Effects of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis

Wendy De Roock, Bart Claes, David Bernasconi, Jef De Schutter, Bart Biesmans, George Fountzilas, Konstantine T Kalogeras, Vassiliki Kotoula, Demetris Papamichael, Pierre Laurent-Puig, Frédérique Penault-Llorca, Philippe Rougier, Bruno Vincenzi, Daniele Santini, Giuseppe Tonini, Federico Cappuzzo, Milo Frattini, Francesca Molinari, Piercarlo Saletti, Sara De Dosso, Miriam Martini, Alberto Bardelli, Salvatore Siena, Andrea Sartore-Bianchi, Josep Tabernero, Teresa Macarulla, Frédéric Di Fiore, Alice Oden Gangloff, Fortunato Ciardiello, Per Pfeiffer, Camilla Qvortrup, Tine Plato Hansen, Eric Van Cutsem, Hubert Piessevaux, Diether Lambrechts, Mauro Delorenzi, Sabine Tejpar

Summary

Background Following the discovery that mutant *KRAS* is associated with resistance to anti-epidermal growth factor receptor (EGFR) antibodies, the tumours of patients with metastatic colorectal cancer are now profiled for seven *KRAS* mutations before receiving cetuximab or panitumumab. However, most patients with *KRAS* wild-type tumours still do not respond. We studied the effect of other downstream mutations on the efficacy of cetuximab in, to our knowledge, the largest cohort to date of patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab plus chemotherapy in the pre-*KRAS* selection era.

Methods 1022 tumour DNA samples (73 from fresh-frozen and 949 from formalin-fixed, paraffin-embedded tissue) from patients treated with cetuximab between 2001 and 2008 were gathered from 11 centres in seven European countries. 773 primary tumour samples had sufficient quality DNA and were included in mutation frequency analyses; mass spectrometry genotyping of tumour samples for *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* was done centrally. We analysed objective response, progression-free survival (PFS), and overall survival in molecularly defined subgroups of the 649 chemotherapy-refractory patients treated with cetuximab plus chemotherapy.

Findings 40·0% (299/747) of the tumours harboured a *KRAS* mutation, 14·5% (108/743) harboured a *PIK3CA* mutation (of which 68·5% [74/108] were located in exon 9 and 20·4% [22/108] in exon 20), 4·7% (36/761) harboured a *BRAF* mutation, and 2·6% (17/644) harboured an *NRAS* mutation. *KRAS* mutants did not derive benefit compared with wild types, with a response rate of 6·7% (17/253) versus 35·8% (126/352; odds ratio [OR] 0·13, 95% CI 0·07–0·22; p<0·0001), a median PFS of 12 weeks versus 24 weeks (hazard ratio [HR] 1·98, 1·66–2·36; p<0·0001), and a median overall survival of 32 weeks versus 50 weeks (1·75, 1·47–2·09; p<0·0001). In *KRAS* wild types, carriers of *BRAF* and *NRAS* mutations had a significantly lower response rate than did *BRAF* and *NRAS* wild types, with a response rate of 8·3% (2/24) in carriers of *BRAF* mutations versus 38·0% in *BRAF* wild types (124/326; OR 0·15, 95% CI 0·02–0·51; p=0·0012); and 7·7% (1/13) in carriers of *NRAS* mutations versus 38·1% in *NRAS* wild types (110/289; OR 0·14, 0·007–0·70; p=0·013). *PIK3CA* exon 9 mutations had no effect, whereas exon 20 mutations were associated with a worse outcome compared with wild types, with a response rate of 0·0% (0/9) versus 36·8% (121/329; OR 0·00, 0·00–0·89; p=0·029), a median PFS of 11·5 weeks versus 24 weeks (HR 2·52, 1·33–4·78; p=0·013), and a median overall survival of 34 weeks versus 51 weeks (3·29, 1·60–6·74; p=0·0057). Multivariate analysis and conditional inference trees confirmed that, if *KRAS* is not mutated, assessing *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations (in that order) gives additional information about outcome. Objective response rates in our series were 24·4% in the unselected population, 36·3% in the *KRAS* wild-type selected population, and 41·2% in the *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* exon 20 wild-type population.

Interpretation While confirming the negative effect of *KRAS* mutations on outcome after cetuximab, we show that *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations are significantly associated with a low response rate. Objective response rates could be improved by additional genotyping of *BRAF*, *NRAS*, and *PIK3CA* exon 20 mutations in a *KRAS* wild-type population.

Funding Belgian Federation against Cancer (Stichting tegen Kanker).

Introduction

There has been a recent and rapid development in biological agents targeted against components of receptor tyrosine kinase signalling cascades in the treatment of cancer. In the field of metastatic colorectal cancer the use of monoclonal antibodies against the epidermal growth factor receptor (EGFR), such as cetuximab or panitumumab, has been implemented in clinical practice since 2004. Experience with anti-EGFR monoclonal antibodies has been a textbook example of how genetic

Lancet Oncol 2010; 11: 753–62

Published Online July 8, 2010 DOI:10.1016/S1470-2045(10)70130-3

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Centre for Human Genetics, KU Leuven, Leuven, Belgium (W De Roock MD, J De Schutter MSc, B Biesmans MSc, Prof S Tejpar MD); **Vesalius Research Centre, KU Leuven and VIB, Leuven, Belgium** (B Claes MSc, Prof D Lambrechts PhD); **Swiss Institute of Bioinformatics, Lausanne, Switzerland** (D Bernasconi MSc, M Delorenzi PhD); **Hellenic Cooperative Oncology Group (HeCOG) and Aristotle University of Thessaloniki School of Medicine, Thessaloniki, Greece** (Prof G Fountzilas MD, K T Kalogeras MD, V Kotoula MD); **B O Cyprus Oncology Centre, Nicosia, Cyprus** (D Papamichael FRCP); **Université Paris Descartes, INSERM UMR-S775, Paris, France** (Prof P Laurent-Puig MD); **Departement de Pathologie, Centre De Lutte Contre le Cancer Jean Perrin and Equipe d'accueil EA 4233, Université d'Auvergne, Clermont-Ferrand, France** (Prof F Penault-Llorca MD); **Assistance Publique Hôpitaux Paris, Hôpital Ambroise Paré, Boulogne Billancourt, France**; (Prof P Rougier MD); **Medical Oncology University Campus Bio-Medico of Rome, Italy** (B Vincenzi MD, D Santini MD, Prof G Tonini MD); **Division of Medical Oncology, Ospedale Civile, Livorno, Italy** (F Cappuzzo MD); **Laboratory of**

Molecular Diagnostic, Institute of Pathology, Locarno, Switzerland (M Frattini PhD, F Molinari PhD); **Oncology Institute of Southern Switzerland, San Giovanni Hospital, Bellinzona, Switzerland** (P Saletti MD, S De Dosso MD); **Laboratory of Molecular Genetics, Institute for Cancer Research and Treatment, University of Turin Medical School, Candiolo, Turin, Italy** (M Martini PhD, Prof A Bardelli PhD); **Italian Foundation for Cancer Research, Institute of Molecular Oncology, Milan, Italy** (A Bardelli); **The Falck Division of Medical Oncology, Ospedale Niguarda Ca' Granda, Milan, Italy** (Prof S Siena MD, A Sartore-Bianchi MD); **Medical Oncology Department, Vall d'Hebron University Hospital, Barcelona, Spain** (Prof J Tabernero MD, T Macarulla MD); **Digestive Oncology Unit, Department of Hepato-Gastroenterology, Rouen University Hospital, Rouen Cedex, France, and Inserm U614, Faculty of Medicine, Institute for Biomedical Research, Rouen, France** (F Di Fiore MD, A O Gangloff MD); **Division of Medical Oncology, Department of Experimental and Clinical Medicine, Second University of Naples, Italy** (Prof F Ciardiello MD); **Department of Oncology, Odense University Hospital and Institute of Clinical Research, University of Southern Denmark, Denmark** (C Qvortrup MD, Prof P Pfeiffer MD); **Department of Pathology, Odense University Hospital, Denmark** (T P Hansen MD); **Department of Digestive Oncology, University Hospital Gasthuisberg, KU Leuven, Leuven, Belgium** (Prof E Van Cutsem MD, S Tejpar); **Service de Gastro-entérologie, Cliniques Universitaires Saint-Luc, UCL, Brussels, Belgium** (Prof H Piessevaux MD); **and Département de Formation et Recherche, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland** (M Delorenzi PhD)

Correspondence to:
Prof Sabine Tejpar, Digestive Oncology Unit, University Hospital Gasthuisberg, Herestraat 49, B-3000, Leuven, Belgium
sabine.tejpar@uzleuven.be

profiling of individual tumours can lead to personalised medicine. Although initial response rates of about 10% were seen with cetuximab monotherapy in patients with heavily pretreated metastatic colorectal cancer,[1] it subsequently became clear that tumours without mutations in codon 12 or 13 of the *KRAS* gene responded in 13–17% of cases, whereas only 0–1·2% of the *KRAS* mutant tumours did.[2,3] Although the *KRAS* wild-type state seems to be a condition for response, most patients with *KRAS* codon 12 and 13 wild-type tumours do not respond to anti-EGFR monoclonal antibodies.[4] Mutations in other downstream effectors of the EGFR signalling pathway, such as BRAF, NRAS, and PI3 kinase, might also have a negative effect on response to anti-EGFR antibodies.[5–7]

We designed a study to centrally genotype tumour samples for mutations in four downstream components of the *EGFR* signalling pathway (*KRAS*, *PIK3CA* [which encodes the catalytic subunit of PI3 kinase], *BRAF*, and *NRAS*) using high-throughput mass spectrometric mutation profiling in what is, to our knowledge, the largest cohort to date of patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab and chemotherapy. Since all patients were treated in the pre-*KRAS* selection era, patients were unselected, avoiding any bias of over-representation or under-representation of molecular subgroups. We aimed to use the results of this mutation profiling to provide a clear picture of the efficacy of cetuximab in metastatic colorectal cancer in the chemotherapy-refractory setting according to the presence or absence of activating mutations in *KRAS*, *PIK3CA*, *BRAF*, and *NRAS*. We intended that the algorithms we used would help physicians to predict the efficacy of cetuximab before treatment.

Methods

Data collection

European investigators who had published data for series of patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab were contacted to provide tumour DNA or one slide of formalin-fixed paraffin-embedded (FFPE) tissue. Clinical data were collected in a standardised format. This retrospective study was undertaken after approval by the local ethics review boards.

1022 tumour DNA samples (73 from fresh-frozen and 949 from FFPE tumour tissue) were gathered from 11 centres in seven European countries. Only 773 primary tumour samples with sufficient quality DNA were included for the mutation frequency analysis. For the outcome analysis, we focused on 649 chemotherapy-refractory patients treated with cetuximab plus chemotherapy to study effects related directly to cetuximab. All patients received cetuximab-based treatment between 2001 and 2008, which is the period when cetuximab was on the market without any restrictions in terms of *KRAS* mutation status. Patients treated with cetuximab or panitumumab monotherapy were not included because they were too few for a meaningful subgroup analysis. Patient and sample characteristics are given in table 1, table 2, and webappendix pp 3–7.

Procedures

Since times for radiological assessment for progression varied between the different investigators, progression-free survival (PFS) was not the best endpoint to choose. Neither was overall survival, because the reported effect of molecular markers on overall survival in single-arm studies is potentially a prognostic (independent of any specific treatment) and not a true predictive (attributable to the specific treatment, in this case cetuximab) effect. However, objective response is a robust measurement based on strict criteria, and is independent of time of assessment. Moreover, tumour shrinkage and objective response are correlated with long-term outcome for cetuximab.[8] Therefore, we chose objective response as the main endpoint and PFS and overall survival as secondary endpoints. WHO criteria (HeCOG series) or Response Evaluation Criteria in Solid Tumours (RECIST; all other series) were used to classify tumour response. Objective response was defined as complete or partial response; non-response as stable or progressive disease. PFS was calculated from the start of cetuximab to clinical or radiological progression or death from any cause. Patients who did not meet these criteria were censored at the date of the last administration or of the last scan. Overall survival was defined as the period from the start of cetuximab treatment until death from any cause or last follow-up.

Samples were genotyped centrally to avoid discrepancies inherent in the use of different methods. We queried the COSMIC database[9] for mutations in *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* occurring in colorectal cancer, and selected the most frequent mutations per gene (webappendix pp 9–14). We covered 97·7% of the *KRAS* mutations, 81·2% of the *PIK3CA* mutations, and 97·0% of the *BRAF* mutations described. At the time of genotyping, only six mutations in *NRAS* were reported in the COSMIC database, all arising in codon 12, 13, or 61. Based on the extensive homology between *KRAS* and *NRAS*,[10] we expected *NRAS* to be mutated similarly to *KRAS* in these mutation hotspot regions. Consequently, we aimed to also cover codon 12, 13, and 61 mutations that were not reported in the COSMIC database. Genomic positions of the mutated nucleotides were downloaded from Ensembl, and 200 bp upstream and downstream sequences were used for primer design with the Sequenom MassARRAY Assay Design 3·1 software using default parameters. Multiplex PCR was done in a 5 µL volume containing 5–10 ng DNA. Detailed methods regarding the Sequenom MALDI-TOF MassARRAY multiplex PCR and genotyping can be found in the webappendix p 1. Serial dilutions of DNA from different cell lines were used to determine the sensitivity of Sequenom mutation detection, which was shown to be between 5% and 15%, depending on the

	Samples
Number of samples with DNA of sufficient quality	886/1022 (86·7%)
Type of tissue sample	
Primary tumour	773/886 (87·3%)
Metastasis	111/886 (12·5%)
Missing	2/886 (0·2%)
Total number of samples* from patients with chemotherapy-refractory tumours	727/886 (80·0%)
Anti-EGFR treatment received in 708 patients refractory to chemotherapy	
Panitumumab monotherapy	15/708 (2·1%)
Cetuximab monotherapy	42/708 (5·9%)
Cetuximab plus chemotherapy†	649/708 (91·7%)
Missing	2/708 (0·3%)

Data are n/N (%) unless otherwise indicated. EGFR=epidermal growth factor receptor. *19 patients contributed two samples. †The exact chemotherapy regimens are given in webappendix pp 3–7.

Table 1: **Sample characteristics**

	Patients
Sex	
Male	377 (58·1%)
Female	272 (41·9%)
Median age (years; range)	61 (22–86)
Number of previous chemotherapy regimens	
1*	84 (12·9%)
2	320 (49·3%)
3	156 (24·0%)
4	60 (9·2%)
≥5	25 (3·9%)
Unknown	4 (0·6%)
Worst skin toxicity seen (NCI–CTC)	
0	91 (14·0%)
1	239 (36·8%)
2	207 (31·9%)
3	47 (7·2%)
4	2 (0·3%)
Missing	63 (9·7%)
Type of tissue sample	
Primary tumour	558 (86·0%)
Metastasis	89 (13·7%)
Missing	2 (0·3%)
Response rate	146 (22·5%)
Median PFS (weeks; IQR)	18 (9–30)
Median overall survival (weeks; IQR)	39 (22–64)

Data are n (%) unless otherwise indicated. NCI–CTC=National Cancer Institute–Common Toxicity Criteria. PFS=progression-free survival. *Refractory to the chemotherapeutic agent administered with cetuximab.

Table 2: **Characteristics of 649 chemotherapy-refractory patients treated with cetuximab plus chemotherapy**

For **Ensembl** see http://www.ensembl.org/index.html

See **Online** for webappendix

mutation (webappendix p 8). Genotyping results were further validated independently in a subset of the samples by direct sequencing or allele-specific PCR (*KRAS* codon 12 and p.G13D; *BRAF* p.V600E) or by the DxS PI3K Mutation Test Kit (DxS, Manchester, UK) for *PIK3CA* p.H1047R, p.E542K, p.E545D, and p.E545K mutations (webappendix p 1).

DNA of a sample was considered of sufficient quality when more than 75% of mutations were reliably genotyped. 136 samples had DNA of insufficient quality and were omitted (table 1). A sample was considered wild type for a given gene when the most frequently mutated sites in this gene (webappendix pp 9–14) did not show a mutation. When an assay for a frequent mutation in one gene did not succeed and no other mutations in the gene were found, no mutation status was assigned to the sample.

Statistical analysis

About a third of the samples included in the outcome analysis were from patients included in previous reports regarding the effect of molecular alterations on the efficacy of anti-EGFR monoclonal antibodies (webappendix pp 3–7). However, we did all statistical analyses using de-novo genotyping results that were obtained centrally. Comparisons between qualitative variables were analysed using Fisher's exact test. Logistic regression with likelihood ratio test was used to assess univariate and multivariate analyses (adjusted by age [as a continuous variable], sex [male *vs* female], number of previous chemotherapy lines and centre) with a binary endpoint. As our aim was to provide physicians with pre-treatment markers to predict efficacy of cetuximab, we did not include skin toxicity. In the *KRAS* wild-type population we had a case of quasi-complete separation of the covariate *PIK3CA* exon 20 with respect to the dependent variable objective response. In this situation, maximum likelihood for the logistic regression does not exist, and an estimated odds ratio (OR) for this covariate is not defined. Therefore, we did Fisher's exact test to compute OR, 95% CI, and p value for *PIK3CA* exon 20 in the univariate analysis. In the multivariate analysis, OR and 95% CIs were not estimable. Survival analysis was done with the Kaplan-Meier survival function followed by log-rank test, and with the Cox proportional hazard regression with the same clinical covariates as in the logistic regression. Conditional inference trees were implemented and were fitted using a method recently proposed by Hothorn and colleagues.[11] The statistical internal stop criterion in this method ensures that interpretations drawn from such trees are valid in a statistical sense—ie, with appropriate control of type I errors. Patients with missing mutation status for any gene were excluded from this analysis. To show the relative effect of each mutation on objective response, a receiver operating characteristic (ROC) curve was constructed. All p values were two-sided, and significance was assumed for a p value of less than 0·05. Analyses were done with R and SPSS version 16.0.2 for Windows. All analyses were preplanned in the statistical analysis plan.

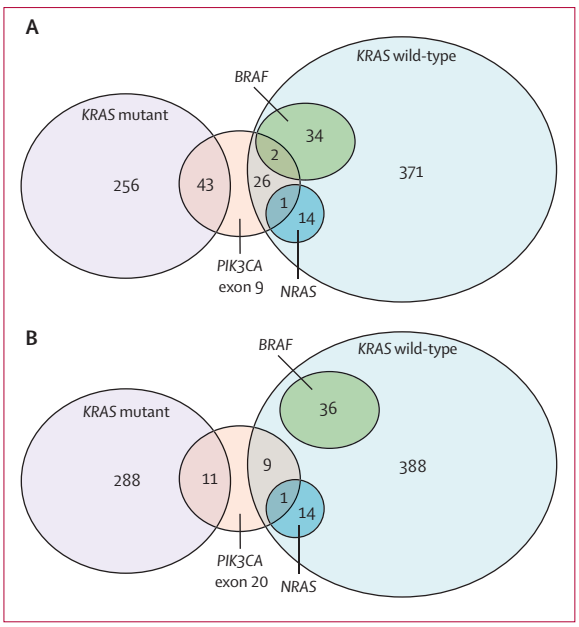

Figure 1: **Associations between mutations**
Absolute numbers of *KRAS* wild type, *KRAS* mutant, *BRAF* mutant, *NRAS* mutant, *PIK3CA* exon 9 mutant samples (A), and *PIK3CA* exon 20 mutant (B) samples are shown.

Role of the funding source
The funding source had no role in study design, data collection, data analysis, data interpretation, or the writing of the report. WDR, DB, MD, and ST had full access to all data in the study. The corresponding author had a final responsibility to submit the manuscript for publication.

Results
KRAS mutation status could not be assigned to 26 of 773 (3·4%) primary tumour samples with sufficient quality DNA. 40·0% (299/747) harboured a *KRAS* mutation (webappendix pp 9–10): 36·3% (271/747) in codon 12 or 13, 2·1% (16/747) in codon 61, and 2·0% (15/747) in codon 146. We also identified one codon 59 mutant tumour. Four tumours had a double *KRAS* mutation (p.G12V+p.G12S, p.A146T+p.Q61L, and twice p.G12V+p.A146T).

PIK3CA mutation status could not be assigned to 30 of 773 (3·9%) samples. 14·5% (108/743) of samples harboured a *PIK3CA* mutation (webappendix pp 11–12), most of which were located in exon 9 (74/108; 68·5%) or exon 20 (22/108; 20·4%). Five samples had a double *PIK3CA* mutation (p.E81K+p.H1047L, p.R93W+p.H1047R, p.E545K+p.E542K, p.E545K+p.H1047L, and p.G1049S+p.E545K). These mutation rates are similar to those described in other series of stage I–III and stage IV colorectal cancer (webappendix pp 15–16).[12–14] There was a strong association between *PIK3CA* exon 9 and *KRAS* mutations (43/292 [14·7%] in *KRAS* mutants *vs* 29/429 [6·8%] in *KRAS* wild types; p=0·0006; figure 1A), whereas this association was not found for *PIK3CA* exon 20 mutations (11/292 [3·8%] in *KRAS* mutants *vs* 10/429 [2·3%] in *KRAS* wild types; p=0·27; figure 1B).

BRAF mutation status could not be assigned to 12 of 773 (1·6%) samples. 4·7% (36/761) of samples harboured a *BRAF* mutation (webappendix p 13). 35 p.V600E mutations were found, and one p.D549G mutation. The absolute *BRAF* mutation frequency was comparable to the frequency in the COSMIC database (webappendix p 13).[9]

NRAS mutation status could not be assigned to 129 of 773 (16·7%) samples. 2·6% (17/644) of samples harboured an *NRAS* mutation (webappendix p 14). Eight of these *NRAS* mutant tumours carried a mutation not described in the COSMIC database. Although *NRAS* is a *Ras* gene closely related to *KRAS*,[10] most *NRAS* mutations occurred in codon 61, rather than in codon 12 or 13.

KRAS and *BRAF* mutations were mutually exclusive, as were *KRAS* and *NRAS* mutations, and *BRAF* and *NRAS* mutations (figure 1). 20·2% (59/292) of *KRAS* mutant tumours harboured a *PIK3CA* mutation (of which 11/292 [3·8%] exon 20 and 43/292 [14·7%] exon 9 mutations).

For the outcome analyses we focused on the 649 chemotherapy-refractory patients treated with cetuximab plus chemotherapy. *KRAS* mutation status could be assigned to 630 of 649 (97·1%) samples. For 25 of 630 (4·0%), no response data were available. Compared with carriers of wild-type *KRAS*, patients with *KRAS* mutant tumours (codon 12 [n=183], 13 [n=47], 59 [n=1], 61 [n=13], and 146 [n=9] taken together) had a significantly lower response rate (6·7% [17/253] *vs* 35·8% [126/352]; OR 0·13, 95% CI 0·07–0·22; p<0·0001), lower disease control rate (49·0% [124/253] *vs* 74·1% [261/352]; OR 0·335, 0·237–0·471; p<0·0001), and shorter median PFS (12 *vs* 24 weeks; hazard ratio [HR] 1·98, 95% CI 1·66–2·36; p<0·0001) and overall survival (32 *vs* 50 weeks; HR 1·75, 1·47–2·09; p<0·0001). Patients with the more rare codon 61 mutant tumours had a significantly lower response rate than did wild types (0/13 [0·0%] *vs* 123/345 [35·7%]; p=0·0055), but this was not the case for patients with codon 146 mutant tumours (2/11 [18·2%] *vs* 101/274 [36·9%]; p=0·34). Although almost 20% of *KRAS*-mutant tumours harboured a *PIK3CA* mutation, neither *PIK3CA* exon 20 nor exon 9 mutation statuses had a significant effect on objective response, disease control, PFS, or overall survival in patients with *KRAS*-mutant tumours (data not shown).

Since anti-EGFR monoclonal antibodies are now only prescribed in patients with *KRAS* wild-type tumours, we assessed the value of additional information on the *PIK3CA*, *BRAF*, and *NRAS* status in the *KRAS* wild-type population only.

PIK3CA mutation status could be assigned to 356 of 370 (96·2%) *KRAS* wild-type samples. For 4·8% (17/356) no response data were available. Compared with carriers of wild-type *PIK3CA*, patients with mutant *PIK3CA* as a whole had a significantly lower response rate (17·7% [6/34] *vs* 37·7% [115/305]; OR 0·35, 95% CI 0·13–0·83; p=0·015) and disease control rate (55·9% [19/34] *vs* 77·0% [234/304];

OR 0·38, 0·18–0·80; p=0·011). There was no significant difference in PFS and overall survival between patients with mutant *PIK3CA* and carriers of wild-type *PIK3CA* (median PFS 18 *vs* 24 weeks, HR 1·30, 95% CI 0·91–1·86; p=0·17; and median overall survival 39 *vs* 51 weeks; HR 1·41, 0·96–2·06; p=0·09).

As different biological effects have been suggested for *PIK3CA* exon 9 (helical domain) and exon 20 (kinase domain) mutations,[15-17] all analyses involving *PIK3CA* were also done in these two subgroups separately. Compared with *PIK3CA* wild type, *PIK3CA* exon 20 mutations had a negative effect on objective response (0·0% [0/9] *vs* 36·8% [121/329], Fisher's exact test estimated OR 0·00, 95% CI 0·00–0·89; p=0·029), disease control (33·3% [3/9] *vs* 76·0% [250/329]; OR 0·158, 0·0327–0·613; p=0·0078), PFS (median 11·5 *vs* 24 weeks, HR 2·52, 1·33–4·78; p=0·013), and overall survival (median 34 *vs* 51 weeks; HR 3·29, 1·60–6·74; p=0·0057). *PI3KCA* exon 9 mutations had no significant effect on response rate (28·6% [6/21] *vs* 36·3% [115/317] in wild types; OR 0·70, 0·25–1·78; p=0·47), disease control rate (66·7% [14/21] *vs* 75·4% [239/317] in wild types; OR 0·65, 0·26–1·78; p=0·39), median PFS (23·5 *vs* 24 weeks, HR 1·11, 0·72–1·71; p=0·65), and median overall survival (46 *vs* 51 weeks in wild types; HR 1·30, 0·82–2·05; p=0·28).

BRAF mutation status could be assigned to 368 of 370 (99·5%) *KRAS* wild-type samples. For 4·6% (17/368), no

	Unselected population			*KRAS* wild-type population		
	Mutant/wild type (n)	Adjusted odds ratio (95% CI)	LRT p value	Mutant/wild type (n)	Adjusted odds ratio (95% CI)	LRT p value
KRAS	202/291	0·098 (0·050–0·179)	<0·0001	NA	NA	NA
PIK3CA exon 9	45/448	0·515 (0·177–1·30)	0·17	17/274	0·468 (0·133–1·43)	0·19
PIK3CA exon 20	17/476	0·103 (0·0052–0·642)	0·011	8/283	0*	0·0008
BRAF	21/472	0·119 (0·018–0·438)	0·0005	21/270	0·109 (0·0165–0·410)	0·0004
NRAS	13/480	0·105 (0·0056–0·582)	0·0065	13/278	0·0867 (0·00443–0·511)	0·0040

LRT=likelihood ratio test. NA=not applicable. *95% CI were not calculated due to a quasi-complete separation of the covariate PIK3CA exon 20 with respect to the dependent variable objective response. Odds ratio (OR) values are adjusted by age, sex, number of previous chemotherapy lines, and centre. Centre was borderline significant in the *KRAS* wild-type population (p=0·051). The number of previous chemotherapy lines was significant in the unselected population (OR=0·768, 95 % CI 0·590–0·988; p=0·040).

Table 3: **Multivariate logistic regression analysis of objective response in unselected and *KRAS* wild-type population**

	Unselected population			*KRAS* wild-type population		
	Mutant/wild type (n)	Adjusted HR (95% CI)	LRT p value	Mutant/wild type (n)	Adjusted HR (95% CI)	LRT p value
KRAS	204/303	1·96 (1·58–2·43)	<0·0001	NA	NA	NA
PIK3CA exon 9	47/460	1·03 (0·73–1·45)	0·86	18/285	1·23 (0·72–2·11)	0·46
PIK3CA exon 20	18/489	1·16 (0·66–2·03)	0·62	9/294	3·30 (1·46–7·45)	0·012
BRAF	24/483	2·93 (1·85–4·65)	<0·0001	24/279	3·35 (2·08–5·39)	<0·0001
NRAS	13/494	1·82 (1·01–3·30)	0·068	13/290	1·98 (1·08–3·62)	0·042

LRT=likelihood ratio test. NA=not applicable. Hazard ratios (HR) are adjusted by age, sex, number of previous chemotherapy lines, and centre. Sex (HR 0·81, 95% CI 0·66–0·99; p=0·041), the number of previous chemotherapy lines (HR 1·23, 1·11–1·35; p<0·0001) and centre (p=0·0019) were significant covariates in the unselected population. In the *KRAS* wild-type population, only the number of previous chemotherapy lines was significant (HR 1·18, 1·03–1·34; p=0·016).

Table 4: **Multivariate Cox regression analysis of overall survival in unselected and *KRAS* wild-type population**

	Unselected population			*KRAS* wild-type population		
	Mutant/wild type (n)	Adjusted HR (95% CI)	LRT p value	Mutant/wild type (n)	Adjusted HR (95% CI)	LRT p value
KRAS	199/288	2·54 (2·04–3·16)	<0·0001	NA	NA	NA
PIK3CA exon 9	46/441	1·22 (0·88–1·69)	0·25	18/270	1·28 (0·77–2·14)	0·36
PIK3CA exon 20	18/469	1·15 (0·69–1·91)	0·59	9/279	2·27 (1·10–4·66)	0·042
BRAF	23/464	3·82 (2·38–6·12)	<0·0001	23/264	4·01 (2·46–6·53)	<0·0001
NRAS	13/474	1·79 (1·00–3·20)	0·068	13/275	1·81 (1·00–3·28)	0·069

LRT=likelihood ratio test. Hazard ratios (HR) are adjusted by age, sex, number of previous chemotherapy lines, and centre. Both the number of previous chemotherapy lines (HR 1·14, 1·02–1·26; p=0·018) and centre (p=0·0060) were significant covariates in the unselected population, whereas in the *KRAS* wild-type population only centre was borderline significant (p=0·056). NA=not applicable.

Table 5: **Multivariate Cox regression analysis of progression-free survival in unselected and *KRAS* wild-type population**

response data was available. Compared with *BRAF* wild types, *BRAF* mutants had a significantly lower response rate (8·3% [2/24] *vs* 38·0% [124/326] for wild types; OR 0·15, 95% CI 0·02–0·51; p=0·0012) and disease-control rate (37·5% [9/24] *vs* 77·3% [252/326]; OR 0·176, 0·071–0·41; p<0·0001), and shorter PFS (median 8 *vs* 26 weeks in wild types; HR 3·74, 95% CI 2·44–5·75; p<0·0001) and overall survival (median 26 *vs* 54 weeks in wild types, HR 3·03, 1·98–4·63; p<0·0001). One of the two *BRAF* mutants that responded had a p.D594G mutation; the other had a p.V600E mutation, present in low copy number in the tumour.

NRAS mutation status could be assigned to 315 of 370 (85·1%) *KRAS* wild-type samples. For 4·4% (14/315), no response data were available. *NRAS* mutants had a significantly lower response rate (7·7% [1/13] *vs* 38·1% [110/289]; OR 0·14, 95% CI 0·007–0·70; p=0·013) than did wild types. There was no significant difference between *NRAS* wild types and mutants in disease control rate (53·8% [7/13] *vs* 77·9% [225/289]; OR 0·332, 0·11–1·06; p=0·063), median PFS (14 *vs* 26 weeks; HR 1·82, 1·04–3·18; p=0·055), and median overall survival (38 *vs* 50 weeks; HR 1·89, 1·05–3·39; p=0·051).

Multivariate analyses for objective response, disease control, overall survival, and PFS were done using the mutation status of *KRAS*, *PIK3CA* exon 20, *PIK3CA* exon 9, *BRAF*, and *NRAS*, and age, sex, number of previous chemotherapy lines, and centre as covariates. The significant associations between outcome and *KRAS*, *PIK3CA* exon 20, and *BRAF* mutation status was confirmed in multivariate analyses (tables 3, 4, and 5, and webappendix p 17). Compared with wild types, *NRAS* mutations were significantly associated with lower response and disease control rates, and shorter overall survival, whereas no significant difference in PFS was seen. *PIK3CA* exon 9 mutation status was not retained in the multivariate analysis for any of the outcomes.

The recursive partitioning algorithm uses p values to choose the covariate that will be used to split the population, since p values are a measure that combines strength and incidence. Therefore, conditional inference trees (figure 2) provide a clear representation of the effect of each of the different mutations in clinical practice. The same mutations were retained as in the multivariate analysis for objective response and PFS. For overall survival, *NRAS* was not retained. *PIK3CA* exon 9 mutation status was

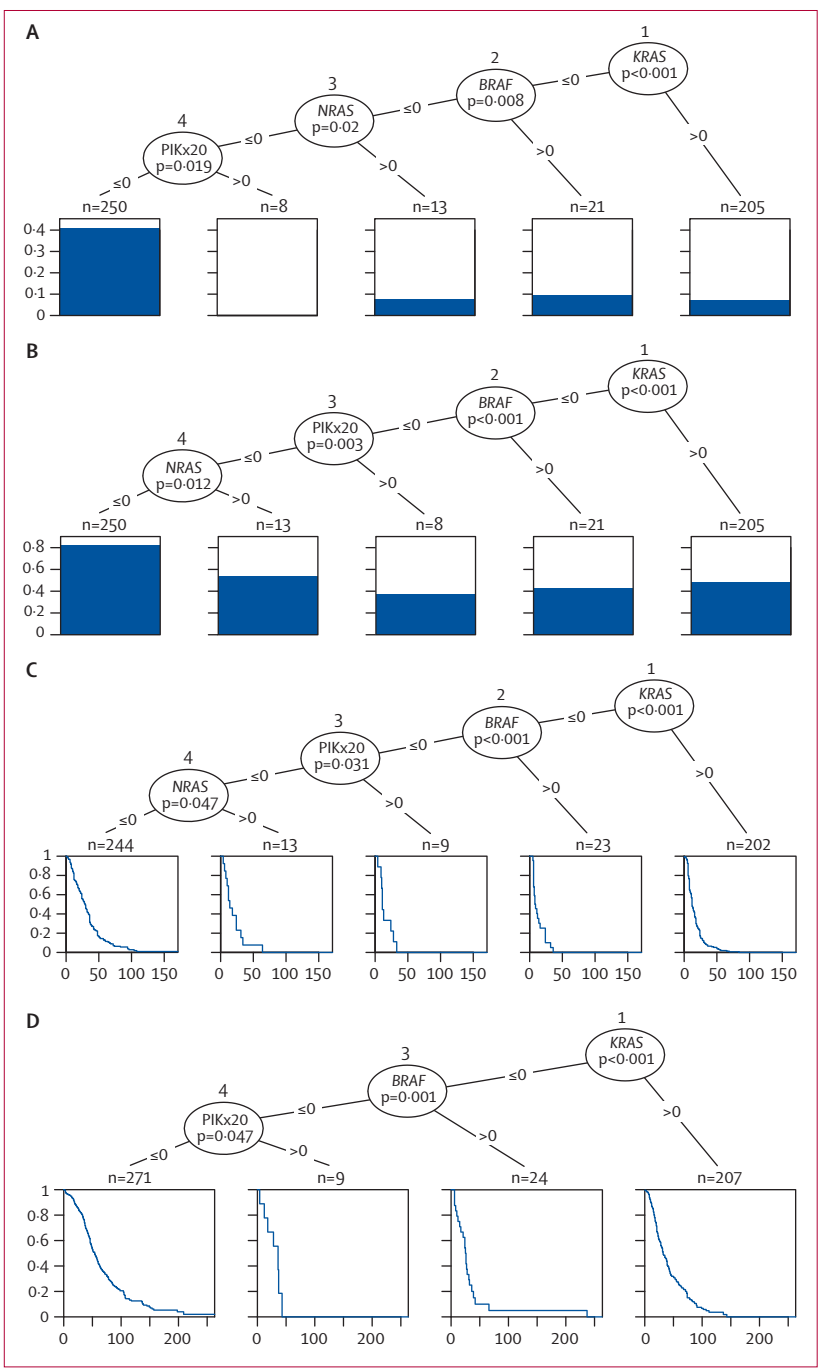

Figure 2: **Conditional inference trees for objective response (A), disease control (B), progression-free survival (C), and overall survival (D)**
p values correspond to permutation test.[11] (A) The fraction of objective responders in each terminal node is indicated by the height of the blue bar. The tree shows that to best predict response to cetuximab, *KRAS* mutations should be assessed first (36·3% response rate), *BRAF* second (38·4% response rate), *NRAS* third (39·9% response rate), and *PIK3CA* exon 20 fourth (41·2% response rate). *PIK3CA* exon 9 mutation state is not retained in the model. (B) The tree shows that to best predict disease control, *KRAS* mutations should be assessed first (76·7% disease control), *BRAF* second (79·3% disease control), *PIK3CA* exon 20 third (80·6% disease control), and *NRAS* fourth (82·0% disease control). *PIK3CA* exon 9 mutation state is not retained in the model. (C) Kaplan-Meier estimates of the progression-free survival time (in weeks) are shown in the terminal nodes. The tree shows that to best predict progression-free survival (PFS), *KRAS* mutations should be assessed first (median PFS 24 weeks; range 24–29), *BRAF* second (median PFS 28 weeks; range 24–30), *PIK3CA* exon 20 third (median PFS 28 weeks; range 24–31), and *NRAS* fourth (median PFS 29 weeks; 24–32). *PIK3CA* exon 9 mutation state is not retained in the model. (D) Kaplan-Meier estimates of the overall survival time (in weeks) are shown in the terminal nodes. The tree shows that to best predict overall survival, *KRAS* mutations should be assessed first (median overall survival 49 weeks; range 46–55), *BRAF* second (median overall survival 52 weeks; range 47–58), and *PIK3CA* exon 20 third (median overall survival 54 weeks; range 48–59). *NRAS* and *PIK3CA* exon 9 mutation state are not retained in the model. PIKx20=*PIK3CA* exon 20. >0=mutant state. ≤0=wild-type state.

included in the analysis, but was not retained in the trees. The fact that the results were in line with the multivariate statistical analysis suggests there was no overfitting.

Discussion

Following the discovery that mutant *KRAS* is associated with resistance to anti-EGFR monoclonal antibodies, tumours of all patients with metastatic colorectal cancer are now profiled for seven *KRAS* codon 12 and 13 mutations before receiving cetuximab or panitumumab.[4,18,19] However, up to 65% of patients with *KRAS* wild-type tumours are resistant to anti-EGFR monoclonal antibodies.[4] In, to our knowledge, the largest series to date, we studied the effects of not only *KRAS*, but also of *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in patients with chemotherapy-refractory metastatic colorectal cancer.

In addition to confirming the lack of benefit of cetuximab in patients with common *KRAS* mutations, the sample size of our series allowed us to study the effect of the rare *KRAS* codon 61 and codon 146 mutations. Since patients with codon 61 mutant tumours have a lower response rate than wild types and, because the mutation incidence (2%) is similar to some codon 12 mutations, we suggest that codon 61 should be included in *KRAS* mutation testing. By contrast with a previous report based on one patient,[20] codon 146 mutations do not affect cetuximab efficacy. The co-occurrence of codon 146 mutations with other *KRAS* mutations is an additional indication that this might not be an important oncogenic codon. The one sample harbouring both *KRAS* p.G12V and p.G12S mutations possibly represents a polyclonal tumour.

We report for the first time the association between *KRAS* mutations and *PIK3CA* exon 9 (but not exon 20) mutations in colorectal cancer. This is in line with the finding that the gain of function induced by *PIK3CA* exon 20 mutations (coding for the kinase domain) is independent of Ras binding in vitro, whereas that of exon 9 mutations (helical domain) requires Ras–GTP interaction.[15] Moreover, our data suggest that only *PIK3CA* exon 20 mutations are associated with worse outcome after cetuximab, and might represent a different biological subgroup, which is also suggested by in-vitro data.[15] The small p values in the outcome analyses indicate a resistance effect, but because of the wide confidence intervals, due to the low numbers of patients with *PIK3CA* exon 20 mutations (n=9), the size of the effect is uncertain, and these data need to be independently confirmed. By contrast, for exon 9, the wide confidence intervals together with the high p values suggest a weak effect or no resistance. Although until now the effect of *PIK3CA* mutations as a whole has been reported in metastatic colorectal cancer,[21,22] it makes little sense to interpret the estimate of the effect of *PIK3CA* when exon 9 and exon 20 mutations are considered together if, as our data suggest, these mutations have different effects on cetuximab efficacy. The estimated *PIK3CA* effect is otherwise a mean effect, which depends on the relative proportions of exon 9 and exon 20 mutations and is difficult to interpret. These data seem to reconcile the previous conflicting reports obtained by members of the consortium with respect to the role of *PIK3CA* mutations. Specifically, the cohort reported by Sartore-Bianchi and colleagues[21] contained more exon 20 mutations (10%) and fewer exon 9 mutations (3·6%) than did other series, including the series by Prenen and colleagues[22] (webappendix pp 15–16), leading to different results.

We confirm that *KRAS* and *BRAF* mutations are mutually exclusive. We further confirm the inefficacy of cetuximab in patients with *BRAF* mutant metastatic colorectal cancer, which has been suggested in smaller series.[5–7] The only *BRAF* non-p.V600E mutation (p.D594G) that we identified was found in the tumour of a patient who had an objective response. This is in accordance with the observation in cell lines that a p.D594G mutation cannot directly activate MAPK signalling, and that p.D594G mutants have low levels of phospho-MAP2K (MEK) and are resistant to MAP2K inhibition.[23] Of note, the p.V600E mutation was present in low copy number in the tumour of the only *BRAF* p.V600E mutant responder. The mutation prevalence of *BRAF* is heavily dependent on the patient population studied, as the mutation confers such a bad prognosis in metastatic disease[14,24,25] that *BRAF*-mutant populations decline in later lines of therapy. The prevalence of *BRAF* mutations seen here (4·7%) is comparable with the mutation frequency seen in another series of heavily pretreated metastatic colorectal cancer,[6] but lower than described in the first-line metastatic setting (7·9%),[24] which mirrors the frequency in the adjuvant

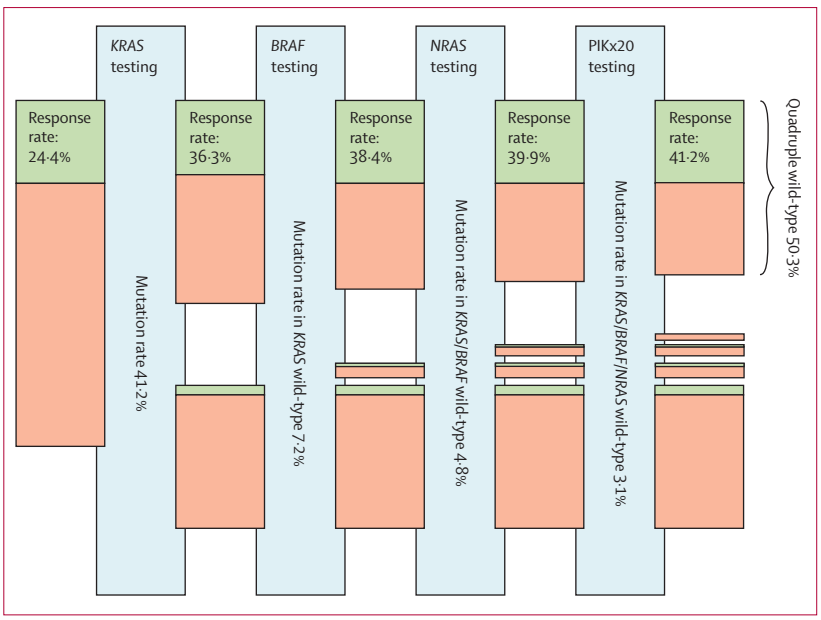

Figure 3: **The improvement in response prediction gained by assessing the mutation status of each gene**
Patients with missing data for any of the markers studied in this analysis were omitted from the start. The green bars represent responders; the orange bars non-responders. Bottom bars represent mutant tumours; upper bars wild-type tumours. The size of the bars is in agreement with the corresponding percentages. PIKx20=*PIK3CA* exon 20.

setting.[26] Because of the strong prognostic effect of a *BRAF* mutation, overall survival is not a good endpoint to assess its possible predictive effect in our study. Objective response, on the other hand, gives a good estimate of the treatment effect,[8] and our results show a significantly lower response rate for *BRAF* mutants in the chemotherapy-refractory setting (8% *vs* 38% for *BRAF* wild types). Recently, it was suggested that *BRAF* mutants could gain some benefit from adding cetuximab to leucovorin, fluorouracil, and irinotecan in first-line metastatic colorectal cancer compared with chemotherapy alone,[25] but the sample size was too small to draw conclusions. However, this seems very unlikely, since the absolute benefit from cetuximab treatment remains very small for patients with chemotherapy-refractory metastatic colorectal cancer with a *BRAF*-mutant tumour, compared with the *BRAF* wild-type population.

NRAS is closely related to *KRAS*.[10] Our results confirm the inefficacy of cetuximab in *NRAS*-mutant metastatic colorectal cancer, which was already suggested in a smaller series.[7] NRAS mutations are significantly associated with lower response and disease control rates in the multivariate analyses and are retained in the conditional inference trees. Because the number of patients with NRAS mutations is low, the magnitude of the effect on survival is still unclear, as shown by the large confidence intervals. Our data are supported by a recent analysis by Peeters and colleagues.[27] In a randomised clinical trial setting, they showed that *NRAS* mutations are associated with lack of response to the other EGFR inhibitor, panitumumab. *NRAS* mutations do not occur very frequently in colorectal cancer (2·64%), but nor do some *KRAS* codon 12 mutations that are routinely being tested in clinical practice (eg, p.G12S occurs at a frequency of 2·54% and p.G12R at 0·67%). Based on these data, we propose to include *NRAS* mutation genotyping to aid the prediction of response to cetuximab.

The conditional inference tree for response (figure 2A) provides a clear representation of the effect of each of the different mutations: *KRAS* should be tested first, and if *KRAS* is not mutated, *BRAF* second, *NRAS* third, and *PIK3CA* exon 20 fourth. *PIK3CA* exon 9 mutation status does not seem to add relevant information for the clinician in the decision-making process. Assessment of these mutations could result in improvements in objective response rates: objective response rates in our series were 24·4% in the unselected population, 36·3% in the *KRAS* wild-type selected population, and 41·2% in the *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* exon 20 wild-type population (figure 3).

Our aim was to bring us another step closer to personalised medicine. The AUC estimate for response of 0·735 (figure 4) after negative selection for the four different genotypes shows that a large part of the benefit in quadruple wild-type patients remains unclear. Other potentially negative factors should be studied, such as loss of expression of phosphatase and tensin homologue (PTEN; which normally inhibits PI3-kinase-initiated signalling). It has been suggested that loss of PTEN expression, as measured by immunohistochemistry, is associated with a lack of benefit of cetuximab in patients with chemotherapy-refractory metastatic colorectal cancer. Some report an effect on response rate,[5] whereas others only report an effect on overall survival.[6] However, as no validated antibody, threshold, or protocol is available yet for PTEN expression analysis by immunohistochemistry on FFPE tissue, PTEN expression data cannot be used legitimately at this point in outcome analyses. Positive factors leading to EGFR dependency of the tumour need to be identified to further refine response prediction. One

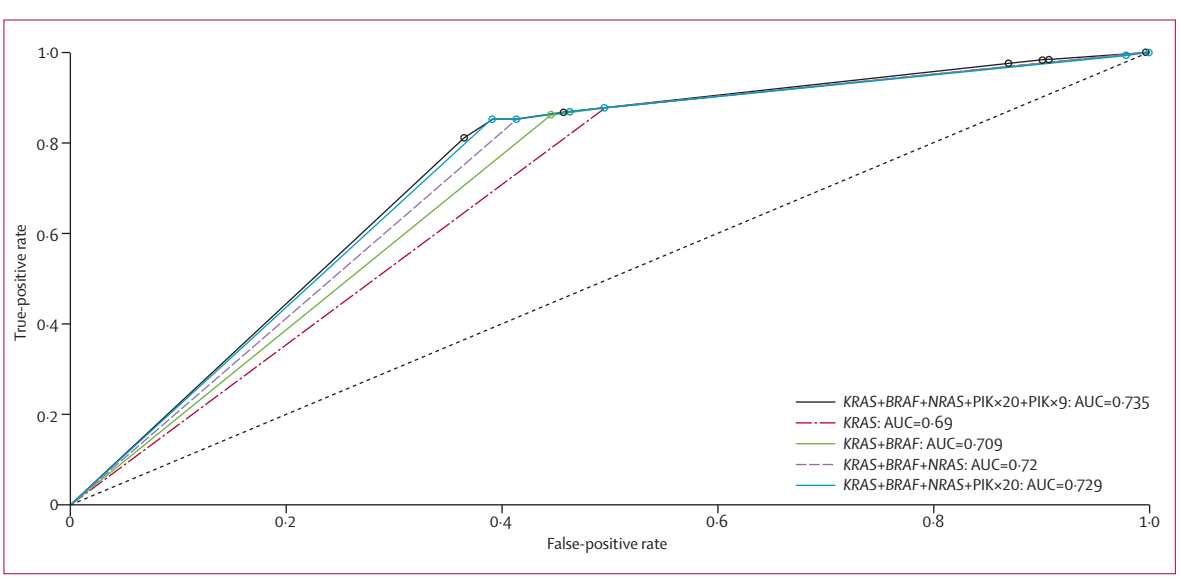

Figure 4: **Receiver-operating characteristic curve for objective response**
PIKx9=*PIK3CA* exon 9. PIKx20=*PIK3CA* exon 20.

good positive predictor of response would be of much more interest than four or more negative predictors, since this would enable physicians to better target a drug to a potentially benefiting population. Increased *EGFR* copy number has been found to be associated with tumour response,[6,28,29] and the level of sensitivity to cetuximab was shown to be proportional to the level of mRNA expression in the tumour of two EGFR ligands, epiregulin and amphiregulin.[30,31] Cetuximab might also exert anti-tumour effects through antibody-dependent cell-mediated cytotoxicity in which the Fc fragments of IgG antibodies interact with Fc-γ receptors (FCGR) expressed by innate immune cells. FCGR 2A and 3A polymorphisms might also be associated with better clinical outcome after cetuximab, but the reports on this topic are not totally concordant.[32–34] The data for these potential positive predictors of benefit to cetuximab need to be further investigated and validated.

Since we retrospectively genotyped samples from patients in single-arm studies, we cannot determine whether the mutations we assessed have prognostic or true predictive effects, attributable to a modifying effect on cetuximab sensitivity. In the randomised CO.17 trial *KRAS* was shown not to be a strong prognostic factor in chemotherapy-refractory metastatic colorectal cancer.[3] However, in the randomised MRC FOCUS trial in first-line metastatic colorectal cancer, mutation in *KRAS* was a poor prognostic factor for overall survival.[24] *PIK3CA* mutations as a whole were associated with shorter cancer-specific survival in patients with *KRAS* wild-type tumours in a series of stage I–III colorectal cancers,[12] but exon 9 and 20 mutations were not studied separately. In breast cancer, a distinctive prognostic role of exon 20 and exon 9 mutations has been described.[18,19] It is clear that randomised controlled trials with prospectively planned biomarker analyses are needed to distinguish between the prognostic and predictive effects of these mutations.

In conclusion, our results provide a clear picture of the efficacy of cetuximab plus chemotherapy in patients with chemotherapy-refractory metastatic colorectal cancer according to the presence or absence of activating *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations. The gain in information from genotyping tumour samples for these mutations will have to be weighed against the cost and the feasibility of genotyping FFPE tumour samples before therapy. Advances in genotyping assays, preferably dedicated drug-specific multiplex assays combining positive and negative molecular predictors, are within our reach, and will provide the necessary advance for true personalised medicine.

Contributors

ST had responsibility for the integrity and accuracy of a data. ST, HP, WDR, and MD were responsible for the study concept and design. ST, HP, WDR, MD, and DB drafted the report. ST and WDR were responsible for data collection. MD, HP, and DB did the statistical analyses. ST, HP, WDR, and MD did the data analysis. BC, JDS, BB, WDR, and DL were responsible for Sequenom assay design and genotyping. All authors revised the report. ST, DL, BC, JDS, and BB were involved with administrative, technical, or material support. GF, KTK, VK, DP, PL-P, FP-L, PR, BV, DS, GT, FC, MF, FM, PS, SDD, MM, AB, SS, AS-B, JT, TM, FDF, AO-G, FCi, PP, CQ, TPH, EVC, and ST provided tumour samples.

Conflicts of interest

DP has received honoraria for participation on a Merck Serono advisory board. PL-P has been a consultant for Merck Serono and Amgen, received grants from Merck Serono and Myriad Genetics, and has travel and accommodations expenses covered or reimbursed by Merck Serono and Amgen. FP-L was a member of the advisory board for Merck Serono and Amgen, and her institution has received grants from Merck Serono and Roche. PR is a member of the advisory board of Merck Serono, a consultant for Pfizer and Sanofi-Aventis, and has received honoraria from Merck Serono and grants from Roche, and travel and accommodation expenses covered or reimbursed from Merck Serono and Amgen. JT is a member of the advisory board of Merck Serono, Amgen, Imclone, and Bristol-Myers Squibb, has received payment for the development of educational presentations by Merck Serono and Amgen, and has received travel and accommodation expenses covered or reimbursed from Merck Serono and Amgen. AB has received honoraria from Amgen and Roche and travel and accommodation expenses from Bristol-Myers Squibb. AS-B has received speaker fees and honoraria for contributing to medical advisory panels from Roche and Amgen. SS has received honoraria for contributing to advisory boards for Amgen, Roche, Merck Serono, Celgene, and AstraZeneca. FDF has received honoraria from Merck Serono and Amgen. FCi has received honoraria from Merck Serono and Roche. EVC has received grants from Merck Serono and Amgen. ST has received research grants from Merck Serono. All other authors declared no conflicts of interest.

Acknowledgments

WDR and BC are supported by a PhD grant from the institute for the Promotion of Innovation through Science and Technology in Flanders, Belgium (IWT-Vlaanderen). Translational research on the part of the Hellenic Cooperative Oncology Group was supported by research grant HE_R6ER/05. FC and FCi are supported by Associazione Italiana Ricerca Cancro (AIRC). PL-P, FP-L, and PR thank INCA 2009-1-RT-03. MF received grants from Oncosuisse and from the Tessin Foundation for Cancer Research (Fondazione Ticinese per la Ricerca sul Cancro). The laboratory of AB was supported by AIRC, Regione Piemonte. AS-B and SS are supported by AIRC and Oncologia Ca' Granda Onlus (OCGO) Fondazione. ST and EVC are senior clinical investigators of the Fund for Scientific Research, Flanders, Belgium (Fonds Wetenschappelijk Onderzoek–Vlaanderen). DL, ST, and EVC received research grants from the Belgian Federation against Cancer (Stichting tegen Kanker), and DL and ST received grants from the Belgian National Cancer Plan (Nationaal Kankerplan). ST also received a grant from the Fund for Scientific Research, Flanders, Belgium (Fonds Wetenschappelijk Onderzoek – Vlaanderen). MD was supported by the Swiss National Center of Competence in Research (NCCR) in Molecular Oncology of the Swiss National Science Foundation, and by grants from the MEDIC foundation. This work was funded by personal academic grants.

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