Original Study

Long-term Survival Update and Extended RAS Mutational Analysis of the CAIRO2 Trial: Addition of Cetuximab to CAPOX/Bevacizumab in Metastatic Colorectal Cancer

Sanne ten Hoorn,[1,2,3] Linda Mol,[4] Dirkje W. Sommeijer,[1,5,6] Lisanne Nijman,[1,2,3] Tom van den Bosch,[1,2,3,7] Tim R. de Back,[1,2,3] Bauke Ylstra,[8] Erik van Dijk,[8] Carel J.M. van Noesel,[9] Roy J. Reinten,[9] Iris D. Nagtegaal,[10] Miriam Koopman,[11] Cornelis J.A. Punt,[12,*] Louis Vermeulen[1,2,3,*]

Abstract

We assessed whether anti-EGFR addition to anti-VEGF therapy could still be an treatment option for a subgroup of patients with metastatic colorectal cancer. Retrospective updated survival and mutational analysis were performed (CAIRO2 trial, n=736). No benefit of anti-EGFR addition was observed within the subgroup, however, compared to the original trial an increase of 6.5 months overall survival was seen.

Background: Here we present updated survival of the CAIRO2 trial and assessed whether the addition of anti-EGFR to anti-VEGF therapy could still be an effective treatment option for patients with extended *RAS*/*BRAF* wildtype and left-sided metastatic colorectal cancer (mCRC). **Materials and Methods:** Retrospective updated survival and extended *RAS* and *BRAF* V600E mutational analysis were performed in the CAIRO2 trial, a multicenter, randomized phase III trial on the effect of adding cetuximab to a combination of capecitabine, oxaliplatin (CAPOX), and bevacizumab in mCRC. **Results:** Updated survival analysis confirmed that the addition of cetuximab did not provide a benefit on either progression free (PFS) or overall survival (OS) in the intention-to-treat population. With the extended mutational analyses 31 *KRAS*, 31 *NRAS* and 12 *BRAF* V600E additional mutations were found. No benefit of the addition of cetuximab was observed within the extended wildtype group, even when selecting only left-sided tumors (PFS HR 0.96, p = 0.7775). However, compared to the original trial an increase of 6.5 months was seen for patients with both extended wildtype and left-sided tumors (median OS 28.6 months). **Conclusion:** Adding cetuximab to CAPOX and bevacizumab does not provide clinical benefit in patients with mCRC, even in the extended wildtype group with left-sided tumors. However, in the extended wildtype group we did observe clinically relevant higher survival compared to the initial trial report, indicating that it is important to analyze a broader panel of *RAS* and *BRAF* variants using more recent sequencing techniques when assessing survival benefit after anti-EGFR therapy.

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[1]Amsterdam UMC location University of Amsterdam, Center for Experimental and Molecular Medicine, Laboratory for Experimental Oncology and Radiobiology, Amsterdam, The Netherlands
[2]Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam, The Netherlands
[3]Oncode Institute, Utrecht, The Netherlands
[4]Clinical Research Department, Netherlands Comprehensive Cancer Center (IKNL), Nijmegen, The Netherlands
[5]Amsterdam UMC location University of Amsterdam, Department of Medical Oncology, Amsterdam, The Netherlands
[6]Flevohospital, Department of Internal Medicine, Almere, The Netherlands
[7]Amsterdam UMC location University of Amsterdam, Amsterdam Gastroenterology & Metabolism, Amsterdam, The Netherlands
[8]Amsterdam UMC location Vrije Universiteit Amsterdam, Department of Pathology, Cancer Center Amsterdam, Amsterdam, The Netherlands
[9]Amsterdam UMC location University of Amsterdam, Department of Pathology, Amsterdam, The Netherlands
[10]Radboud Institute for Molecular Life Sciences, Department of Pathology, Radboud University Medical Center, Nijmegen, The Netherlands
[11]Department of Medical Oncology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[12]Department of Epidemiology, Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht University, Utrecht, The Netherlands

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Address for correspondence. Louis Vermeulen MD, PhD, Amsterdam University Medical Centers – University of Amsterdam, Center for Experimental Molecular Medicine (CEMM), Lab. for Experimental Oncology and Radiobiology (LEXOR), Meibergdreef 9, Amsterdam, The Netherlands, 1105
E-mail contact: l.vermeulen@amsterdamumc.nl

* Shared senior and corresponding authorship

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Introduction

For patients with mismatch repair proficient (pMMR) metastatic colorectal cancer (mCRC), standard first-line treatment is cytotoxic chemotherapy combined with targeted agents directed against either the epidermal growth factor receptor (anti-EGFR) or against the vascular endothelial growth factor (anti-VEGF). Even though improvement in survival has been shown with these regimens in clinical trials, this effect is less clear in daily practice and 5-year survival rates remain poor.[1-3] CRC is a heterogeneous disease and inhibition of a single signal-transduction pathway is unlikely to provide the most beneficial treatment results.[4] Therefore, a combination of targeted agents might be a effective strategy.

Although preclinical and early clinical studies have suggested that the dual EGFR/VEGF inhibition will increase antitumor activity,[5-8] this was not confirmed in the CAIRO2 study. This randomized phase III study of capecitabine, oxaliplatin, and anti-VEGF with or without anti-EGFR therapy (cetuximab) in mCRC resulted in an unexpected significantly shorter progression-free survival (PFS) and inferior quality of life in the intention-to-treat population (ITT).[9] Similar results were obtained in two other trials with comparable design.[10,11] Together these results have ruled out the combined use of anti-VEGF and anti-EGFR monoclonal antibodies with chemotherapy as treatment option for mCRC. However, it has not been explored wether this combination may be benificial for a specific subgroup of patients with extended mutational characterization.

It is known that anti-EGFR is only effective in a subgroup of mCRC patients. Activating *KRAS, NRAS* and *BRAF* V600E mutations have been associated with primary resistance against anti-EGFR and even an inverse outcome following anti-EGFR treatment.[12,13] In the CAIRO2 trial, the worst PFS was indeed seen in cetuximab treated patients with a *KRAS* (exon 2, codon 12 or 13) mutation. However, we currently know that mutational variants other than *KRAS* exon 2 (codon 12 or 13) mutations and sideness are also predictive for resistance to anti-EGFR treatment.[14] We hypothesized that we could identify a subgroup for whom the combined use of anti-VEGF and anti-EGFR will be effective. We therefore assessed the effect of the addition of cetuximab in the CAIRO2 trial in the extended *RAS* and *BRAF* V600E wildtype group.

Methods

Study Design and Participants

The CAIRO2 study was an open label, multicenter, randomized phase III trial conducted in 79 centers in the Netherlands (ClinicalTrials.gov identifier: NCT00208546).[9] Patients with previously untreated mCRC were randomly assigned to receive treatment with capecitabine, oxaliplatin and bevacizumab with (CBC group) or without the addition of cetuximab (CB group). Extensive information on inclusion criteria, randomization process and treatment schedules has been described previously.[9] Between June 2005 and December 2006 755 patients were randomized and 736 were eligible for the ITT population (368 in each treatment group).

The primary endpoint of the CAIRO2 trial was PFS. Secondary endpoints were overall survival (OS), tumor response (RECIST), duration of response, quality of life and safety. From patients of whom resected tumor tissue was available DNA was extracted for *KRAS* (exon 2) mutational analysis. Patients were not selected for *KRAS* wildtype status, as data on the predictive value of *KRAS* mutation status for the outcome of anti-EGFR therapy were not available at the start of the CAIRO2 study.

The survival data were updated using the NKR (Dutch cancer registry). Updated survival data was obtained from all 736 patients of the ITT population in June 2020.

The CAIRO2 trial was approved by the national ethics committee on research involving human subjects Arnhem–Nijmegen and conducted in agreement with the declaration of Helsinki. Written informed consent was obtained from all patients.

Mutation Analysis of KRAS, NRAS, *and* BRAF *V600E*

In the primary analysis of the CAIRO2 study *KRAS* (exon 2) and *BRAF* V600E mutation analysis was performed on primary or metastatic samples by a pyrosequencing approach for 528 (*KRAS*) and 520 (*BRAF* V600E) patients from which formalin-fixed paraffin-embedded (FFPE) tissue was available.[9,15]

For the extended mutational analysis of the current study all patients with available primary tumor tissue and previously reported *KRAS* exon 2 wildtype and *BRAF* V600E wildtype tumors were included. *KRAS* (exon 2,3,4), *NRAS* (exon 2,3,4) and BRAF (exon 15) were analyzed (Supplementary Table 1). Two different methods were used for mutational analysis. For 58 samples whole exome sequencing (WES) data was available,[16] the remaining 215 wildtype samples with available DNA were analyzed using a next generation sequencing (NGS) custom panel.

The methods for the WES are described in Smeets et al. 2018.[16] Briefly, DNA libraries were prepared using the KAPA library preparation kit (KAPA Biosystems), according to the manufacturer's instructions. Libraries were quantified using the Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit. After confirmation of successful library construction, whole exome enrichment was performed using the SeqCapV3 exome enrichment kit (Roche) following the manufacturer's instructions. The enriched libraries were sequenced on a HiSeq2500, paired end 100bp. Target coverage was 60x. For those samples where median coverage of the published data was below 60x, additional sequencing was performed on a HiSeq4000, paired end 150 bp. All sequencing data was reanalyzed to specifically call *KRAS, NRAS* and *BRAF* mutations in the same regions as the custom NGS panel (Supplementary Table 1). Sequencing reads were aligned to the human reference genome hg19 using bwa mem version 0.7.10. Picard tools version 1.111 MarkDuplicates was used for duplicate marking. Variant calling was performed using Mutect2 in tumor-only mode and SnpEff version 4.3t was used for gene annotation. Variants were then manually inspected for presence of *KRAS* (exon 2, 3 and 4), *NRAS* (exon 2, 3 and 4) or *BRAF* V600E mutations in the same regions as the custom targeted NGS panel.

NGS was performed using a custom targeted NGS amplicon panel from the pathology department of *KRAS* (exon 2, 3 and 4), *NRAS* (exon 2, 3 and 4) and *BRAF* V600E (Supplementary Table 1). The DNA libraries were prepared using the Ion AmpliSeq Library Kit 2.0 according to the manufacturer's instructions. Libraries were quantified using the Qubit 3.0 Fluorometer. Tumor DNA libraries were sequenced on an Ion 530 chip in the Ion GeneStudio S5

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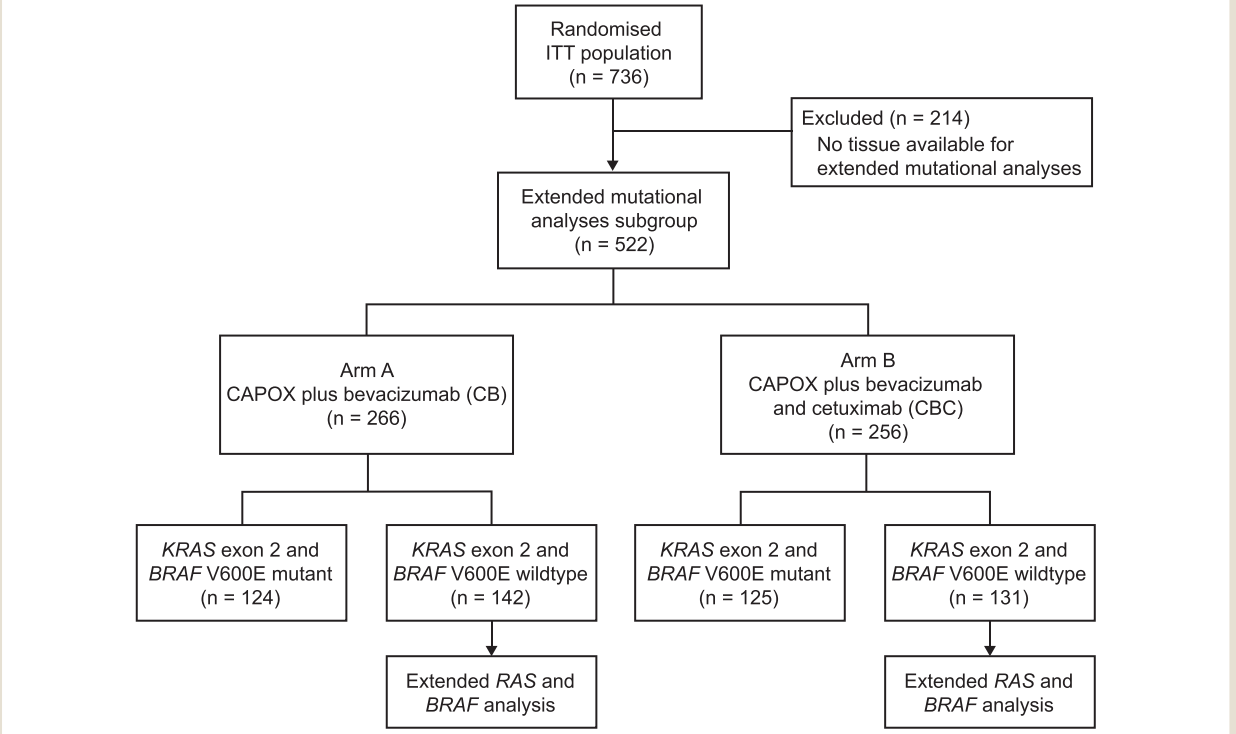
Figure 1 Flow diagram study design.



System (ThermoFisher). The target sequencing depth was 1,500× per amplicon. Sequences were analyzed using SeqNext software v4.1.2 (JSI Medical Systems GmbH, Ettenheim, Germany). For mutation calling a variant allele fraction (VAF) cutoff value of 5% was used.

CMS Classification

Samples were classified into the main molecular subtypes, CMS2/3 and CMS4, using the immunohistochemistry classifier.[17,18] CMS1 is determined based on MMR status (dMMR) and excluded from the analyses reported here due to low numbers (n = 7).

Statistical Analysis

The updated survival analyses were performed on the ITT population (n = 736). For the extended mutational analysis all patients with known mutation status and new extended mutation status were analyzed (n = 522) (Figure 1). Baseline characteristics were compared using the Pearson Chi-squared test for categorical variables and unpaired t-test for continuous variables. Unknowns were excluded for testing variables. The PFS and OS were calculated using the Kaplan-Meier method. Comparisons between the different treatment groups (CB and CBC) were calculated using the log-rank test. The effect of treatment on survival was estimated with the Cox proportional hazards model. All statistical analyses were performed in R (version 4.0.5).

Results

Patients

For all patients in the ITT population (n = 736) updated survival was obtained in June 2020. In the CB group 356 patients (97%) and CBC group 354 patients (96%) had died.

For the extended mutational analysis 522 patients were included in the analysis, of whom 249 with previously known *KRAS* or *BRAF* V600E mutated tumors. 273 patients with wildtype tumors underwent the extended *RAS* and *BRAF* V600E mutational analyses (Figure 1). Baseline characteristics of the extended *RAS* and *BRAF* V600E subgroup were well balanced between the treatment groups and representative for the ITT population (Table 1 and Supplementary Table 2).

Updated Survival

For the updated median OS of the ITT population there was no statistically significant difference between the treatment arms, with 20.3 months (95% CI 18.0-24.1) in the CB group compared with 20.0 months (95% CI 18.3-21.4) in the CBC group (HR 1.14, 95% CI 0.98-1.32), p = 0.0831; Figure 2A and Table 2A). The updated median PFS was 10.6 months (95% CI 9.6-12.2) in the CB group compared with 9.5 months (95% CI 8.5-10.5) in the CBC group (HR 1.12, 95% CI 0.97-1.30), p = 0.123) (Table 2A).

Of the ITT population 52 (7%) of the patients were long-term survivors (>72 months). Baseline characteristics of these long survivors were a slightly younger age (59 versus 62 years), lower

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Table 1 Baseline Characteristics Extended *RAS* and *BRAF* Subgroup

Characteristics	CB Group (n = 266)	CBC Group (n = 256)	*P* Value
Age (years)			0.716
Median (range)	63.8 (57.6-69.5)	63.0 (56.9-69.7)	
Sex (%)			0.255
Male	148 (55.6%)	156 (60.9%)	
Female	118 (44.4%)	100 (39.1%)	
WHO performance status			0.124
0	171 (64.3%)	181 (70.7%)	
1	95 (35.7%)	74 (28.9%)	
No data	0 (0%)	1 (0.4%)	
Serum lactate dehydrogenase level[a]			0.732
0	169 (63.5%)	166 (64.8%)	
1	97 (36.5%)	88 (34.4%)	
No data	0 (0%)	2 (0.8%)	
Previous adjuvant therapy			0.920
No	222 (83.5%)	211 (82.4%)	
Yes	44 (16.5%)	44 (17.2%)	
No data	0 (0%)	1 (0.4%)	
Primary tumor site			0.073
Left-sided	105 (39.5%)	116 (45.3%)	
Right-sided	68 (25.6%)	71 (27.7%)	
Rectum	87 (32.7%)	60 (23.4%)	
No data	6 (2.3%)	9 (3.5%)	
Time of metastasis			0.373
Synchronous	150 (56.4%)	154 (60.2%)	
Metachronous	116 (43.6%)	100 (39.1%)	
No data	0 (0%)	2 (0.8%)	
Resection of primary tumour			
No	257 (96.6%)	246 (96.1%)	1.000
Yes	8 (3.0%)	7 (2.7%)	
No data	1 (0.4%)	3 (1.2%)	

Pearson Chi-squared test used for categorical variables and unpaired t-test used for continuous variables. Unknowns were excluded for testing variables.
Abbreviations: CB = capecitabine and oxaliplatin plus bevacizumab; CBC = capecitabine and oxaliplatin plus bevacizumab and cetuximab; WHO = World health organisation.
[a] Normal (0) or abnormal (1), according to the cutoff values of each individual center.

serum LDH, left-sided primary tumors and a single metastatic site. These patients tolerated more cycles of systemic therapy and had better objective response rates. There was no apparent effect of treatment arm (Supplementary Table 3).

Extended RAS *and* BRAF *V600E Mutational Analysis*

Of the 273 patients with *KRAS* and *BRAF* V600E wildtype tumors and of whom primary tumor FFPE tissue was available for the extended molecular analyses, 269 patients (98.5%) were successfully analyzed. Additional mutations were found in 27.5% (n = 74) of the analyzed tumors. *KRAS* was mutated in 31 (11.4%), *NRAS* in 31 (11.4%) and *BRAF* V600E in 12 (4.4%). *RAS* and *BRAF* V600E mutations were mutually exclusive. Although *KRAS* exon 2 was sequenced during the original trial, we found nine additional patients with a mutation in *KRAS* exon 2 in the previously reported wildtype patients. For *BRAF* V600E we established additional mutations in tumors of 12 patients (Table 3).

Survival of the Extended RAS *and* BRAF *V600E Wildtype Subgroup*

The extended wildtype cohort was defined as those patients with wildtype *RAS* and *BRAF* V600E tumors in both the original as well as the extended mutational analyses (n = 195). In this extended wildtype cohort PFS was not significantly different between the treatment groups (HR 1.01, 95% CI 0.75-1.34, p = 0.9681, with 12.2 months in the CB (95% CI 10.5-15.0) and 12.4 months in the CBC group (95% CI 10.6-13.2). For median OS also no difference was seen for the extended wildtype cohort between the treatment groups (HR 1.01, 95% CI 0.76-1.35, p = 0.9342, with 25.4 months in the CB (95% CI 20.7-30.7) and 25.8 months in the CBC group (95% CI 22.9-32.2) (Table 2B).

As it is known that right-sided primary tumors most probably do not benefit from anti-EGFR treatment,[19] we also calculated the survival of patients with left-sided and extended wildtype tumors (n = 164). Again, no significant difference was seen with both PFS and OS between the treatment groups (PFS: CB 12.8 months versus

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Table 2 Comparing Survival Data

	n	Median PFS	Median OS
A. Total Group Original paper			
CB Group	368	10.7	20.3
CBC Group	368	9.4	19.4
P value[a]		0.01	0.16
Updated survival			
CB Group	368	10.6	20.3
CBC Group	368	9.5	20.0
P value[a]		0.12	0.08
B. Updated survival in subgroup Extended *KRAS/BRAF* Wildtype			
CB Group	105	12.2	25.4
CBC Group	90	12.4	25.8
P value[a]		0.97	0.93
Extended KRAS/BRAF Wildtype and left-sided			
CB Group	92	12.8	26.8
CBC Group	72	12.6	29.2
P value[a]		0.78	0.84
C. Subgroup (treatment arms combined) Original paper			
KRAS Wildtype	316	10.6	22.1
KRAS and *BRAF* Wildtype	266	11.3	24.5
Updated survival			
New *KRAS/BRAF* Mutant	74	8.5	17.7
Extended *KRAS/BRAF* Wildtype	195	12.3	25.6
P value[b]		0.007	0.02
Extended *KRAS/BRAF* Wildtype and left-sided	164	12.6	28.6

Abbreviations: CB = capecitabine and oxaliplatin plus bevacizumab; CBC = capecitabine and oxaliplatin plus bevacizumab and cetuximab; ITT = intention-to-treat population; OS = overall survival; PFS = progression-free survival.
[a] Comparisons between the different treatment groups (CB and CBC), calculated using the log-rank test.
[b] Comparisons between the new *KRAS/BRAF*mutant and extended *KRAS/BRAF* wildtype subgroups, calculated using the log-rank test.

CBC 12.6 months, HR 0.96, 95% CI 0.70-1.31, p = 0.7775; OS CB 26.8 months versus CBC 29.2 months, HR 0.97, 95% CI 0.71-1.33, p = 0.841) (Figure 2B and Table 2B).

However, when combining both treatment groups (as no significant difference between treatment group was observed) median PFS and OS of the extended wildtype subgroup was 3.5 months higher compared to the *KRAS* wildtype subgroup of the original report (25.6 months versus 22.1 months) (Table 2C). Of the 273 patients which were wildtype and underwent extended mutational analyses a significant difference in survival of the extended wildtype subgroup compared to the newly mutated group was observed when the patients with additional mutations were removed (extended wildtype versus new *RAS/BRAF* V600E mutant PFS: 12.3 months versus 8.5 months, p = 0.007; OS: 25.6 versus 17.7 months, p = 0.02 (Table 2C and Supplementary Figure 1). Median survival for patients with previous known *KRAS* or *BRAF*

V600E mutations versus newly found mutations remained unchanged. When restricting to left-sided primary and extended wildtype tumors the OS was even higher, adding another 3.0 months (from 25.6 to 28.6 months) (Table 2C). In total the survival of the extended wildtype and left-sided primary tumor subgroup was 6.5 months higher compared to the original *KRAS* wildtype subgroup.

Prognostic and Predictive Effect of RAS *and* BRAF *V600E Mutation Status in the Total Group of Patients*

We explored the prognostic effect of the mutation status in the total group, combining both treatment groups. Patients with *RAS* and *BRAF* V600E wildtype tumors had significantly better OS compared to patients with either *RAS* or *BRAF* V600E mutations (*RAS/BRAF* wildtype 25.6 months (95% CI 23.0-29.5) versus *RAS* mutation 19.8 months (95% CI 17.7-22.1), HR 1.32, 95% CI 1.09-1.60, p = 0.004; and versus *BRAF* V600E mutation 13.6 months (95% CI 9.61-16.6), p < 0.001). *BRAF* V600E mutations showed the worst survival compared to both *RAS* mutations (HR 1.70, 95% CI 1.27-2.26, p < 0.001) and patients with wildtype tumors (HR 2.27, 95% CI 1.68-3.08, p < 0.001) (Figure 2C).

With regards to the predictive effect, patients with a *RAS* mutation had a significantly worse survival when treated with the addition of cetuximab as compared to the CB group (HR 1.36, 95% CI 1.07-1.75, p = 0.01387) with a median OS of 23.2 months for the CB group (95% CI 19.8-27.7) compared with 18.3 months in

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Figure 2 **Overall survival curves. Six-year overall survival update for (A) the different treatment arms: CB (capecitabine and oxaliplatin plus bevacizumab) and CBC (CB and cetuximab); (B) the extended *RAS* and *BRAF* V600E wildtype and left-sided subgroup compared to patients with a *RAS* or *BRAF* V600E mutation or right-sided tumour; (C) according to RAS and *BRAF* V600E mutational status; (D) according to *RAS* and *BRAF* V600E mutational status and treatment arm.**

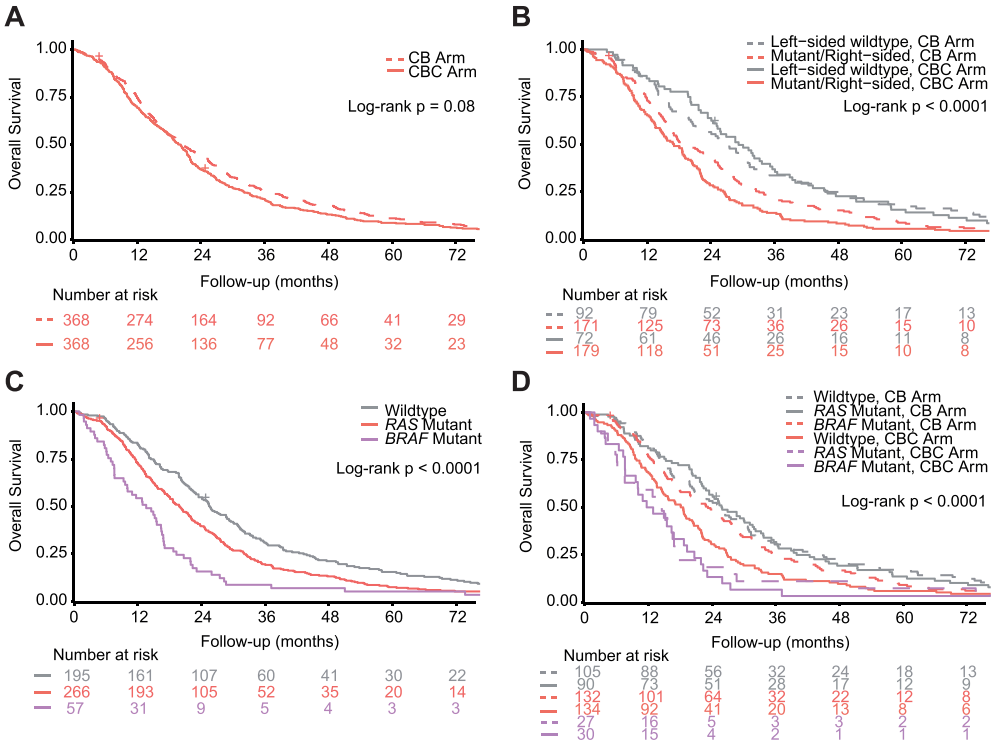

Figure 3 **Overall survival curves for the consensus molecular subtypes. Overall survival stratified by the consensus molecular subtypes (CMS) in the extended *RAS* and *BRAF* Wildtype cohort for (A) CMS2/3 and (B) CMS4. Abbreviations: CB, capecitabine and oxaliplatin plus bevacizumab; CBC, CB and cetuximab.**

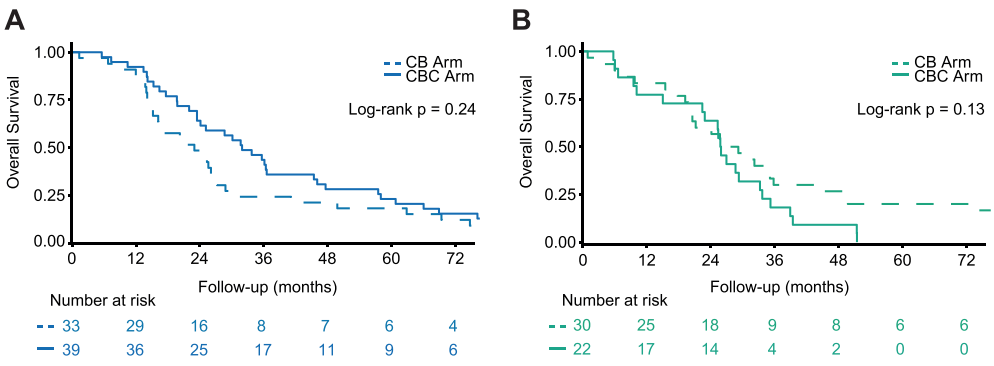

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Table 3 *RAS* and *BRAF* Mutation Status

	Extended (n = 273)		Total (n = 522)		CB Group (n = 266)		CBC Group (n = 256)	
Genotype	n (%)		n (%)		n (%)		n (%)	
Wildtype (*RAS* and *BRAF*)	195	(71.4)	195	(37.4)	105	(39.5)	90	(35.2)
KRAS exon 2 mutant								
KRAS G12A			14	(2.7)	5	(1.9)	9	(3.5)
KRAS G12C	1	(0.4)	21	(4)	11	(4.1)	10	(3.9)
KRAS G12D	1	(0.4)	67	(12.8)	36	(13.5)	31	(12.1)
KRAS G12E			1	(0.2)	1	(0.4)	0	(0)
KRAS G12R			5	(1)	1	(0.4)	4	(1.6)
KRAS G12S	2	(0.7)	10	(1.9)	5	(1.9)	5	(2)
KRAS G12V	1	(0.4)	56	(10.7)	33	(12.4)	23	(9)
KRAS G13C			1	(0.2)	0	(0)	1	(0.4)
KRAS G13D	2	(0.7)	35	(6.7)	18	(6.8)	17	(6.6)
KRAS G13E			1	(0.2)	1	(0.4)	0	(0)
KRAS V14L	1	(0.4)	1	(0.2)	1	(0.4)	0	(0)
KRAS Q22K	1	(0.4)	1	(0.2)	1	(0.4)	0	(0)
KRAS exon 3 mutant								
KRAS A59E	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
KRAS Q61H	5	(1.8)	5	(1)	1	(0.4)	4	(1.6)
KRAS Q61R	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
KRAS exon 4 mutant								
KRAS A146T	10	(3.7)	10	(1.9)	4	(1.5)	6	(2.3)
KRAS A146V	2	(0.7)	2	(0.4)	1	(0.4)	1	(0.4)
KRAS K117N	3	(1.1)	3	(0.6)	1	(0.4)	2	(0.8)
KRAS Total	31	(11.4)	235	(45)	120	(45.1)	115	(44.9)
NRAS exon 2 mutant								
NRAS G12A	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS G12C	1	(0.4)	1	(0.2)	1	(0.4)	0	(0)
NRAS G12D	8	(2.9)	8	(1.5)	3	(1.1)	5	(2)
NRAS G12S	1	(0.4)	1	(0.2)	1	(0.4)	0	(0)
NRAS G12V	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS G13R	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS G13V	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS exon 3 mutant								
NRAS Q61A	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS Q61H	3	(1.1)	3	(0.6)	2	(0.8)	1	(0.4)
NRAS Q61K	9	(3.3)	9	(1.7)	4	(1.5)	5	(2)
NRAS Q61L	3	(1.1)	3	(0.6)	1	(0.4)	2	(0.8)
NRAS Q61R	1	(0.4)	1	(0.2)	0	(0)	1	(0.4)
NRAS Total	31	(11.4)	31	(5.9)	12	(4.5)	19	(7.4)
BRAF mutant (V600E)	12	(4.4)	57	(10.9)	27	(10.2)	30	(11.7)
Unknown	4	(1.5)	4	(0.8)	2	(0.8)	2	(0.8)

Abbreviations: CB = capecitabine and oxaliplatin plus bevacizumab; CBC = capecitabine and oxaliplatin plus bevacizumab and cetuximab.

the CBC group (95% CI 14.7-20.6). No treatment interaction was seen for *BRAF* V600E mutations (HR 1.03, 95% CI 0.61-1.75, p = 0.9088) (Figure 2D).

Predictive Effect of the CMS

As the extended *RAS* and *BRAF* V600E wildtype subgroup did not show a benefit from the combination of anti-EGFR and anti-VEGF, we explored the predictive effect of the consensus molecular subtypes (CMSs). In the extended wildtype group there were 72 CMS2/3 and 51 CMS4 patients. For CMS2/3 patients there was a numerically but statistically non-significant increased median OS in the CBC group of 31.9 months versus 23.0 months in the CB group (HR 0.75, 95% CI 0.46-1.21, p = 0.246). No treatment

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effect was seen for CMS4 (HR 0.78, 95% CI 0.56-1.07, p = 0.127) (Figure 3).

Discussion

In this updated survival analysis and extended analysis of *RAS/BRAF* V600E mutation status of the CAIRO2 study we show that the addition of anti-EGFR to first-line treatment with anti-VEGF and CAPOX does not improve outcome in mCRC patients. However, survival was substantially higher compared to the original trial when only patients with extended *RAS/BRAF* V600E wildtype and left-sided tumors were considered.

For the population with an anti-EGFR resistant *RAS* mutant genotype, the detrimental effect on survival by the addition of anti-EGFR treatment was confirmed.[20,21] Patients with *RAS* mutated tumors in the control arm with chemotherapy plus bevacizumab showed a similar OS compared to patients with wildtype tumors, implying that the significant lower median OS for the *RAS* mutant tumors could be attributed to the detrimental effect of cetuximab in *RAS* mutated tumors. Hence, not only a lack of response but worse survival is seen when treating *RAS* mutated tumors with anti-EGFR therapy. In the PACCE trial addition of panitumumab to chemotherapy plus bevacizumab also resulted in a decrease in PFS and excess toxicity.[10] The same holds true for the study of Saltz et al. where patients with *KRAS* mutant tumors showed inferior PFS when cetuximab was added to chemotherapy and bevacizumab.[11]

Patients harboring a *BRAF* V600E mutation are known to be characterized by a dismal prognosis.[22] In our study we confirmed the poor prognosis of these patients, compared to patients with *RAS* mutated and *RAS/BRAF* wildtype tumors. However, no predictive effect was observed in this trial for the addition of cetuximab. Even though both *RAS* and *BRAF* V600E mutations activate the pathway downstream of EGFR thereby causing resistance to anti-EGFR therapy, there appears to be a difference in the clinical effect dependent on the specific mutation. Patients with *BRAF* V600E mutated tumors show no difference in survival between the two treatment combinations, but patients with *RAS* mutated tumors had worse survival with the addition of cetuximab compared to the control arm.

Interestingly we discovered additional *RAS* exon 2 and *BRAF* V600E mutations in tumors which were classified as wildtype in the primary mutational analyses. This can be explained by the improved quality and efficiency of DNA sequencing and increased accuracy of software for calling of mutations between the primary analyses in 2008 and the current analyses. When removing the patients with the newly found mutations from the wildtype subgroup we showed an important increase in survival of the extended wildtype subgroup. These findings imply that older mutational analyses might underestimate the number of mutants and if retrospective analyses are performed using older mutation data it should be considered to re-analyze the wildtype cohort for additional mutations.

Two possible explanations for the lack of benefit in our extended wildtype subgroup might involve the negative interaction of anti-EGFR when combined with capecitabine.[23] Firstly, the often-decreased total dose intensity and hence efficacy due to increased toxicity from capecitabine-containing regimens. A second, speculative, hypothesis is decreased cytotoxic activity of capecitabine as the required metabolic activation is reduced due to cetuximab-induced G1 arrest.[23] Therefore, it might be interesting to repeat a similar study in an infusional-5-FU or irinotecan-based chemotherapy regimen combined with anti-EGFR. Another interesting step could be to expand the mutational panel with other mutations associated with anti-EGFR resistance, including *HRAS, PIK3CA* exon 20, *PTEN, MAP2K1* and amplifications involving *MET, ERBB2, KRAS* and also including amplifications which increase the sensitivity to anti-EGFR therapy e.g. *EGFR* and *IRS2*.[12,14,24-26] We also did not account for possible secondary resistance, which can occur during treatment with anti-EGFR.[14,25,27] Therefore, we cannot fully exclude that dual EGFR/VEGF inhibition may still be beneficial in a small subset.

Interestingly, in a small subgroup analysis we showed the predictive potential of stratifying the extended wildtype group according to the CMS, with a numerically difference in median OS for CMS2/3 tumors treated with the combination of anti-EGFR and anti-VEGF as compared to no addition of anti-EGFR. However, the sample size of this small subgroup was too small to show any significant effects, which we have shown before in this CAIRO2 wildtype cohort for the original *KRAS* mutations.[18] There could also have been a switch from CMS2 to CMS4 subtype, contributing to acquired cetuximab resistance which was not accounted for in this analysis as we only classified primary tumor tissue prior to treatment.[28] More recently we have shown that the efficacy of anti-EGFR in the different subtypes is dependent on the chemotherapy backbone. Efficacy using an oxaliplatin backbone, as used in the CAIRO2 study, was restricted to left-sided CMS2/3 tumors, which is in line with our results.[29]

In conclusion, based on the here reported retrospective analysis of the CAIRO2 trial, adding cetuximab to CAPOX and bevacizumab does not provide any clinical benefit in the updated survival analyses and extended wildtype group. However, in the extended wildtype group we did observe a clinically relevant higher survival compared to the initial trial report, indicating that it is important to analyze a broader panel of *RAS* and *BRAF* variants using more recent sequencing techniques when assessing survival benefit after anti-EGFR therapy.

Data Availability

The WES data are deposited at the EMBL-EBI under accession code EGAS00001002617 and EGAS00001006319 (additional sequencing for samples with low coverage). The data are available under restricted access.

Clinical Practice Points

- There was no subgroup who benefitted from the combined addition of anti-EGFR and anti-VEGF to a chemotherapy backbone in metastatic colorectal cancer in the CAIRO2 trial. Using more recent sequencing techniques, multiple additional *RAS* and *BRAF* mutations were detected and the extended wildtype left-sided subgroup had superior overall survival (28.6 months) compared to the original trial report. This indicates that it is important to analyze a broader panel of *RAS* and *BRAF* variants using state of the art sequencing techniques when assessing survival benefit after anti-EGFR therapy.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clcc.2022.11.006.

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