference in PFS (p=0.09), patients with *KRAS*-mutated tumors had significantly worse OS than *KRAS* WT patients (p=0.008). The authors concluded that *KRAS* mutations are associated with poor prognosis in advanced CRC [79].

Stremitzer et al. (2012) investigated the influence of the *KRAS* mutation status on recurrence-free survival (RFS) and OS in patients with resectable colorectal cancer liver metastases receiving neo-adjuvant chemotherapy including bevacizumab before liver resection. *KRAS* mutations were found in 25% of these patients (15/60). Among these patients, 8, 4 and 3 patients had a mutation in codon 12, 13, and 61, respectively. When they compared the *KRAS* WT patients to *KRAS*-mutated patients, a significant difference in median RFS (12.4 vs. 5.3 months, p=0.037) and median OS (not reached by time of analysis

(median follow-up 37.5 months) vs. 31.8 months, p=0.011) was found. The authors concluded that *KRAS* mutations had a negative prognostic effect on RFS and OS [80].

Vauthey et al. (2013) studied the prognostic impact of the *RAS* (*KRAS* and *NRAS*) mutation status in 193 patients that had curative resection of colorectal cancer liver metastases after single-regimen chemotherapy. *RAS* mutations were found in 34 patients (18%). Among these patients, 29, 3, and 2 patients harbored mutations at codon 12, 61, and 13, respectively. A significant difference in 3-year overall survival rate was seen between *RAS* WT patients and *RAS*-mutated patients (81% vs. 52.2%, p=0.002). These results indicate that *RAS* mutation status is an independent predictor of OS after resection of colorectal liver metastases. In addition, compared to *RAS* WT patients, *RAS*-mutated patients had a significantly shorter 3-year lung RFS rate (34.6% vs. 59.3%, p<0.001), but there was no significant difference in 3-year liver RFS rate (43.8% vs. 50.2%, p=0.181) [81].

Yaeger et al. (2014) studied the effect of *RAS* mutations on OS in a cohort of 918 mCRC patients. The mutation status of *KRAS* and *NRAS* was assessed in codon 12, 13, 61, 117, and 146. *RAS* mutations were found in 441 cases, including 394 *KRAS* exon 2-mutated cases, 19 *KRAS* exon 3-mutated cases, 10 *KRAS* exon 4-mutated cases, 8 *NRAS* exon 2-mutated cases, and 10 *NRAS* exon 3-mutated cases. A worse OS was associated with the occurrence of these mutations. Among *RAS* WT patients, the median OS was 81 months, while the median OS was only 47 months in *RAS*-mutated patients (p<0.001) [82].

Mise et al. (2014) evaluated whether the mutation status of *RAS* has an impact on survival in 184 patients undergoing liver resection for colorectal liver metastases. They studied mutations in *KRAS* and *NRAS* codon 12, 13, 61, and 146. *RAS* mutations were detected in 38 patients (21%), 32 patients had mutations in *KRAS*, 6 patients had mutations in *NRAS*. The authors found that the 5-year OS rate was significantly higher in *RAS* WT patients compared to *RAS*-mutated patients (61.6% vs. 23.2%, p<0.001) [83].

In the aforementioned study of Schirripa et al. (2015) (see section 5.3 Cetuximab- or panitumumab-based therapy) the prognostic role of *KRAS* and *NRAS* mutation status was studied in a cohort of mCRC patients, most of them were not treated with anti-EGFR therapy. Compared to all *RAS* WT patients who had a median OS of 42.7 months, a significantly shorter OS was seen in patients with mutations in *NRAS* (25.6 months, p=0.0013) and *KRAS* (30.2 months, p=0.0015). These results suggest a potential negative prognostic role of *RAS* mutations in mCRC patients [78].

Most of these studies suggest a negative prognostic role of *RAS* mutations. However, looking at the treatment arms that do not contain anti-EGFR therapy in table 3, survival results of both *RAS*-mutated and *RAS* WT patients are comparable in some studies [67, 69]. In sum, no conclusions can be drawn on

this theme. It seems that the prognostic value of new *RAS* mutations is depending on the treatment that patients are receiving.

7. Considerations regarding *RAS* evaluation

Since *RAS* evaluation is affected by many factors, we will report the most important considerations regarding *RAS* mutation analysis in this part. First, the quality and origin of the starting material is important. DNA is usually isolated from formalin-fixed, paraffin-embedded (FFPE) material of the primary tumor. Therefore, the quality of DNA is often suboptimal due to chemical degradation of DNA in FFPE samples, cold ischemia, or delayed fixation [84, 85]. Ideally, fresh frozen tissue should be used, but unfortunately, frozen material is often lacking.

Next to the quality of DNA, variable handling before DNA extraction can also affect the results of the mutation analysis. Microdissection of the tumor tissue increases the purity of tumoral DNA but this technique is labor intensive and therefore not often performed. In the current FDA-approved assay, microdissection is only recommended for patients where less than 20% of the cells are cancerous. In addition, estimation of the percentage of tumoral cells in the specimen holds substantial interobserver variation [86, 87].

Another important consideration is whether a single biopsy of the primary tumor is sufficient for mutation analysis as intratumor heterogeneity has been shown [88]. A possible solution are liquid biopsies, consisting of circulating cell-free DNA and circulating tumor cells present in the blood of cancer patients. It has been reported that these liquid biopsies reflect the total systemic tumor burden [89] and it is possible to detect mutations in these liquid biopsies of patients with advanced cancer [90-95]. However, further research needs to be performed before liquid biopsies can be implemented in the clinic.

In addition, the used detection platforms may also affect the results of *RAS* mutation analysis. Sanger sequencing has been used for many years, but this technique has a sensitivity of only 20% [4, 96]. Currently, the only FDA-approved test for analyzing mutations in *KRAS* codon 12 or 13 uses the Scorpion Amplified Refractory Mutation System (ARMS) polymerase chain reaction methodology with a reported sensitivity of approximately 1% to 5%. More sensitive technologies, such as digital PCR, BEAMing, and many next generation sequencing platforms reach a sensitivity up to 0.1% [1, 87, 97]. There is a growing need for these sensitive platforms in order to serially monitor tumor burden and the emergence of acquired resistance mutations in liquid biopsies. However, it remains to be questioned which methodologies should be used for mutation analysis. The existing commercial *KRAS* mutation kits cannot be used for mutation analysis of *KRAS* exon 4 and *NRAS* exon 2, 3, and 4. Adding these exons to existing

kits will take a lot of time and money. Therefore, it is likely that targeted panels, sequenced through next generation sequencing will soon replace the traditional Sanger sequencing and allele-specific methods that are clinically used at this moment. In future, these panels can be quickly adapted when new negative predictive mutations are detected.

Another important consideration is the lower limit of detection of mutation that has a clinical relevance in the treatment of patients with anti-EGFR therapy. In other words, how many mutant alleles have to be present in order to predict unresponsiveness to anti-EGFR therapy? Laurent-Puig et al. (2015) used picodroplet digital procedures to perform a mutation analysis on the tumor tissue of CRC patients. They found an inverse correlation between the proportion of mutated DNA and the frequency of response to anti-EGFR therapy. However, patients with less than 1% of mutant *KRAS* alleles did respond to anti-EGFR therapy and had similar PFS and OS results as patients with wild-type *KRAS* tumors [4]. Therefore, it seems to be needless to exclude these patients for anti-EGFR therapy. However, large prospective studies are needed to perform further research on this theme and to translate these findings in clinical settings. At this moment, the predictive value of low frequency *RAS* mutations remains unclear. It is possible that these patients first respond to therapy and develop resistance after a few months of treatment. The existence of acquired resistance has already been shown by Diaz et al. (2012) who showed that 38% of patients that were initially classified as having wild-type tumors, developed detectable *KRAS* mutations in serum during or after treatment [5]. Acquired resistance can be caused by a few cancer cells harboring *RAS* mutations that expand during treatment, while wild-type cells are dying. On the other hand, acquired resistance can also be caused by new *RAS* mutations that arise during treatment [5, 37].

Finally, currently, there is no recommendation on the appropriate timing of *RAS* mutation determination, except that the mutation analysis needs to be done before the start of anti-EGFR therapy. In the NCCN Guidelines it is also recommended not to perform *KRAS/NRAS* genotyping at the early stage I, II or III disease, as anti-EGFR agents are only used in the treatment of metastatic colorectal cancer [11]. We suggest to perform *RAS* mutation analysis on fresh tumor tissue, preferably from metastatic origin, obtained just before the start of anti-EGFR treatment. In this way, analyzing old tumor tissue of which the tumor characteristics have possibly been changed over time due to chemotherapy or evolution of the tumor is avoided and a real-time reflection of the metastasis is obtained.

8. Discussion and conclusion

Anti-EGFR therapy significantly improves the clinical outcomes of patients with mCRC. The main drawback associated with this therapy is the occurrence of resistance. Many patients are resistant to this

therapy and almost all patients develop resistance within a few months after treatment start. In addition, anti-EGFR therapy is also associated with high costs and harmful side effects [3]. Despite the restriction of this therapy to patients that are WT for *KRAS* exon 2, up to 60% of these patients do not respond to cetuximab or panitumumab [18, 19]. Improving patient selection might lead to better survival outcomes and quality of life for these patients.

In the last five years, a lot of research has been performed on the so-called new *RAS* mutations, as they might predict responsiveness to anti-EGFR therapy. The NCCN and EMA Guidelines have been updated recommending the restriction of anti-EGFR therapy to all *RAS* WT patients [11, 22, 23]. Recently, the FDA guidelines have also been changed reflecting the predictive role of all *RAS* mutations [47, 51, 52].

One aspect that impedes the study of the different *RAS* mutations is their low frequency. The majority of *RAS* mutations occur in codon 12 (23.9%) and 13 (3.6%) in exon 2 of *KRAS*, but the restriction of anti-EGFR therapy to *KRAS* exon 2 WT mCRC patients has already been recommended since 2008. Less common mutations occur in codon 12 (2.1%) of exon 2 and codon 61 (3.6%) of exon 3 of *NRAS* and in codon 61 (1.4%) of exon 3 and codon 146 (3.3%) of exon 4 of *KRAS* [98]. However, regarding the aforementioned studies, new *RAS* mutations were detected in 15 to 26% of patients (Table 1), which accounts for a considerable number of mCRC patients. Here, we looked only at the studies in which the mutation status of *KRAS* exon 3 and 4 and *NRAS* exon 2, 3, and 4 were all analyzed in a *KRAS* exon 2 WT population, as the amount of new *RAS*-mutated patients in those populations are clinically important.

Regarding response to anti-EGFR therapy in different studies, it has been shown that the RR is lower in all *RAS*-mutated patients compared to all *RAS* WT patients (Table 2). Some studies reported no response in all analyzed *RAS*-mutated patients [62, 72] or in all *NRAS*-mutated patients [21]. Other studies reported sporadic cases with a mutation in *RAS* that showed partial response [21, 48, 55, 73, 78].

The survival results in all studies generally showed a difference in PFS and OS in patients with new *RAS* mutations compared to all *RAS* WT patients that were treated with anti-EGFR therapy (Table 3). In the re-analysis of the PRIME trial, the addition of panitumumab to FOLFOX4 seemed to be detrimental for *RAS*-mutated patients [20]. This was also the case in the OPUS trial where cetuximab was added to FOLFOX4 [67]. Furthermore, in the PEAK and FIRE-3 trial, the addition of anti-EGFR therapy to chemotherapy in *RAS*-mutated patients generally resulted in worse outcome than the addition of bevacizumab to chemotherapy [63, 74].

The results of all these studies indicate that anti-EGFR therapy should be restricted to all *RAS* WT mCRC patients. Although the frequency of different mutations in the population is minor, the frequency of all *RAS* mutations together reaches 15% to 26% in a subgroup of patients that have no mutations in *KRAS*

exon 2. Most of these patients showed no response to anti-EGFR therapy and had poor survival results. Although there was a very small subgroup of patients that showed response to treatment, none of these patients should be treated with anti-EGFR therapy in order to improve treatment possibilities in the vast majority of patients with new *RAS* mutations. This is a relatively large group of patients which are exposed to unnecessary toxicities and costs. If these patients can immediately be treated with another and possibly more effective therapy, the survival and quality of life of many mCRC patients might increase.

In conclusion, it is advised to perform a mutation analysis on *KRAS* and *NRAS* codons 12 and 13 (exon 2), 59 and 61 (exon 3), and 117 and 146 (exon 4) on the tissue of all mCRC patients before treatment with anti-EGFR therapy. The used mutation detection platform is of minor importance, as long as there is enough expertise and the methodology is sensitive enough to detect mutations at an allele frequency threshold of ≤5%. We advise to work with this threshold until the predictive value of low frequency *RAS* mutations is clear. We believe that a lot of patients will benefit from the expanded mutation analysis, as patients with new *RAS* mutations will not be exposed to unnecessary toxicities and costs. However, it is clear that even with extended *RAS* testing, some patients will still not respond to anti-EGFR therapy. For those patients, further research is necessary in order to identify other biomarkers.

Compliance with Ethical Standards:

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685 Table 1. Overview of all new *RAS* mutations and their frequency detected in different studies

Study	Population	New *RAS* mutations analyzed Gene, codon (or exon)	No.	Mutation %	Total %
Loupakis et al. [62]	*KRAS* exon 2 WT	*KRAS*, 61	7	8	9
		KRAS, 146	1	1	
De Roock et al. [48]	Unselected	*KRAS*, 59	1	0,001	
		KRAS, 61	16	2,1	*KRAS*: 4,101
		KRAS, 146	15	2	
		NRAS, 12, 13, 61	17	2,6	*NRAS*: 2,6
FIRE-3 [63]	*KRAS* exon 2 WT	*KRAS*, 61, 146; *NRAS*, 12, 13, 59, 61, 117, 146	65	16	16
OPUS [67]	*KRAS* exon 2 WT	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3, 4	31	26	26
CRYSTAL [70]	*KRAS* exon 2 WT	*KRAS*, 59, 61, 117, 146; *NRAS*, 12, 13, 59, 61, 117, 146	63	15	15
Peeters et al. [21]	Unselected	*KRAS*, 61	7	2,5	7,5
		NRAS, 12, 13, 61	14	5	
PRIME [20]	*KRAS* exon 2 WT	*KRAS*, 61	24	3,7	16,8
		KRAS, 117, 146	36	5,6	
		NRAS, 12, 13	22	3,4	
		NRAS, 61	26	4,1	
		NRAS, 117, 146	0	0	
André et al. [72]	*KRAS* exon 2 WT	*KRAS*, 59	1	1,7	15,1
		KRAS, 61	3	5	
		KRAS, 117, 146	0	0	
		NRAS, 12	1	1,7	
		NRAS, 13	1	1,7	
		NRAS, 61	3	5	
20020408 [73]	*KRAS* exon 2 WT	*KRAS* exon 4	9	3,7	4,5
		NRAS exon 4	2	0,8	
PEAK [74]	*KRAS* exon 2 WT	*KRAS*, 59, 61	9	4,1	23,1
		KRAS, 117, 146	17	7,7	
		NRAS, 12, 13	12	5,4	
		NRAS, 59, 61	13	5,9	
		NRAS, 117, 146	0	0	
20050181 [76]	*KRAS* exon 2 WT	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3, 4	107	18	18
Molinari et al. [55]	Unselected	*KRAS*, 60	1	1	4
		KRAS, 61	3	3	
Sorich et al. [77]	*KRAS* exon 2 WT	*KRAS*, 59, 61	NA	4,3	19,9
		KRAS, 117, 146	NA	6,7	
		NRAS, 12, 13	NA	3,8	
		NRAS, 59, 61	NA	4,8	
		NRAS, 117, 146	NA	0,5	
Schirripa et al. [78]	Unselected	*KRAS*, 61	16	2	7,9
		NRAS, 12	14	1,8	
		NRAS, 13	6	0,7	
		NRAS, 61	27	3,4	

686 No.: Number of patients harboring the specified mutation, Mutation %: Percentage of patients harboring the specified mutation, Total %:
687 Percentage of patients harboring one of the new *RAS* mutations per study, WT: wild-type, NA: data not available

Table 2. Response rates of evaluable new *RAS*-mutated patients in different studies

Study	***RAS* mutations analyzed** Gene, codon (or exon)	Treatment (line of treatment)	***RAS* MT** No.	***RAS* WT** No.	***RAS* MT** RR (%)	***RAS* WT** RR (%)	Remarks
Loupakis et al. [62]	*KRAS*, 61, 146	cmab + irinotecan (advanced lines)	8	68	0	32,4	p=0,096
De Roock et al. [48]	*KRAS*, 12, 13, 59, 61, 146	cmab + CT (advanced lines)	253	352	6,7	35,8*	p<0,0001
	KRAS, 61		13	352	0	35,8*	
	KRAS, 146		11	352	18,2	35,8*	PR in 2 *KRAS* 146 MT patients
	NRAS, 12, 13, 61		13	289	7,7	38,1	p=0,013; R in 1 *NRAS* MT patient
FIRE-3 [63]	*KRAS*, 61, 146; *NRAS*, 12, 13, 61, 117, 146	FOLFIRI + bmab	31	171	58	60	
		FOLFIRI + cmab (first-line)	34	171	38	65	
OPUS [67]	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3, 4	FOLFOX4	16	49	43,8	28,6	
		FOLFOX4 + cmab (first-line)	15	38	53,3	57,9	
CRYSTAL [70]	*KRAS*, 59, 61, 117, 146; *NRAS*, 12, 13, 59, 61, 117, 146	FOLFIRI	31	189	35,5	38,6	
		FOLFIRI + cmab (first-line)	32	178	34,4	66,3	
Peeters et al. [21]	*KRAS*, 12, 13, 61	pmab MT (advanced lines)	109	126	1	17	PR in 1/6 codon 61 MT patients
	NRAS, 12, 13, 61		9	126	0	17	
André et al. [72]	*KRAS*, 12, 59, 61; NRAS, 12, 13, 61	irinotecan + pmab (third-line)	15	45	0	46,3	
20020408 [73]	*KRAS* and *NRAS*, exon 2, 3, 4	pmab (advanced lines)	95	72	1	15	PR in 1 *KRAS* exon 4 MT patient
Molinari et al. [55]	*KRAS*, 12, 13	cmab- or pmab-based regimen (first- or advances lines)	43	64	4,7	28,1	PR in 2 *KRAS* G13D MT patients
	KRAS, 60		1	64	100	28,1	
	KRAS, 61		3	64	0	28,1	also other mutations present
Schirripa et al. [78]	*NRAS*, 12, 13, 61	cmab + irinotecan, or cmab MT, or pmab MT (advanced lines)	8	NA	12,5	NA	PR in 1 patient

WT: wild-type; MT: mutated; NA: data not available; cmab: cetuximab; pmab: panitumumab; bmab: bevacizumab; MT: monotherapy; RR: response rate; PR: partial response; R: response; CT: chemotherapy; No.: Number of patients harboring the specified mutation(s); * compared to *KRAS* exon 2 WT patients instead of all *RAS* WT patients

Table 3. Survival outcomes of new *RAS*-mutated patients and all *RAS* WT patients in different studies

Study	new *RAS* mutations analyzed Gene, codon (or exon)	Treatment (line of treatment)	New *RAS* MT No.	*RAS* WT No.	New *RAS* MT PFS (months)	New *RAS* MT OS (months)	all *RAS* WT PFS (months)	all *RAS* WT OS (months)
Loupakis et al. [62]	*KRAS*, 61, 146	Irinotecan + cmab	8	68	3,8	9,7	5,1	14,7
		(advanced lines)			*p(PFS)=0,028; p(OS)=0,390*			
De Roock et al. [48]	*KRAS*, 12, 13, 59, 61, 146	CT + cmab	253	352	3	8	6	12,5
					p(PFS and OS)<0,0001			
	NRAS, 12, 13, 61	CT + cmab	13	289	3,5	9,5	6,5	12,5
		(advanced lines)			*p(PFS)=0,055; p(OS)=0,051*			
FIRE-3 [63]	*KRAS*, 61, 146; *NRAS*, 12, 13, 61, 117, 146	FOLFIRI + bmab	31	171	12,2	20,6	10,2	25,6
		FOLFIRI + cmab	34	171	6,1	16,4	10,4	33,1
		(first-line)			*p=0,004*	*p=0,57*	*p=0,54*	*p=0,011*
OPUS [67]	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3, 4	FOLFOX4	16	49	7,4	17,8	5,8	17,8
		FOLFOX4 + cmab	15	38	7,5	18,4	12	19,8
		(first-line)			*p=0,60*	*p=0,86*	*p=0,062*	*p=0,80*
CRYSTAL [70]	*KRAS*, 59, 61, 117, 146; *NRAS*, 12, 13, 59, 61, 117, 146	FOLFIRI	31	189	6,9	20,7	8,4	20,2
		FOLFIRI + cmab	32	178	7,2	18,2	11,4	28,4
		(first-line)			p=0,56	p=0,5	p=0,0002	p=0,0024
PRIME [20]	*KRAS*, 61, 117, 146; *NRAS*, 12, 13, 61	FOLFOX4	57	253	8	17,8	7,9	20,2
		FOLFOX4 + pmab	51	259	7,3	17,1	10,1	26
		(first-line)			*p=0,33*	*p=0,12*	*p=0,004*	*p=0,04*
André et al. [72]	*KRAS*, 12, 59, 61; *NRAS*, 12, 13, 61	irinotecan + pmab	15	45	1,9	4,6	8,7	15,8
		(third-line)			*NA*	*NA*	*NA*	*NA*
PEAK [74]	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3	mFOLFOX6 + bmab	27	82	8,9	16,6	9,5	28,9
		mFOLFOX6 + pmab	24	88	7,8	27	13	41,3
		(first-line)			*p=0,318*	*p=0,020*	*p=0,029*	*p=0,058*
20050181 [76]	*KRAS*, exon 3, 4; *NRAS*, exon 2, 3, 4	FOLFIRI	294	213	* 4,0	* 11,1	4,6	13,9
		FOLFIRI + pmab	299	208	* 4,8	* 11,8	6,4	16,2
		(second-line)			*p=0,14*	*p=0,34*	*p=0,007*	*p=0,08*

NA: data not available; WT: wild-type; MT: mutated; PFS: progression-free survival; OS: overall survival; cmab: cetuximab; pmab: panitumumab, bmab: bevacuzimab; CT: chemotherapy; No.: Number of patients harboring the specified mutation(s); *: all *RAS*-mutated patients instead of only new-*RAS* mutated patients

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Figure 1. EGFR pathway. 1) Normal state, the EGFR pathway has been presented upon binding of a ligand to EGFR; 2) the EGFR pathway is blocked by anti-EGFR therapy, which inhibits cell proliferation and survival; 3) a mutation in *RAS* (star) causes the constitutive activation of the EGFR pathway resulting in cell proliferation and survival, despite the blocking of EGFR by anti-EGFR therapy.