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Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab

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ABSTRACT

Background: Anti-EGFR monoclonal antibodies in metastatic colorectal cancer (mCRC) treatment are only effective in patients with *KRAS* wild type tumours. Here we assess the predictive value of other potential relevant markers involved in the epidermal growth factor receptor (EGFR) signalling pathways for response to cetuximab-based treatment.

Materials and methods: Formalin-fixed paraffin-embedded colorectal cancer tissue of the primary tumour was obtained from 559 mCRC patients treated with chemotherapy and bevacizumab with or without cetuximab (phase III CAIRO2 study). DNA was isolated for mutation analysis of *BRAF* (V600E), *KRAS* (codon 12 and 13) and *PIK3CA* (exon 9 and 20). Tissue microarray's (TMA's) were constructed for the assessment of *EGFR* and *HER2* gene copy number (GCN), and EGFR and PTEN protein expression. The results of these markers, individually or in combination, were correlated with progression-free survival (PFS) and overall survival (OS) in the subgroup of patients with a *KRAS* wild type tumour treated in the cetuximab-arm. *KRAS* wild type patients treated without cetuximab were used as a control group.

Results: A total of 208 tumours (39.4%) contained a *KRAS* mutation, 8.7% a *BRAF* mutation and 9.9% a *PIK3CA* mutation. Loss of PTEN expression and the presence EGFR protein expression were observed in 42.0% and 61.7% of the samples, respectively. An increased *EGFR* GCN was observed in 15.3% of the samples, and 11.5% of the evaluable samples contained an increased *HER2* GCN. In *KRAS* wild type patients treated with cetuximab a *BRAF* mutation was significantly and independently associated with PFS and OS. In patients treated without cetuximab the PFS and OS were also associated with the *BRAF* genotype. No prognostic or predictive value was observed for any of the other markers when tested individually or in combination.

Conclusions: *BRAF* genotype is correlated with PFS and OS in *KRAS* wild type mCRC patients, which is independent of cetuximab treatment. *PIK3CA* mutation, loss of PTEN expression, EGFR GCN and *HER2* GCN have no predictive value for response to treatment with cetuximab, neither individually nor in combination with other markers.

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1. Introduction

With the introduction of targeted agents the number of therapeutic options for patients with metastatic colorectal cancer (mCRC) has increased. Two classes of targeted agents have shown efficacy in mCRC.[1] Bevacizumab is a monoclonal antibody against the vascular endothelial growth factor (VEGF) and is effective in combination with chemotherapy in the first-line treatment of mCRC.[2–4] The other class of targeted agents consists of monoclonal antibodies directed against the epidermal growth factor receptor (EGFR): the chimeric cetuximab and the fully human panitumumab. Cetuximab is effective as monotherapy and in combination with irinotecan in late-line treatment[5,6] but shows only modest efficacy in combination with chemotherapy in the first-line treatment.[7] Panitumumab is effective as monotherapy in late-line mCRC treatment.[8] The EGFR monoclonal antibodies exert their effect by inhibition of binding of the ligand to EGFR and the prevention of subsequent dimerization of the receptor which leads to the activation of several intracellular signal transduction pathways stimulating cell proliferation, neoangiogenesis and inhibiting apoptosis.[9] The most important signal transduction pathways involved in this process are the RAS/MAPK pathway and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway.

Since only a subset of patients respond to these agents, predictive markers are needed to avoid unnecessary toxicity and healthcare costs. Patients with a tumour harbouring a mutation in codon 12 and 13 of the *KRAS* gene are resistant to treatment with anti-EGFR monoclonal antibodies.[10–12] Subsequently, several other tissue markers involved in one of the pathways downstream of EGFR have been suggested as a predictive marker of response to EGFR-antibodies in mCRC patients, although the results are conflicting.[13]

In the current study the predictive role of a *BRAF* mutation, a *PIK3CA* mutation, loss of PTEN expression, EGFR protein expression and EGFR and *HER2* gene copy number (GCN) changes are assessed for progression-free survival (PFS) and overall survival (OS) in the subgroup of patients with a *KRAS* wild type tumour treated in a phase III CAIRO2 trial with chemotherapy plus bevacizumab with or without cetuximab.

2. Materials and methods

2.1. Patient population

All patients included in this study participated in the CAIRO2 trial (CKTO 2005-02) of the Dutch Colorectal Cancer Group (DCCG). In this multicentre phase III trial 755 mCRC patients were randomised between first-line treatment with capecitabine, oxaliplatin and bevacizumab or the same schedule with the addition of weekly cetuximab.[14,15] All cycles were given every 3 weeks. Inclusion criteria included a histologically proven mCRC with irresectable distant metastases, the presence of at least one measurable lesion, World Health Organisation (WHO) performance status 0 or 1 and adequate organ functions. The primary end-point of the study was progression-free survival (PFS). Secondary end-points included overall survival (OS) and response rate.

2.2. Tissue collection

Suitable formalin-fixed paraffin-embedded (FFPE) tumour samples obtained at the resection of the primary tumour were collected from 559 primary tumours from more than 50 different Dutch laboratories of pathology. The written informed consent required for all patients before study entry also included translational research on tumour tissue. The histology of all tumour samples was centrally reviewed by a pathologist (IDN, JHJMvK).

2.3. Immunohistochemical staining

In order to analyse a large number of tumour specimens efficiently, tissue microarray's (TMA's) were constructed for immunohistochemistry staining and fluorescence *in situ* hybridisation assays. Of each FFPE tumour tissue block one 2 mm punch containing a high percentage of tumour cells was taken.

EGFR expression was determined by immunohistochemistry using the EGFR PharmDx kit (Dako Corporation, Glostrup, Denmark) diluted at 1:100 according to the manufacturer's instructions. Both for EGFR and for PTEN staining Powervision (Immunologic) was used as a second antibody and Power DAB was used for visualisation. According to the PharmDx kit guideline tumours showing >1% membranous EGFR stained cells were considered positive.

The PTEN expression status was determined by immunohistochemistry using the monoclonal anti-mouse anti-human PTEN antibody by Dako (Dako Corporation, Carpinteria, CA) at a dilution of 1:100 according to the manufacturers' instructions. Stromal tissue and endothelial cells were used as a positive internal control. Per tumour sample the percentage of cells with positive staining was assessed by three independent investigators (JT, MD, LL) and in case of discrepancy, a definite result was generated based on the expertise of a fourth investigator (JHJMvK, IDN).

2.4. Fluorescence in situ hybridisation (FISH)

The *EGFR* and *HER2* GCN were assessed by FISH analysis of the *EGFR* and *HER2* locus and centromeres performed following standard procedures. The *EGFR* locus probe and chromosome seven centromere probe were used according to the manufacturers' protocol (Vysis-Abbott Molecular Diagnostics). For *HER2* GCN assessment the *HER2* locus probe and the centromere chromosome 17 probes were obtained from Kreatech Diagnostics (Amsterdam, The Netherlands). The number of locus and centromere signals was counted in a minimum of 40 cells per tumour sample. An increased GCN was defined as an average of three or more locus copies per nucleus, or a locus to centromere ratio of two or more.

2.5. DNA extraction

Genomic DNA was extracted from 4 to 8 manually microdissected 50 μm sections of FFPE tissue as previously described.[16] DNA concentration was determined at 260 nm using the

Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA). DNA quality was assessed by performing a multiplex PCR using four primer sets, resulting in fragments of 100, 200, 300 and 400 base pairs (bp).[17]

2.6. Mutation analysis

The *KRAS* mutation status was assessed as previously described[16] and the *BRAF* V600E and *PIK3CA* mutation status were assessed in duplicate by sequencing analysis.

For *BRAF* mutation analysis, exon 15 was amplified using a 50 μl reaction mixture, containing 0.2 μM primer each (Table 1); dATP, dCTP, dGTP and dTTP (GE Healthcare, The Netherlands) at 500 μM each; 20 mM $(NH_4)_2SO_4$; 75 mM Tris–HCl (pH 9.0); 0.01% Tween 20; 0.625 U of Thermoperfect Taq polymerase (Integro, Leuvenheim, The Netherlands) and 50 ng of template DNA, were used to generate a 197 bp PCR product. The PCR conditions were as follows: 92 °C for 5 min; 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s (35 cycles) and 72 °C for 10 min.

For *PIK3CA* mutation detection exon 9 and exon 20 were screened. For exon 9 a 50 μl reaction mixture, containing 0.2 μM primer each (Table 1); dATP, dCTP, dGTP and dTTP (GE Healthcare, The Netherlands) at 500 μM each; 50 mM KCl; 10 mM Tris–HCl (pH 8.3); 2.5 mM $MgCl_2$; 0.625 U of AmpliTaq Gold polymerase (Applied Biosystems, USA) and 50 ng of template DNA, was used to generate a 269 bp PCR product. The PCR conditions were as follows: 94 °C for 5 min; 94 °C for 1 min, 57 °C for 45 s, 72 °C for 45 s (35 cycles) and 72 °C for 10 min. For *PIK3CA* exon 20 mutation analysis the reaction mixture was identical as used for *PIK3CA* exon 9, with the exception that different designated primers (Table 1) were used and the $MgCl_2$ concentration was lowered to 1.0 mM. A 273 bp PCR product was obtained using the following PCR conditions: 94 °C for 5 min; 94 °C for 1 min, 58 °C for 45 s, 72 °C for 45 s (40 cycles) and 72 °C for 10 min.

All PCR products were purified with MinElute 96 UF PCR Purification Kit (Qiagen, The Netherlands). Subsequently the purified products were sequenced using either the primers used for amplifying the PCR product (*PIK3CA* exon 9 and 20) or the M13 primers (*BRAF*, *KRAS*) with fluorescently labelled terminators (BigDye® Terminators (v 1.1); Applied Biosystems, USA). Sequence results were scored by visual inspection of the chromatograms.

2.7. Statistical analysis

The primary objective was to assess the predictive value for the response to cetuximab treatment of *KRAS* wild type patients of several markers involved in the EGFR pathway. Eligible patients were analysed according to the intention-to-treat principle. Patients alive without recurrence at the time of analysis were censored. The PFS and OS of patients treated without cetuximab were used as the control group. Differences in baseline characteristics were calculated using a Chi-square test. Survival curves were constructed using Kaplan–Meier models and the PFS and OS curves were compared using a Log-Rank test. First, we assessed the effect of all individual markers and next, we evaluated the combined effect of EGFR expression, *EGFR* GCN, *HER2* GCN (receptor alterations), a *KRAS* or *BRAF* mutation (RAS/MAPK activation), PIK3CA mutation or PTEN expression (PI3K/Akt activation) and the combined effect of RAS/MAPK and PI3K/Akt activation.

Finally, several multivariate analyses were performed using a Cox proportional hazards model with the stratification parameters (serum LDH, number of affected organs and previous adjuvant therapy) as covariates. First, we added one single marker to this model. Next, a forward and backward selection model was applied using the stratification parameters, all markers plus the two-way interaction terms between the markers as covariates, since individual alterations in downstream signalling pathways are unlikely to be independent events.

3. Results

3.1. Patient characteristics

A total of 559 patients were evaluable for these analyses, 277 treated with cetuximab and 282 treated without cetuximab. The baseline characteristics of these patients were comparable with the total CAIRO2 study population, except for baseline serum lactate dehydrogenase, which was less frequently above the upper limit of normal in the subgroup included in these analyses compared to the total CAIRO2 population. The median duration of follow-up at the time of these analyses was 34.8 months (95% confidence interval [CI] 33.6–37.3).

In Table 2 the clinical characteristics of patients are presented in relation to the different markers. *BRAF* mutated tumours were more often localised in the colon and *BRAF* wild type tumours were more often present in the rectum. For the other markers no difference in baseline characteristics was detected.

3.2. Marker prevalence

The prevalence of the different markers and their relationship are shown in Table 3. A *KRAS* codon 12 or 13 mutation was

Table 1 – Primers used for Sanger sequencing.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
KRAS	TGTAAAACGACGGCCAGTGGTACTGGTGGAGTATTTGATAGTG	CAGGAAACAGCTATGACCTGGATCATATTCG TCCACAAAA
BRAF	TGT AAA ACG ACG GCC AGT CCT TTA CTT ACT ACA CCT CAG	CAG GAA ACA GCT ATG ACC AAA AAT AGC CTC AAT TCT TAC
PIK3CA exon 9	CTGTGAATCCAGAGGGGAAA	ACATGCTGAGATCAGCCAAAT
PIK3CA exon 20	ATGATGCTTGGCTCTGGAAT	GGTCTTTGCCTGCTGAGAGT

M13 sequence in *Italic*.

Table 2 – Clinical characteristics for the different markers.

	EGFR expression		EGFR GCN		*HER2* GCN		*BRAF*		*PIK3CA*		PTEN expression		*KRAS*	
	Present	Absent	Disomy	Increased	Disomy	Increased	wt	mut	wt	mut	loss	intact	wt	mut
Total (*n*)	322	200	471	85	223	29	473	45	393	43	207	286	320	208
Age (mean-SD)	62.3 (10.8)	62.4 (9.7)	62.3 (10.1)	62.1 (11.3)	61.6 (11.6)	63.5 (10.7)	62.1 (10.5)	64.3 (8.6)	62.9 (9.5)	60.0 (9.7)	63.2 (9.6)	61.7 (10.9)	62.4 (9.5)	62.3 (11.3)
Gender – male (%)	58	59	58	64	63	76	58	44	58	56	57	58	60	55
Treatment arm – cetux.(*n*, %)	49	51	50	48	47	62	49	62	51	47	49	49	51	48
Serum LDH normal (*n*, %)	63	65	63	68	63	62	63	71	62	63	67	62	61	67
WHO PS = 0 (*n*, %)	69	66	68	62	62	69	68	64	68	60	67	71	69	65
No. of affected organs = 1 (%)	43	40	41	42	40	38	43	33	41	42	40	43	45	37
Localisation														
Colon (%)	49	41	45	44	44	42	42*	77*	44	55	49	44	45	47
Rectum (%)	25	28	26	30	26	15	28	8	27	15	23	29	26	24
Rectosigmoid (%)	26	31	28	26	30	42	30	15	28	30	28	27	29	29

GCN = gene copy number; WHO PS = World Health Organisation Performance Status; wt = wild type; mut = mutation.
* $p < 0.05$.

observed in the tumours of 208 out of 529 evaluable patients (39.3%), and a *BRAF* mutation in 45 out of 518 evaluable samples (8.7%). These mutations were mutually exclusive. A total of 43 tumours (32 in exon 9 and 11 in exon 20; 9.9%; Table 4) contained a *PIK3CA* mutation. In 123 samples the *PIK3CA* mutation status was not evaluable, which was partly explained by poor DNA quality with 41.9% of the not evaluable samples contained poor quality DNA with a maximum DNA fragment length of 200 bp, compared to 1.8% poor quality DNA in samples that did generate a result ($p < 0.001$). Loss of PTEN expression was observed in 207 out of 493 samples (42.0%). The consistency of the interpretation of PTEN expression status between the three individual investigators was >80%. Of the 411 samples in which the PTEN expression status and the *PIK3CA* mutation status were assessable, both loss of PTEN and a *PIK3CA* mutation were present in 12 samples (2.9%). The distribution of alterations in PTEN, *PIK3CA*, *BRAF* and *KRAS* is visualised in Fig. 1.

Membranous EGFR protein expression was present in 322 out of 522 samples (61.7%). An increased *EGFR* GCN was observed in 85 of 556 evaluable samples (15.3%), of which 13 had high copy number amplifications (locus/centromere ratio $\geqslant 3$). An increased *HER2* GCN was observed in 29 of 252 evaluable samples (11.5%).

3.3. *Correlation of individual markers with outcome in cetuximab-treated patients*

In Fig. 2A the hazard ratios for all individual markers are given. We previously showed that a *KRAS* mutation is associated with a decreased PFS in patients treated with cetuximab compared to patients with a *KRAS* wild type tumour with a median of 8.1 versus 10.5 months ($p = 0.04$).[14] Since anti-EGFR treatment is currently restricted to patients with a *KRAS* wild type tumour, the current analysis focuses on the 162 patients with a *KRAS* wild type tumour treated with cetuximab (Table 5A). An increased GCN of *EGFR* or *HER2* did not affect the PFS and OS, with a median PFS of 9.5 months in patients with an increased *EGFR* GCN compared to 10.4 months in patients with a normal *EGFR* GCN ($p = 0.19$), and a median PFS of 9.6 months for patients with an increased *HER2* GCN versus 9.7 months for patients with a normal *HER2* GCN ($p = 0.88$). Our results on the predictive and prognostic value of *BRAF* mutation status have been presented earlier as a short communication,[18] and here we update our results. A *BRAF* mutated tumour was associated with a significantly lower PFS and OS in cetuximab-treated patients compared to a *BRAF* wild type tumour (median PFS 6.5 versus 11.4 months; $p = 0.0001$, and median OS 12.9 versus 24.5 months; $p < 0.0001$, respectively) (Fig. 3).

The median PFS was 10.3 months in patients with a *PIK3CA* mutated tumour versus 10.4 months in patients with a *PIK3CA* wild type tumour ($p = 0.50$), and the median OS was 17.6 months in patients with a *PIK3CA* mutated versus 22.4 months in patients with a *PIK3CA* wild type tumour ($p = 0.22$). In patients with a tumour that showed loss of PTEN expression the median PFS was 10.6 months versus 9.4 months in patients whose tumour had an intact PTEN expression ($p = 0.25$), and the median OS was 22.2 months versus 21.0 months ($p = 0.83$), respectively.

Table 3 – Distribution and correlation of different EGFR pathway markers in 559 metastatic colorectal cancer samples.

	EGFR expression present	*EGFR* GCN increased	*HER2* CGN increased	KRAS mutation	BRAF mutation	PIK3CA mutation	PTEN loss
EGFR expression present	X	55/322 17.1%	17/146 11.6%	134/311 43.1%*	29/305 9.5%	23/267 8.6%	110/306 35.9%*
EGFR GCN increased	55/82 67.1%	X	7/35 20.0%	24/83 28.9%*	10/81 12.3%	3/69 4.3%	40/79 50.6%
HER2 CGN increased	17/27 63.0%	7/29 24.1%	X	9/28 32.1%	1/28 3.6%	2/54 3.7%	11/27 40.7%
KRAS mutation	134/198 67.7%*	24/206 11.7%*	9/103 8.7%	X	0*	22/171 12.9%	77/188 41.0%
BRAF mutation	29/43 67.4%	10/45 22.2%	1/18 5.6%	0*	X	1/38 2.6%	15/42 35.7%
PIK3CA mutation	23/42 54.8%	3/42 7.1%	2/22 9.1%	22/43 51.2%	1/43 2.3%	X	12/39 30.8%
PTEN loss	110/207* 53.1%	40/207 19.3%	11/75 14.7%	77/201 38.3%	15/197 7.6%	12/171 7.0%	X
Total	322/522 61.7%	85/556 15.3%	29/252 11.5%	208/528 39.3%	45/518 8.7%	43/436 9.9%	207/493 42.0%

GCN = gene copy number.
* $p < 0.05$

Table 4 – Distribution of the different *PIK3CA* mutations in 436 metastatic colorectal cancer samples.

Exon	Nucleotide	Number of samples	%
Exon 9	1624G>A	9	1.9
	1633G>A	13	2.8
	1634A>G	1	0.2
	1634A>C	4	0.9
	1635G>C	1	0.2
	1636C>A	1	0.2
	1637A>G	2	0.4
	1634A>C; 1636C>A	1	0.2
Exon 20	3130A>G	1	0.2
	3140A>G	8	1.8
	3140A>T	2	0.4
Total		43	9.9

3.4. *Pathway analysis to predict outcome in cetuximab-treated patients*

In order to evaluate the different pathways involved in the response to cetuximab treatment, we combined the individual markers (Table 5A, Figs. 2A and 4). At the level of receptor, the presence of either EGFR expression or an increased *EGFR* or *HER2* GCN (⩾1 receptor alteration) did not influence the PFS and OS in *KRAS* wild type patients; the median PFS was 9.4 months in 40 patients with ⩾1 receptor alteration versus 10.3 months in 16 patients without receptor alterations ($p = 0.59$), and the median OS was 20.2 months in patients with ⩾1 receptor alteration versus 16.3 months in patients without receptor alterations ($p = 0.73$) (Fig. 4A and B).

Activation of the PI3K/Akt pathway was defined as either loss of PTEN expression, or a *PIK3CA* mutation, or both. A total

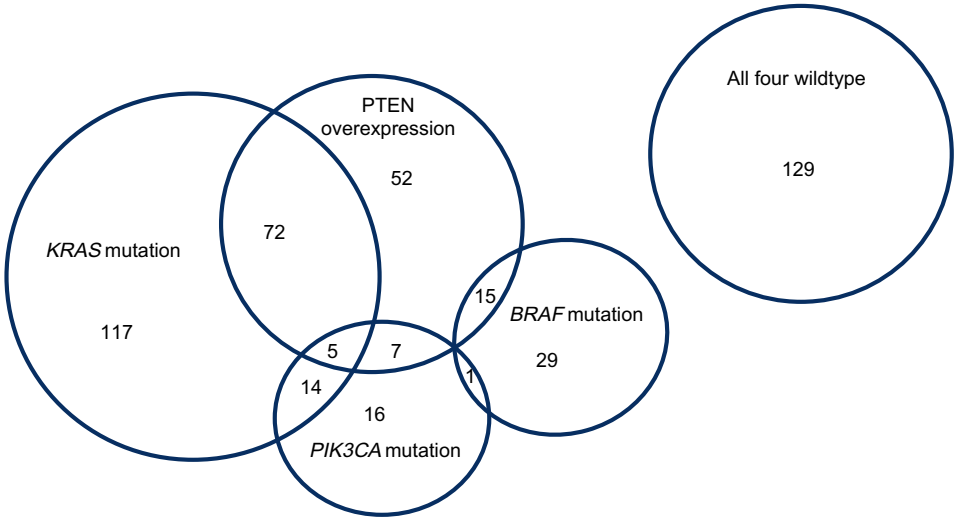

Fig. 1 – Distribution of different molecular alterations in 559 patients, of which 328 patients (58.6%) had a tumour with at least one alteration.

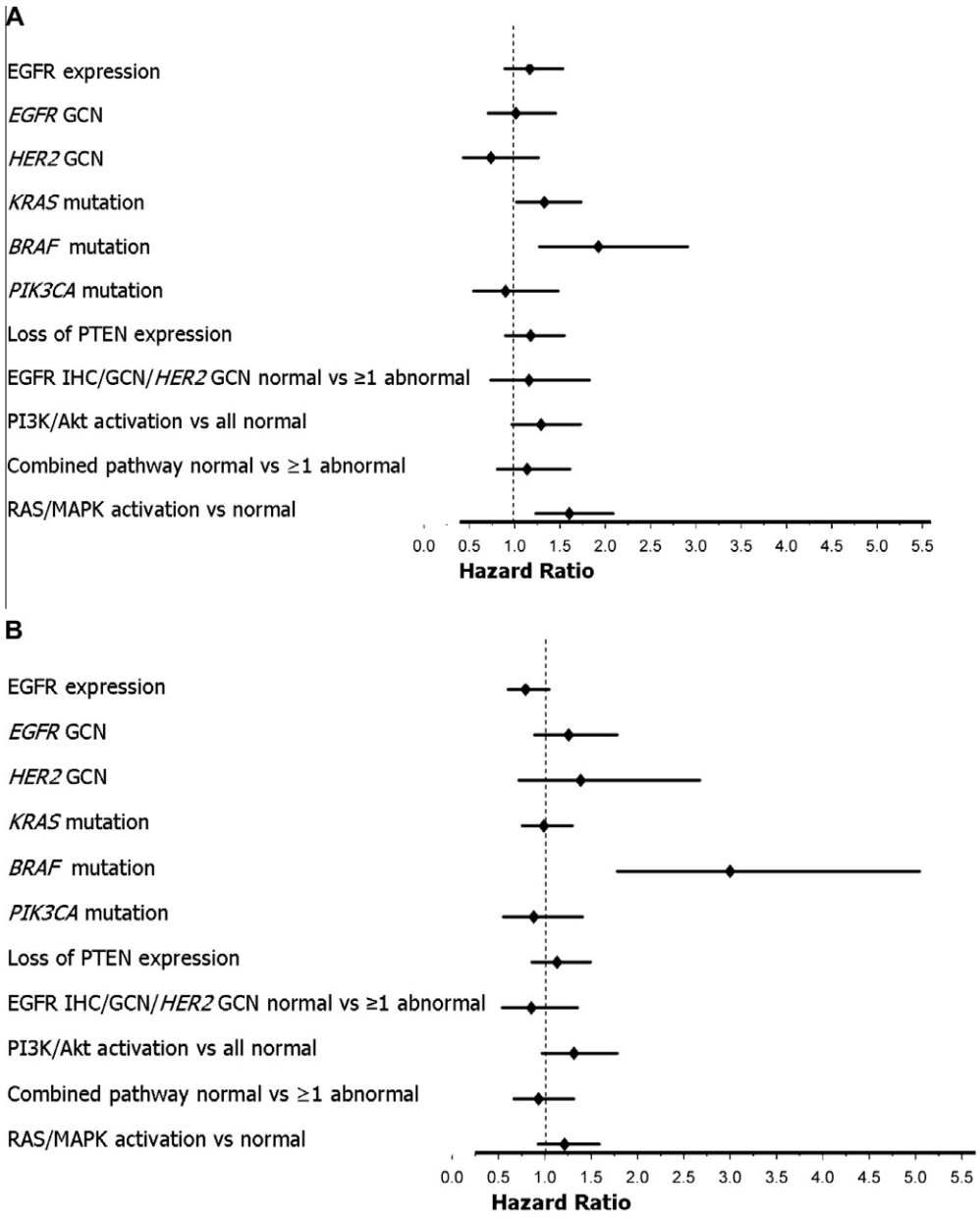

Fig. 2 – Forest plot for the effect of the individual markers on progression-free survival in patients treated with cetuximab (Panel A) and without cetuximab (Panel B)

of 65 patients in the *KRAS* wild type cetuximab group had PI3K/Akt activation and 61 patients had no alteration in the PI3K/Akt pathway. No difference in PFS (median 10.6 months in patients with PI3K/Akt pathway activation versus 9.6 months in patients without PI3K/Akt activation; $p = 0.31$) and OS (median 21.7 months in patients with PI3K/Akt activation versus 22.0 months in patients without PI3K/Akt activation; $p = 0.99$) was observed for these subgroups (Fig. 4C and D).

Activation of the RAS/MAPK pathway was defined as the presence of either a *KRAS* or a *BRAF* mutation. 127 patients with RAS/MAPK pathway activation had a significantly decreased PFS (median 7.3 versus 11.4 months; $p = 0.0004$) and OS (median 15.8 versus 24.1 months; $p < 0.0001$) compared to 121 patients with RAS/MAPK wild type tumours (Fig. 4E and F).

Next, we combined the outcome of both the PI3K/Akt pathway and the RAS/MAPK pathway ('combined pathway activation'). The PFS and OS were compared in 46 'combined pathway' negative patients (e.g. with both an intact PI3K/Akt and an intact RAS/MAPK pathway defined as *PIK3CA*, *KRAS* and *BRAF* wild type en PTEN expression positive) versus 151 'combined pathway' positive patients with one of more downstream pathway alterations (Fig. 4G and H). The median PFS was not significantly different between the negative and positive groups (9.7 months versus 9.3 months; $p = 0.45$), but the median OS was longer in 'combined pathway' negative patients compared to 'combined pathway' positive patients (median of 24.1 months in negative versus 18.9 months in positive patients; $p = 0.038$). To determine whether the effect of this 'combined pathway activation' on OS was solely

Table 5 – Median progression-free and overall survival according to the presence of tumour markers in metastatic colorectal cancer patients with a *KRAS* wild type tumour treated with cetuximab (Panel A), or without cetuximab (Panel B).

EGFR expression	*n*	PFS			OS		
		median	95% CI	*p*-Value	median	95% CI	*p*-Value
(A)							
EGFR expression							
Absent	62	11.2	8.6–12.4	0.26	22.4	19.8–29.3	0.21
Present	85	9.7	7.8–12.3		20.6	15.5–24.1	
EGFR GCN							
Disomy	129	10.4	8.6–12.2	0.19	22.0	19.2–25.4	0.65
Increased	26	9.5	6.4–12.3		21.9	13.4–27.1	
HER 2 GCN							
Disomy	54	9.7	7.1–12.4	0.88	18.6	12.7–24.1	0.43
Increased	9	9.6	7.5–17.1		25.4	14.3–40.0	
BRAF							
Wild type	126	11.4	10.1–12.6	0.0001	24.5	21.7–28.2	<0.0001
Mutation	27	6.5	4.8–9.3		12.9	9.6–18.7	
PIK3CA							
Wild type	119	10.4	9.3–12.2	0.50	22.4	20.2–25.8	0.22
Mutation	11	10.3	4.8–12.5		17.6	14.8–21.0	
Loss	67	10.6	8.2–12.5	0.25	22.2	17.6–26.0	0.83
Intact	74	9.4	7.8–11.4		21.0	16.8–26.0	
Combination of different markers							
EGFR IHC/GCN/*HER2* GCN							
⩾1 Abnormality	40	9.4	7.2–12.6	0.59	20.2	14.4–26.9	0.73
All normal	16	10.3	7.1–12.4		16.3	10.0–28.2	
PI3K/Akt							
Activation	65	10.6	8.8–12.4	0.31	21.7	16.4–25.8	0.99
Normal	61	9.6	7.8–11.4		22.0	18.1–26.9	
RAS/MAPK							
Activation	121	7.3	6.5–8.7	0.0004	15.8	13.1–18.7	<0.0001
Normal	127	11.4	10.1–12.6		24.1	21.4–28.2	
Combined pathway							
All wild type	46	9.7	8.4–12.6	0.45	24.1	18.6–31.7	0.038
⩾1 Abnormality	151	9.3	7.4–10.6		18.9	15.8–21.9	
(B)							
EGFR expression							
Absent	56	9.2	7.4–10.6	0.08	20.7	15.2–27.4	0.87
Present	84	10.6	8.6–13.5		17.2	15.6–24.6	
EGFR GCN							
Disomy	119	10.5	8.6–12.5	0.15	21.2	16.2–25.4	0.19
Increased	28	9.8	8.3–12.8		20.4	16.2–26.0	
HER 2 GCN							
Disomy	59	10.6	8.2–13.5	0.39	24.6	15.6–30.0	0.16
Increased	9	11.7	4.5–15.3		20.3	6.1–28.2	
BRAF							
Wild type	126	10.8	9.3–12.8	<0.0001	23.0	19.2–26.1	0.0002
Mutation	17	5.7	2.6–7.8		12.8	6.1–16.5	
PIK3CA							
Wild type	112	9.7	8.7–12.2	0.88	20.3	16.2–24.6	0.47
Mutation	9	8.3	4.4–14.3		13.1	8.6–31.0	
PTEN							
Loss	53	10.7	9.3–14.3	0.19	23.8	17.7–28.4	0.11
Intact	79	8.4	7.4–10.6		16.7	14.6–22.6	

(continued on the next page)

Table 5 – *continued*

EGFR expression	*n*	PFS			OS		
		median	95% CI	*p*-Value	median	95% CI	*p*-Value
Combination of different markers							
EGFR IHC/GCN/*HER2* GCN							
⩾1 Abnormality	47	12.2	8.6–14.3	0.68	19.8	15.1–25.4	0.47
All normal	14	9.1	8.6–14.3		25.8	12.2	
PI3K/Akt							
Activation	52	10.7	9.2–14.3	0.17	23.0	16.6–28.7	0.23
Normal	61	8.8	7.4–11.4		19.2	15.2–25.4	
RAS/MAPK							
Activation	117	10.7	9.0–12.9	0.16	17.9	15.5–23.8	0.15
Normal	125	10.8	9.5–12.9		23.0	19.2–26.1	
Combined pathway							
All wild type	53	9.6	8.2–13.2	0.70	22.6	16.2–27.4	0.77
⩾1 Abnormality	134	12.2	9.7–13.5		21.7	17.1–26.0	

n = number of patients; IHC = immunohistochemistry; PFS = progression-free survival; GCN = gene copy number; OS = overall survival; CI = confidence interval.
PI3K/Akt activation: either loss of PTEN or *PIK3CA* mutation.
RAS/MAPK: either *KRAS* or *BRAF* mutation.
Combined pathway: *KRAS*, *BRAF*, *PIK3CA* or PTEN abnormality.

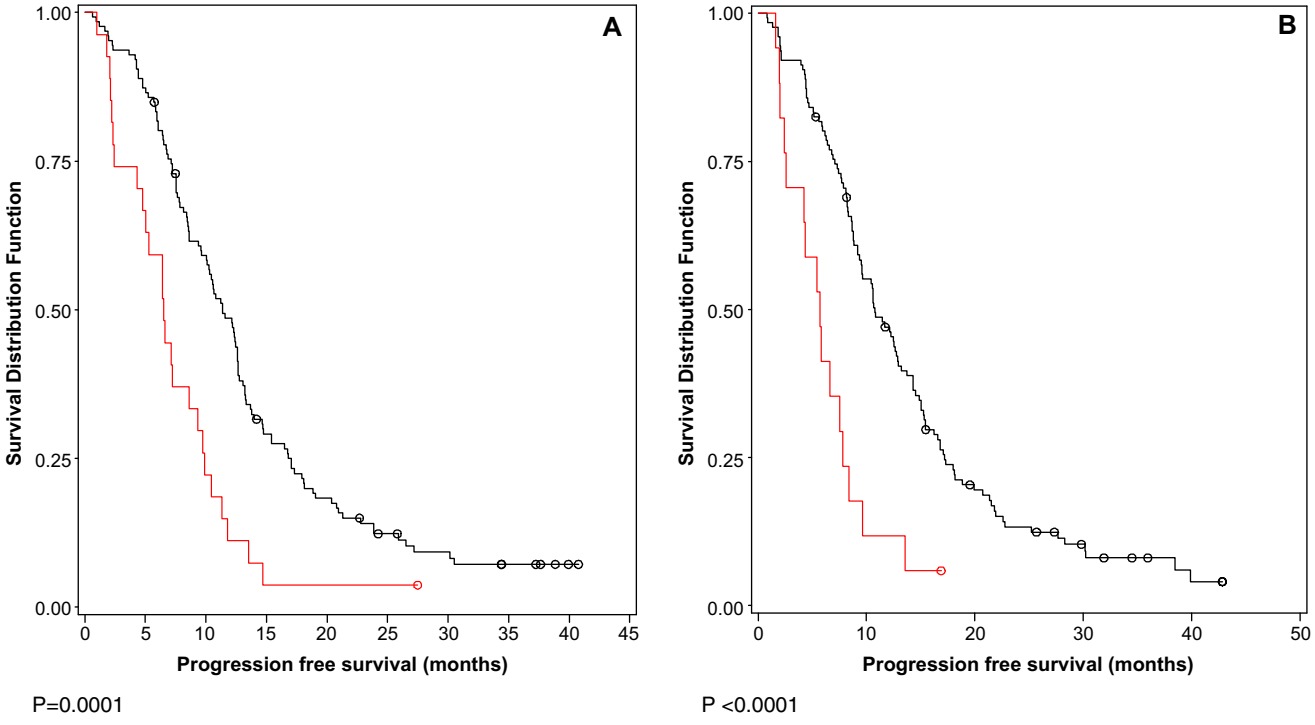

Fig. 3 – Progression-free survival of patients with a *KRAS* wild type tumour and a *BRAF* mutated tumour (red line) versus a *BRAF* wild type tumour (black line) treated with cetuximab (Panel A) and without cetuximab (Panel B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

explained by the effect of RAS/MAPK pathway activation, we performed a multivariate analysis including the stratification parameters, 'combined pathway activation' and 'RAS/MAPK activation' as covariates for the prediction of OS. Only RAS/

MAPK remained strongly associated with OS ($p = 0.0006$), whereas the 'combined pathway activation' was not significantly associated with OS ($p = 0.93$), indicating that the effect of the 'combined pathway activation' in OS was caused by the effect of RAS/MAPK pathway activation.

3.5. *Analysis of individual markers and pathway analysis in patients treated without cetuximab*

In order to be able to differentiate between a predictive effect of cetuximab and a role of the other agents administered to

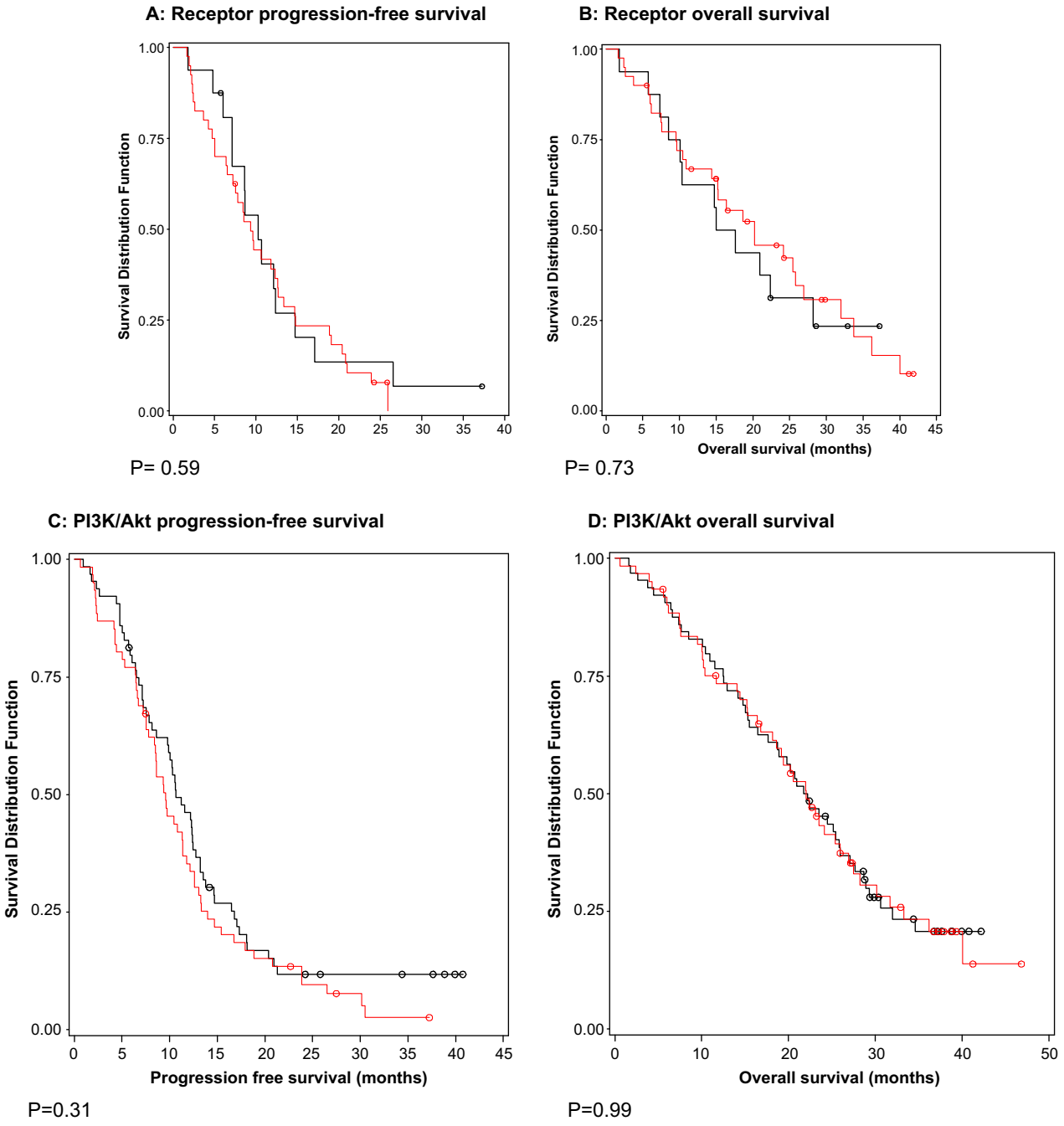

Fig. 4 – Kaplan–Meier survival curves indicating the progression-free (Panel A, C, E and G) and overall survival (Panel B, D, F and H) in 56 *KRAS* wild type cetuximab-treated patients with (red line) or without (black line) ⩾1 receptor abnormality (Panel A and B), in 126 *KRAS* wild type cetuximab-treated patients with (black line) or without (red line) PI3K pathway activation, defined as either a *PIK3CA* mutation or loss of PTEN expression (Panel C and D), in 270 cetuximab-treated patients with (red line) or without (black line) RAS/MAPK activation, defined as either a *KRAS* or a *BRAF* mutation (Panel E and F), and in 197 cetuximab-treated patients with (red line) or without (black line) combined PI3K/Akt and RAS/MAPK pathway activation (Panel G and H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

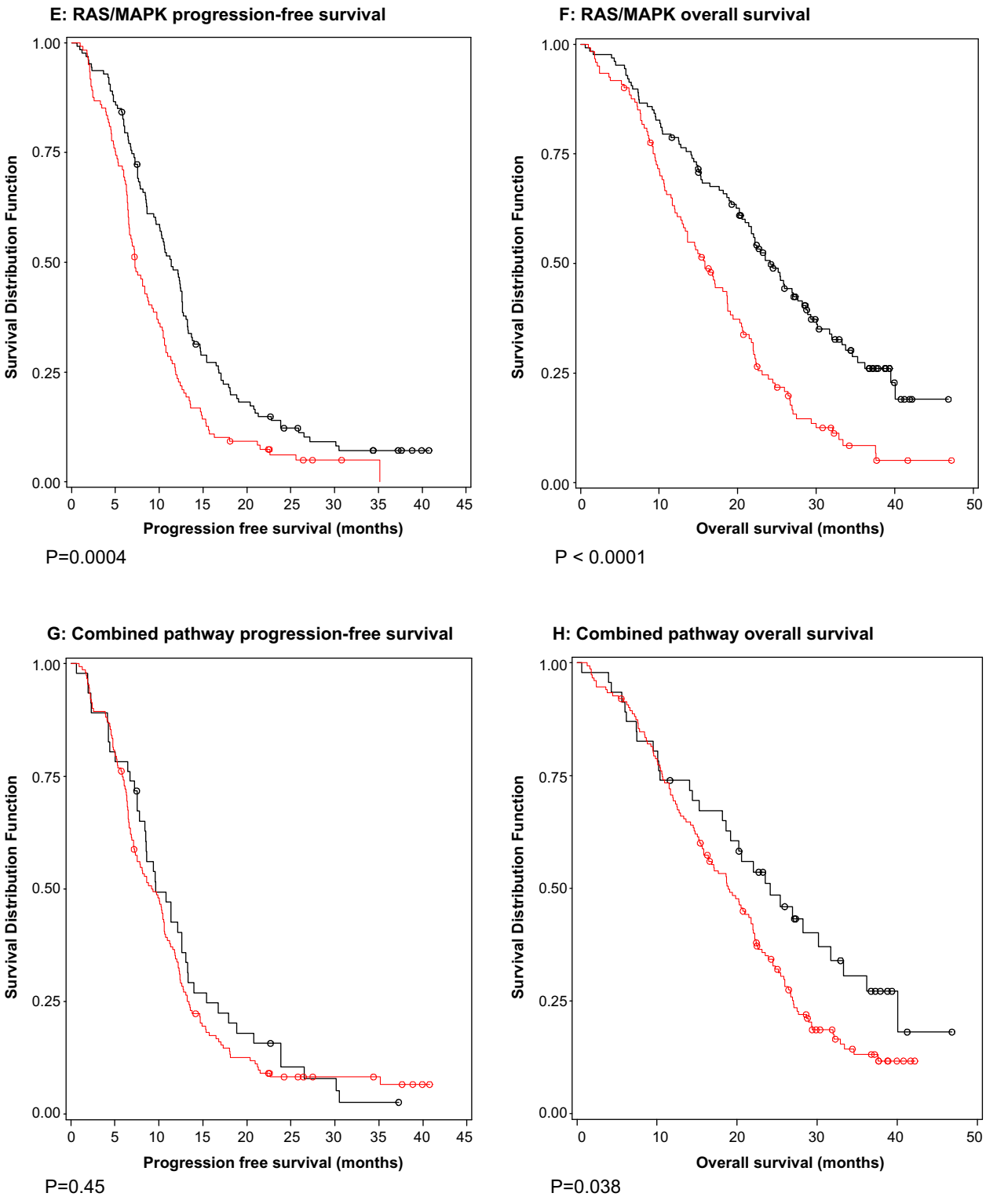

Fig 4. *(continued)*

these patients or even a prognostic effect of the investigated markers, we assessed the influence of all markers on the outcome of patients not treated with cetuximab (Fig. 2B). Also in the *KRAS* wild type subgroup treated without cetuximab a mutation in the *BRAF* oncogene was associated with a significantly reduced PFS and OS compared to *BRAF* wild type (the median PFS was 5.7 versus 10.8 months; $p < 0.0001$, and the median OS was 12.8 versus 23.0 months; $p = 0.0002$). The other markers did not affect PFS or OS in *KRAS* wild type patients treated without cetuximab, neither analysed individ-

ually nor in combination as receptor alteration, PI3K/Akt activation, RAS/MAPK activation or combined pathway activation (Table 5B).

3.6. Multivariate analysis

First, each marker was analysed individually together with the stratification parameters to the CAIRO2 study (serum LDH, the number of affected organs and prior adjuvant chemotherapy) as a predictor for PFS and OS in *KRAS* wild type patients treated with cetuximab. In this analysis, *BRAF* genotype was significantly associated with PFS (HR 2.3; $p = 0.0002$) and OS (HR 3.2; $p < 0.0001$). The same analysis in the total group of cetuximab-treated patients showed a significant association of the *KRAS* (HR 1.3; $p = 0.044$ for PFS and HR 1.5; $p = 0.0065$ for OS) and *BRAF* (HR 2.0; $p = 0.0011$ for PFS and HR 2.4; $p < 0.0001$ for OS) genotype with PFS and OS.

Next, we applied forward and backward selection to a model including the stratification parameters, all individual markers plus the two-way interaction terms between all markers for the prediction of PFS in cetuximab-treated patients. The forward selection model was the best model for the prediction of PFS with a −2 log likelihood ratio of 525.3 (versus 493.8 with backward selection), and the only marker that was significantly associated with PFS in this model was a *BRAF* mutation with a hazard ratio of 2.80 ($p = 0.0026$).

4. Discussion

In this large series of *KRAS* wild type mCRC patients treated with chemotherapy and bevacizumab with or without cetuximab, we observed that a *BRAF* mutation is significantly associated with PFS and OS, which was independent of the treatment with cetuximab. None of the other markers tested were associated with the outcome in *KRAS* wild type patients. Theoretically, patients with intact pathways, defined as a *KRAS*, *BRAF* and *PIK3CA* wildtype and a PTEN-positive tumour, could be expected to have an optimal response to cetuximab treatment. However, we observed that the combined effect on OS of a *BRAF*, *KRAS*, *PIK3CA* or PTEN alteration was fully explained by the effect of *KRAS* and *BRAF* mutations.

Several studies suggested a predictive role for the markers investigated in this study. Although initially the administration of anti-EGFR antibodies was limited to patients whose tumour expresses EGFR, the response to these agents later appeared to be independent of EGFR expression status.[19] *EGFR* GCN changes were shown to be associated with an increased sensitivity to anti-EGFR antibodies,[20–23] which however was not confirmed by others.[24,25] A mutation in exon 9 or 20 of *PIK3CA*, encoding the p110alpha catalytic subunit of phosphatidylinositol-3-kinase (PI3K), leads to a constitutive kinase activity.[26] Although a predictive role of *PIK3CA* mutations on the response to anti-EGFR treatment has been observed,[27,28] several negative reports exist as well.[20,21,29,30] Loss of PTEN expression in the primary tumour[22] or in metastatic tissue[31] has also been associated with resistance to cetuximab, but not by others.[25,27,32] Finally, patients with an upregulation of the PI3K/Akt pathway by either a *PIK3CA* mutation or a PTEN aberration have been suggested to be non-responsive to cetuximab.[24,27] Since several large studies,[29,30] including ours, do not observe any effect of *PIK3CA* or the combination with PTEN, the impact of these markers for clinical practice should be reconsidered. When *PIK3CA* and PTEN are combined with *KRAS* and *BRAF* mutation status, we and others[33] observe a significant effect on survival. However, in our study this effect is fully explained by the effect of *KRAS* and *BRAF* mutation status.

There are several possible explanations for the divergent results of these marker studies. First, differences in expression of the various markers between primary tumours and metastatic sites may have influenced the correlation with clinical outcome of treatment of metastatic tissue. This issue has shown to be relevant for PTEN expression[31,32,34] and *EGFR* GCN,[34,35] but appears to be less relevant for *KRAS*[36] and *BRAF*,[34] although this should be confirmed in larger series. Other issues influencing the consistency of data are heterogeneity of the marker within the tumour, which is known to be the case for *EGFR* GCN[35] and especially relevant in tissue microarray analysis, and inter-observer bias hampering the interpretation of immunohistochemistry and FISH analysis.[37] To overcome this latter problem for PTEN analysis, we had this test performed by multiple investigators, and observed a low and acceptable inter-observer variation. Whereas the *PIK3CA* results were hampered by poor DNA quality, this issue was less of a problem in *KRAS* and *BRAF* analyses, since these amplicons were much smaller. In contrast to *KRAS*,[16] the tumour cell percentage of the individual samples was not associated with the percentage of assessable *PIK3CA* results, since also samples containing a low number of tumour cells did generate an evaluable result.

Most other reports on predictive markers in mCRC are derived from heterogeneous cohorts of patients predominantly treated in late lines, with different chemotherapy schedules, and often concern the use of different anti-EGFR antibodies. We present data on these biomarkers in a large, homogenous population. However, since in our study treatment with cetuximab unexpectedly had a negative outcome,[38] the predictive value of these markers for outcome of cetuximab is difficult to assess.

An activating mutation in *BRAF*, encoding the BRAF protein localised directly downstream of KRAS in the RAS/MAPK pathway, is associated with resistance to treatment with anti-EGFR antibodies.[30,39] However, we and others recently showed that *BRAF* mutations appear to be rather prognostic than predictive since also mCRC patients treated without cetuximab have a markedly reduced survival when their tumour harbours a *BRAF* mutation.[18,28,40] The benefit from any systemic treatment in mCRC patients with a *BRAF* mutated tumour remains therefore uncertain.

Obviously, our model including seven EGFR-related markers is an oversimplified representation of the complex reality of treatment with targeted agents. Other genetic and epigenetic factors related to the tumour or its environment are likely to be involved in response to anti-EGFR targeted agents in mCRC. For example, the expression of the EGFR ligands epiregulin and amphiregulin has been suggested as predictive marker for cetuximab treatment.[41,42] The rapid progress of our knowledge and the increasing availability of molecular techniques paves the way to an individualised

cancer treatment. From our study two important lessons can be drawn. First, biobanking in large clinical trials is essential in the search for predictive markers for response to new agents. Second, the outcome in cetuximab-treated patients with *KRAS* wild type tumours is reduced when their tumour harbours a *BRAF* mutation. Future studies should establish the optimal treatment strategy in patients with a *BRAF* mutated tumour.

Conflict of interest statement

None declared.

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REFERENCES

1. Chau I, Cunningham D. Treatment in advanced colorectal cancer: what, when and how? *Br J Cancer* 2009;**100**(11):1704–19.
2. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;**350**(23):2335–42.
3. Kabbinavar FF, Schulz J, McCleod M, et al. Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. *J Clin Oncol* 2005;**23**(16):3697–705.
4. Saltz LB, Clarke S, Diaz-Rubio E, et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 2008;**26**(12):2013–9.
5. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;**351**(4):337–45.
6. Jonker DJ, O'Callaghan CJ, Karapetis CS, et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;**357**(20):2040–8.
7. van Cutsem E, Kohne CH, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009;**360**(14):1408–17.
8. Van Cutsem E, Peeters M, Siena S, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 2007;**25**(13):1658–64.
9. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res* 2006;**12**(18):5268–72.
10. Amado RG, Wolf M, Peeters M, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;**26**(10):1626–34.
11. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;**359**(17):1757–65.
12. Lievre A, Bachet JB, Boige V, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008;**26**(3):374–9.
13. Siena S, Sartore-Bianchi A, Di NF, Balfour J, Bardelli A. Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. *J Natl Cancer Inst* 2009;**101**(19):1308–24.
14. Tol J, Koopman M, Cats A, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N Engl J Med* 2009;**360**(6):563–72.
15. Tol J, Koopman M, Rodenburg CJ, et al. A randomised phase III study on capecitabine, oxaliplatin and bevacizumab with or without cetuximab in first-line advanced colorectal cancer, the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG). An interim analysis of toxicity. *Ann Oncol* 2008;**19**(4):734–8.
16. Tol J, Dijkstra JR, Vink-Borger ME, et al. High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. *J Cell Mol Med* 2009.
17. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003;**17**(12):2257–317.
18. Tol J, Nagtegaal ID, Punt CJ. BRAF mutation in metastatic colorectal cancer. *N Engl J Med* 2009;**361**(1):98–9.
19. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005;**23**(9):1803–10.
20. Moroni M, Veronese S, Benvenuti S, et al. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005;**6**(5):279–86.
21. Lievre A, Bachet JB, Le CD, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006;**66**(8):3992–5.
22. Frattini M, Saletti P, Romagnani E, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer* 2007;**97**(8):1139–45.
23. Laurent-Puig P, Cayre A, Manceau G, et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol* 2009;**27**(35):5924–30.
24. Perrone F, Lampis A, Orsenigo M, et al. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol* 2009;**20**(1):84–90.
25. Razis E, Briasoulis E, Vrettou E, et al. Potential value of PTEN in predicting cetuximab response in colorectal cancer: an exploratory study. *BMC Cancer* 2008;**8**:234.
26. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;**304**(5670):554.
27. Sartore-Bianchi A, Martini M, Molinari F, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res* 2009;**69**(5):1851–7.
28. Souglakos J, Philips J, Wang R, et al. Prognostic and predictive value of common mutations for treatment response and

survival in patients with metastatic colorectal cancer. *Br J Cancer* 2009;**101**(3):465–72.
29. Prenen H, De SJ, Jacobs B, et al. PIK3CA mutations are not a major determinant of resistance to the epidermal growth factor receptor inhibitor cetuximab in metastatic colorectal cancer. *Clin Cancer Res* 2009;**15**(9):3184–8.
30. Lambrechts D, de Roock W, Prenen H, et al. The role of KRAS, BRAF, NRAS, and PIK3CA mutations as markers of resistance to cetuximab in chemorefractory metastatic colorectal cancer. *J Clin Oncol* 2009;**27**(15s) [Suppl.;abstr 4020].
31. Loupakis F, Pollina L, Stasi I, et al. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. *J Clin Oncol* 2009;**27**(16):2622–9.
32. Bengala C, Bettelli S, Fontana F, et al. EGFR gene copy number, KRAS and BRAF status, PTEN and AKT expression analysis in patients with metastatic colon cancer treated with anti-EGFR monoclonal antibodies ± chemotherapy. *J Clin Oncol* 2009;**27**(Suppl.;abstr e15055).
33. Sartore-Bianchi A, Di NF, Nichelatti M, et al. Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. *PLoS One* 2009;**4**(10):e7287.
34. Molinari F, Martin V, Saletti P, et al. Differing deregulation of EGFR and downstream proteins in primary colorectal cancer and related metastatic sites may be clinically relevant. *Br J Cancer* 2009;**100**(7):1087–94.
35. Ooi A, Takehana T, Li X, et al. Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent in situ hybridization study. *Mod Pathol* 2004;**17**(8):895–904.
36. Italiano A, Hostein I, Soubeyran I, et al. KRAS and BRAF mutational status in primary colorectal tumors and related metastatic sites: biological and clinical implications. *Ann Surg Oncol* 2010.
37. Moroni M, Sartore-Bianchi A, Veronese S, Siena S. EGFR FISH in colorectal cancer: what is the current reality? *Lancet Oncol* 2008;**9**(5):402–3.
38. Punt CJ, Tol J. More is less-combining targeted therapies in metastatic colorectal cancer. *Nat Rev Clin Oncol* 2009;**6**(12):731–3.
39. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008;**26**(35):5705–12.
40. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009;**58**(1):90–6.
41. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007;**25**(22):3230–7.
42. Jacobs B, De RW, Piessevaux H, et al. Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. *J Clin Oncol* 2009;**27**(30):5068–74.