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Vascular endothelial growth factor D expression is a potential biomarker of bevacizumab benefit in colorectal cancer

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Background: Bevacizumab prolongs progression-free survival (PFS) in patients with metastatic colorectal cancer. We analysed the protein expression levels of vascular endothelial growth factor (VEGF) ligands and receptors to determine their prognostic and predictive effects.

Methods: We graded expression of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-R1, and VEGF-R2 to assess whether overexpression predicted bevacizumab resistance in samples from 268 of 471 patients randomised to capecitabine (C), capecitabine and bevacizumab (CB), or CB and mitomycin (CBM) in the MAX trial and extended the analysis to the CAIRO-2 population.

Results: Patients with low expression of VEGF-D (0, 1+) benefited from bevacizumab treatment (PFS hazard ratio (HR) (C *vs* CB + CBM), 0.21; 95% CI, 0.08–0.55; overall survival (OS) HR, 0.35; 95% CI, 0.13–0.90). Patients with higher VEGF-D expression received less benefit (VEGF-D 2+ PFS HR, 0.67; 95% CI, 0.45–1.00; OS HR, 0.82; 95% CI, 0.52–1.30; VEGF-D 3+ PFS HR, 0.77; 95% CI, 0.50–1.17; OS HR, 1.28; 95% CI, 0.79–2.09) (P interaction <0.05). In CAIRO-2, there was no difference in PFS or OS according to VEGF-D expression.

Conclusions: The predictive value of VEGF-D expression for bevacizumab may depend on the chemotherapy backbone used. Further evaluation is required before clinical utilisation.

Angiogenesis is necessary for tumour proliferation and metastasis and presents an attractive target for drug therapy. Circulating angiogenic factors include the vascular endothelial growth factor (VEGF) family (A–D), placental growth factor (PlGF), platelet-derived growth factors (PDGFs), and fibroblast growth factors (FGFs). They bind to a range of receptors, including VEGF receptors (VEGFRs) 1–3 on endothelial and tumour cells, to regulate angiogenesis (Kerbel, 2008). Bevacizumab, a monoclonal antibody that binds to and inactivates VEGF-A, is an antiangiogenic drug that, when used in combination with chemotherapy, improves progression-free survival (PFS) in patients with metastatic colorectal cancer (Hurwitz *et al*, 2013). However, the benefit gained is modest (Hurwitz *et al*, 2005; Saltz *et al*, 2008; Tebbutt *et al*, 2010), highlighting the need to identify responsive and resistant subgroups through biomarker studies.

Although several studies have been undertaken, none has identified or validated a clinically applicable predictive biomarker for bevacizumab efficacy (Lambrechts *et al*, 2013). These studies

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have included: serum or plasma biomarkers such as VEGF-A levels; tissue biomarkers such as VEGF-A, VEGFR-2, and neuropilin-1; levels of circulating endothelial cells; imaging changes, VEGF or VEGFR single-nucleotide polymorphisms (SNPs); and dynamic biomarkers, such as hypertension (Jubb *et al*, 2006; Dowlati *et al*, 2008; Schneider *et al*, 2008; Foernzler *et al*, 2010; Jubb and Harris, 2010; Jubb *et al*, 2011; Van Cutsem *et al*, 2012; Miles *et al*, 2013).

It is recognised that there may be redundancy among family members of angiogenic regulators (Achen and Stacker, 1998; Cao *et al*, 1998; Pan *et al*, 2007; Zhang *et al*, 2010). We hypothesised therefore that related VEGF family members such as VEGF-C and VEGF-D may continue to stimulate angiogenesis despite inhibition of VEGF-A by bevacizumab (Jubb *et al*, 2011).

The AGITG MAX trial was an investigator-initiated study evaluating the effect on PFS of adding bevacizumab to capecitabine chemotherapy as first-line therapy for metastatic colorectal cancer. We evaluated the expression of angiogenesis-related factors (ARFs) VEGF-A to VEGF-D, VEGFR-1, and VEGFR-2 in tumour tissue as predictors of efficacy of bevacizumab by correlating the expression with clinical outcomes in the MAX study and for significant biomarkers attempted to support the result in separate tumour specimens from patients in the CAIRO2 trial (Tol *et al*, 2009).

SUBJECTS AND METHODS

Patients and study design. The primary objective of the phase III MAX study (Tebbutt *et al*, 2010) was to evaluate the effect on PFS of adding bevacizumab with or without mitomycin to capecitabine among patients receiving first-line chemotherapy for metastatic colorectal cancer. Eligible patients were enrolled between July 2005 and June 2007 and randomly assigned to capecitabine (C), capecitabine and bevacizumab (CB), or capecitabine, bevacizumab, and mitomycin (CBM). Patients were evaluated for tumour response or progression every 6 weeks. Treatment was continued until disease progression in the absence of significant toxicity. All patients provided written informed consent for the main study, and most provided additional optional consent for provision of tumour tissue for biomarker analyses. Separate ethics approval for biomarker studies was obtained centrally.

Tumour collection and processing. Formalin-fixed, paraffin-embedded samples of tumour tissue from archival specimens collected at the time of diagnosis were retrieved where possible. Although the majority of specimens (83%) were from the primary tumour, a minority only had specimens from metastatic sites to assess. Fourteen patients had both primary and secondary specimens to compare the expression levels. Tumour blocks were collected and analysed centrally by technicians blinded to trial outcome data.

Guided by a haematoxylin and eosin-stained slide, three representatve 1-mm adjacent tumour cores were extracted from each patient's tumour section and assembled into a recipient block using a Beecher mark II tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Cores from a renal cortex specimen were also inserted on each tissue microarray (TMA) for orientation and to serve as a positive control for staining.

Immunohistochemical analysis. Four 1-μm sections were cut from each TMA and mounted on individual microscope slides. The sections were deparaffinised by baking at 60 °C for 1 h, followed by two washes each with xylene and then with ethanol. Slides were rehydrated with a wash in deionised water. Antigen retrieval was performed for 30 min at 100 °C using Citrate (Labvision, Fremont, CA, USA) or for 60 min with Dako target retrieval solution (Dako, Glostrup, Denmark). No antigen retrieval was required for the anti-VEGF-B antibody. After cooling for 20 min, slides were washed in tap water and then twice in trisethanolamine-buffered saline (Dako) with 0.05% Tween 20 detergent (TBS-T). Endogenous peroxidase was quenched by incubating the slides in 3% hydrogen peroxide for 10 min at room temperature, and the slides were washed twice more with TBS-T.

Slides were incubated with primary antibody at room temperature or at 37 °C at an optimised concentration (Supplementary Table S1). Slides were washed twice more in TBS-T before exposure to the appropriate secondary antibody at room temperature, again washed twice in TBS-T, and incubated with chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St Louis, MO, USA) for 5–15 min until a signal developed. They were then washed in water and counterstained with haematoxylin and Scott's solution (20 g $MgSO_4$ and 3.5 g $NaHCO_3$ per litre DH_20). Following further washes in water, slides were coated with CC/Mount (Sigma-Aldrich) aqueous mounting medium and coverslips were applied. For each antibody, a negative control was prepared by parallel staining of each slide with an appropriate subclass control antibody.

Scoring was performed independently by two investigators, who were blinded to treatment allocation and outcome. Grading of intensity and extent of staining, in tumour cells but not adjacent stroma, was 0 = negative; 1 = weak widespread/very limited moderate staining (i.e., <25% of cells); 2 = moderate widespread; and 3 = strong widespread staining (Supplementary Figure S1). Where possible, the patient's metastatic tissue rather than primary tumour was assessed for expression. For the purpose of this analysis, when there was a discrepancy in the scores for a given patient, it was resolved by consensus on a second review by two scorers.

Statistical analysis. Statistical analyses were in accordance with a protocol for statistical analysis developed with blinding to treatment allocation and patient outcomes. No prior information was available regarding the distribution of these biomarkers. The final choice of cut points for each biomarker (0, 1+; 2+; 3+) was based on a pooled distribution of biomarkers in all the three treatment groups. Tests for each biomarker considered these as ordered categories.

All patients for whom data on biomarker expression were available were included in the analysis. PFS, the primary end point, was defined as the time from randomisation until documented evidence of disease progression according to the Response Evaluation Criteria in Solid Tumours (RECIST, version 1.0), occurrence of new disease, or death from any cause. The secondary end point was OS, defined as the time from randomisation until death from any cause.

Each of the six biomarkers was initially analysed individually. The PFS of patients according to biomarker expression (0, 1+ *vs* 2+ *vs* 3+) and treatment group were summarised in Kaplan–Meier curves, and the differences between these groups were compared in a log-rank test. A proportional-hazards model with biomarker expression, a treatment covariate (C *vs* CB and CBM), and their interaction was used to assess whether increasing biomarker expression predicted resistance to bevacizumab. Each analysis was adjusted for baseline clinicopathological factors, using the same variables identified to be significant in multivariate models of the intention-to-treat MAX population.

Multivariate proportional-hazards analysis with treatment, all six biomarkers, and their individual treatment-by-biomarker interactions assessed the predictive values of these biomarkers simultaneously. Only statistically significant biomarkers and the biomarker interactions ($P < .05$) were retained in the final multivariate model. A global assessment of the predictive values of all biomarkers combined was tested using the log-likelihood ratio test to compare this multivariate model with another model with treatment and the expression of all six biomarkers only.

The reported *P*-values were adjusted for the effects of all six biomarkers and their interactions with treatment; $P<0.05$ indicated that overexpression of biomarkers predicts resistance to bevacizumab. These *P*-values were not adjusted for multiple comparisons. Similar methods were adopted in assessing the predictive values of these biomarkers for OS. All reported *P*-values were two-sided and not adjusted for multiple comparisons.

Secondary cohort. We attempted to support the findings for VEGF-D expression with an independent patient population from the CAIRO2 trial (Tol *et al*, 2009). In this study, 251 patients with metastatic colorectal cancer were randomised to capecitabine, oxaliplatin, and bevacizumab (control arm) or the same regimen plus weekly cetuximab (experimental arm). Only tumour samples, in the form of a TMA, from patients in the control arm (all treated with bevacizumab) were analysed, using the same methods as for the MAX samples. We hypothesised that, if there was a prognostic difference in the VEGF-D 0–1+ compared with 2–3+ patients in PFS and OS, this would support but not validate our findings.

RESULTS

Characteristics of the patients. Of the 471 randomised patients, 389 consented to the biomarker analysis study, from whom 268 tumour specimens were available for examination of VEGF and VEGFR expression (57% of the study population) (Figure 1). They were representative of the study population (Supplementary Table S2). The median follow-up time for these patients was 30.6 months (range 1.2–42.4 months). Tumour specimens from the remaining patients could not be retrieved or were unsuitable for TMA construction. The grading of staining intensity and its distribution for each biomarker is illustrated in Supplementary Figure S2. The concordance between the independent scorers was determined by weighted Kappa score and was >0.79 for each biomarker. Fourteen patients had matched primary and secondary specimens allowing comparison of tissue expression between each, with good-to-very-good concordant expression (weighted Kappa scores >0.70).

Progression-free survival. When each of the six biomarkers was examined singly, only VEGF-D predicted the effect of bevacizumab on PFS (Table 1). Among patients with VEGF-D expression scores of 0 or 1+, the median PFS was 5.8 months in the C group and 16.8 months in the CB + CBM group ($P=0.0001$; Figure 2A, Table 2). Among patients with VEGF-D expression 2+, the median PFS was 6.0 months in the C group and 8.8 months in the CB + CBM group ($P=0.05$). Among patients with VEGF-D expression 3+, the median PFS was 7.0 months in the C group and 9.0 months in the CB + CBM group ($P=0.22$; Figure 2A, Table 2). The additional benefit of bevacizumab was significantly greater among the patients with lower expression of VEGF-D than among those with higher expression of VEGF-D ($P=0.02$ for the interaction between VEGF-D expression and treatment group; Figure 3). The interaction remained significant after multivariate adjustments for baseline factors (performance status, prior resection of the primary tumour, number of organ sites of metastasis, baseline serum alkaline phosphatase, and baseline serum bilirubin). For the other biomarkers, the interactions between biomarker expression and treatment were not significant (Table 1). In the step-down multivariable analysis with a treatment covariate and individual treatment-by-biomarker interactions, only treatment, VEGF-D, and treatment-by-VEGF-D interaction remained significant. In a step-down multivariable model where only significant predictors were retained, treatment, VEGF-D, and treatment-by-VEGF-D interaction were the only predictors. However, the global interaction was not significant for PFS (log-likelihood $\chi^2=8.23$ (with six degrees of freedom); $P=0.22$).

Overall survival. Among patients with VEGF-D expression 0 or 1+, the median OS was 18.9 months in the C group and the median OS was not reached in the CB + CBM group ($P=0.03$; Figure 2B, Table 2). Among patients with VEGF-D expression 2+, the median OS was 20.6 months in the C group and 21.6 months in the CB + CBM group ($P=0.40$). Among patients with VEGF-D expression 3+, the median OS was 24.5 months in the C group and 19.4 months in the CB + CBM group ($P=0.32$). Therefore, the additional benefit of bevacizumab for OS was only evident in patients with lower expression of VEGF-D ($P=0.01$ for the interaction between VEGF-D expression and the assigned treatment; Figure 2B). The interaction remained significant after multivariate adjustments for baseline clinicopathological factors (performance status, prior resection of the primary tumour, prior radiotherapy, baseline serum alkaline phosphatase, and baseline serum neutrophils). The interaction between VEGF-A expression and assigned treatments was not significant (Table 1). For the other biomarkers, the interactions between biomarker expression and assigned treatments were also significant when examined singly for VEGF-B, VEGF-C, VEGFR-1, and VEGFR-2.

In the step-down multivariable model with the treatment and individual treatment-by-biomarker interactions, only treatment, VEGFR-1, and treatment by VEGFR-1 interaction remained

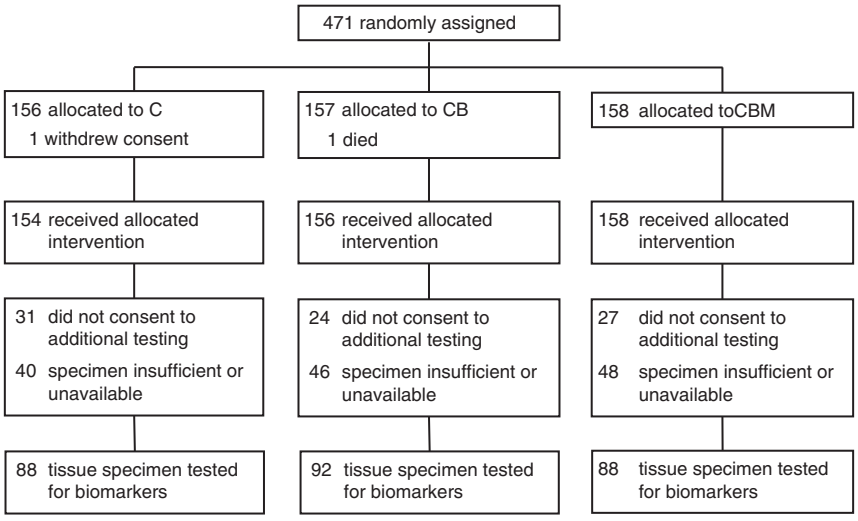

Figure 1. CONSORT diagram.

Table 1. Treatment effect for progression-free and overall survival according to the level of expression for each biomarker

		Treatment effect (C *vs* CB + CBM)[a]							
		0, 1+		2+		3+	Interaction P[b]		
Biomarker	*n*	HR (95% CI)	*n*	HR (95% CI)	*n*	HR (95% CI)	*P*[c]	*P*[d]	*P*[e]
Progression-free survival									
VEGF-A	64	0.44 (0.26–0.76)	141	0.64 (0.44–0.93)	62	0.84 (0.48–1.48)	0.15	0.22	0.30
VEGF-B	105	0.47 (0.30–0.73)	91	0.80 (0.51–1.24)	71	0.80 (0.46–1.38)	0.11	0.16	0.69
VEGF-C	113	0.55 (0.37–0.83)	83	0.60 (0.36–1.00)	70	0.72 (0.43–1.21)	0.40	0.78	0.19
VEGF-D	32	0.22 (0.08–0.55)	117	0.67 (0.45–1.00)	110	0.77 (0.50–1.17)	0.02	0.04	0.04
VEGFR-1	85	0.42 (0.26–0.68)	89	0.95 (0.57–1.57)	87	0.65 (0.41–1.04)	0.21	0.49	0.55
VEGFR-2	101	0.51 (0.33–0.79)	102	0.60 (0.37–0.96)	62	0.84 (0.50–1.44)	0.19	0.35	0.95
Overall survival									
VEGF-A	64	1.00 (0.53–1.86)	141	0.75 (0.49–1.14)	62	1.18 (0.63–2.21)	0.74	0.98	0.86
VEGF-B	105	0.55 (0.33–0.91)	91	1.12 (0.67–1.85)	71	1.30 (0.71–2.38)	0.02	0.004	0.46
VEGF-C	113	0.56 (0.36–0.89)	83	1.18 (0.65–2.16)	70	1.40 (0.75–2.58)	0.02	0.05	0.36
VEGF-D	32	0.35 (0.13–0.90)	117	0.82 (0.52–1.30)	110	1.28 (0.79–2.09)	0.01	0.02	0.24
VEGFR-1	85	0.41 (0.24–0.69)	89	1.37 (0.78–2.40)	87	1.53 (0.86–2.73)	0.001	0.002	0.06
VEGFR-2	101	0.48 (0.30–0.79)	102	1.12 (0.66–1.90)	62	1.67 (0.87–3.21)	0.003	0.004	0.93

Abbreviations: C = apecitabine; CB = capecitabine and bevacizumab; CBM = capecitabine, bevacizumab, and mitomycin; CI = confidence interval; HR = hazard ratio; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor.

[a]Scores for treatment effect (0, 1+; 2+; 3+) are based on the staining intensity of the antibody in tumour samples.

[b]*P* indicates the level of significance for the interaction between treatment (C *vs* CB + CBM) and the biomarker.

[c]Analysis unadjusted.

[d]Analysis adjusted for baseline clinicopathological characteristics.

[e]Analysis adjusted for other biomarkers.

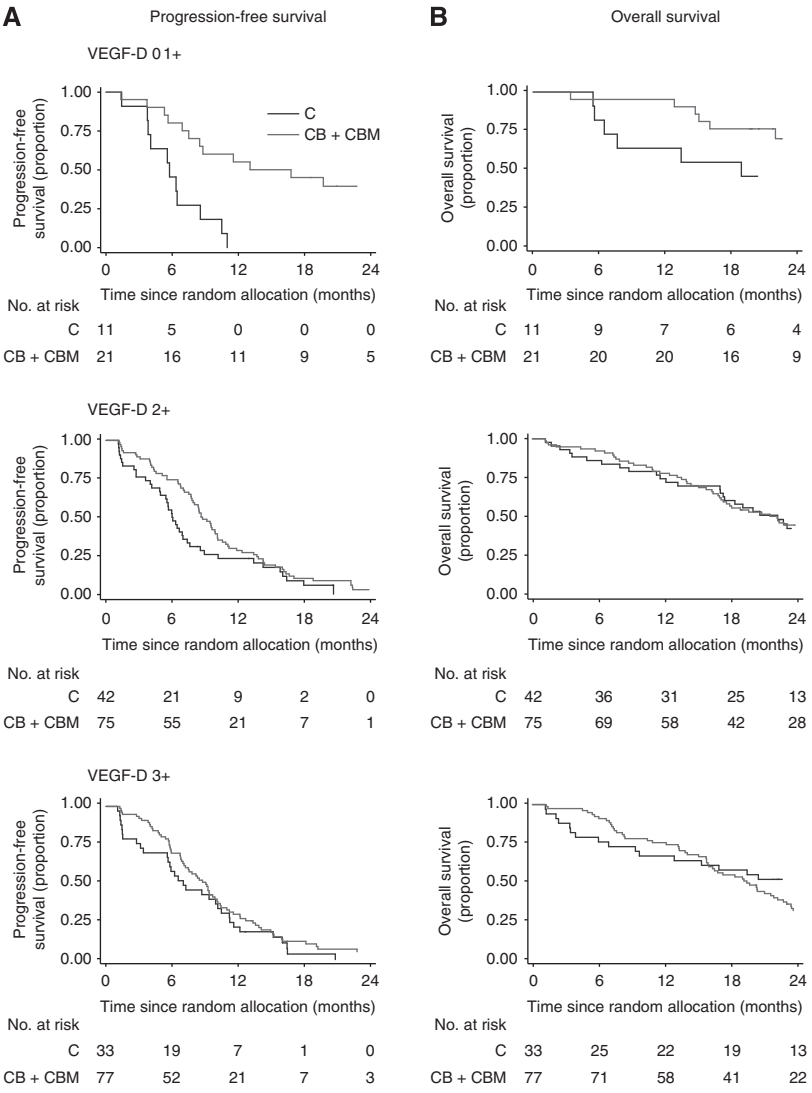

Figure 2. Kaplan–Meier curves for (**A**) PFS and (**B**) OS according to VEGF-D expression in unadjusted analyses.

significant. Even with adjustment for multiple comparisons, the global assessment of treatment by VEGFR-1 interaction remained significant for OS (Figure 4). The global test of interaction with all six biomarkers combined was significant for OS (log-likelihood $\chi^2 = 15.12$ (with six degrees of freedom); $P = 0.02$).

Sensitivity analysis. C was compared with CB and C with CBM for VEGF-D only (Supplementary Figure S2). Among patients with VEGF-D expression scores of 0 or 1+, the median PFS was 11.5 months in the CB group and was not reached in the CBM group. Among patients with VEGF-D expression 2+, the median PFS was 9.5 months in the CB group and 8.4 months in the CBM group. Among patients with VEGF-D expression 3+, the median PFS was 8.8 months in the CB group and 9.3 months in the CBM group. The additional benefit of bevacizumab was consistently significantly greater among the patients with lower expression of VEGF-D than among those with higher expression of VEGF-D ($P = 0.03$ for the interaction between VEGF-D expression and the C *vs* CB treatment comparison and $P = 0.04$ for the interaction between VEGF-D expression and the C *vs* CBM treatment comparison).Additionally, there is no difference in the treatment outcome between CB *vs* CBM, and no significant interaction between VEGF-D and treatment (CB *vs* CBM) for PFS ($P = 0.94$) and OS ($P = 0.62$).

Outcomes of CAIRO2 patients according to VEGF-D expression. VEGF-D expression of 0 or 1+, 2+, and 3+ were recorded for 25, 126, and 100 patients, respectively. The median PFS for patients with VEGF-D expression 0 or 1+, 2+, and 3+ was 10.1 (95% CI, 7.3–14.8), 9.7 (95% CI, 7.5–11.4), and 9.8 (95% CI, 6.9–11.8) months, respectively ($P = 0.35$). The median OS for patients with VEGF-D expression 0 or 1+, 2+, and 3+ was 22.0 (95% CI, 12.5–26.9), 21.7 (95% CI, 18.6–23.4), and 21.7 (95% CI,16.9–25.4) months, respectively ($P = 0.67$).

Table 2. Effect of VEGF-D on benefit of bevacizumab on response rate, progression-free survival, and overall survival

VEGF-D expression	Treatment	Response rate (%)	Median progression-free survival (months)	Median overall survival (months)
0, 1	C	36	5.8	18.9
0, 1	CB + CBM	33	16.8	NR
2	C	31	6.0	20.6
2	CB + CBM	34	8.8	21.6
3	C	52	7.0	24.6
3	CB + CBM	52	9.0	19.4

Abbreviations: C = capecitabine; CB = capecitabine and bevacizumab; CBM = capecitabine, bevacizumab, and mitomycin; NR = not recorded; VEGF = vascular endothelial growth factor.

DISCUSSION

In the MAX study, high expression of VEGF-D shown on immunohistochemistry predicted resistance to bevacizumab. The PFS benefit of bevacizumab was greater for patients with lower expression of VEGF-D than those with higher expression.

This study represents a comprehensive evaluation of tumoural expression of VEGF ligands and receptors in colorectal cancer patients treated with bevacizumab. The study used a large cohort of tumour samples (57%). Other randomised phase III colorectal bevacizumab biomarker studies tested not >34% of patient samples (Jubb *et al*, 2006; Foernzler *et al*, 2010). Expression levels of biomarkers were scored by two independent reviewers, one of whom was an anatomical pathologist, blinded to trial allocation and treatment outcome. There was good inter-rater agreement, with weighted Kappa >0.79 between the two scorers. The investigators remained blinded to trial allocation and treatment outcome when deciding the appropriate categorisation of ARFs into different expression groups of 0, 1+; 2+; and 3+.

This study also has several limitations. First, the optimal methods for examining these novel biomarker expression levels are not well established. There are conflicting reports on the rate of discordance between primary and metastatic tissue in the expression of VEGF-A levels (Cascinu *et al*, 2000; Kuramochi *et al*, 2006; Jubb and Harris, 2010). No study has examined the rate of discordance in the expression of the other ARFs. Most biological samples in this study were from archived primary colorectal tumours (83%) although expression levels between paired primary and secondary tumours (14 samples) had at least good-to-very-good concordant expression. Second, there is currently no consistent and validated scoring system to define the expression levels of ARFs as assessed by immunohistochemistry, which, moreover, is a semiquantitative technique (Jubb *et al*, 2006; Schneider *et al*, 2008; Foernzler *et al*, 2010). Although commercially available antibodies with prior published use with immunohistochemistry were used in the analysis, their specificity for epitopes was not tested independently. In determining the ARF expression in tumours using immunohistochemistry, we used a simplified scoring system aimed at easy future replication and clinical use. Furthermore, in correlation of ARF expression in tumour with response as an outcome, the number of samples in each group was relatively small, which limits its interpretability

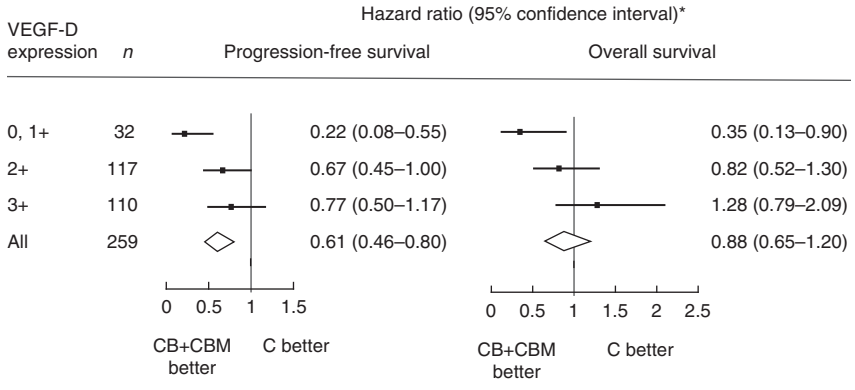

Figure 3. Forest plots for (**A**) PFS and (**B**) OS for groups with different levels of VEGF-D expression. *Hazard ratio for all patients in the MAX trial, including those whose tumour samples were not analysed.

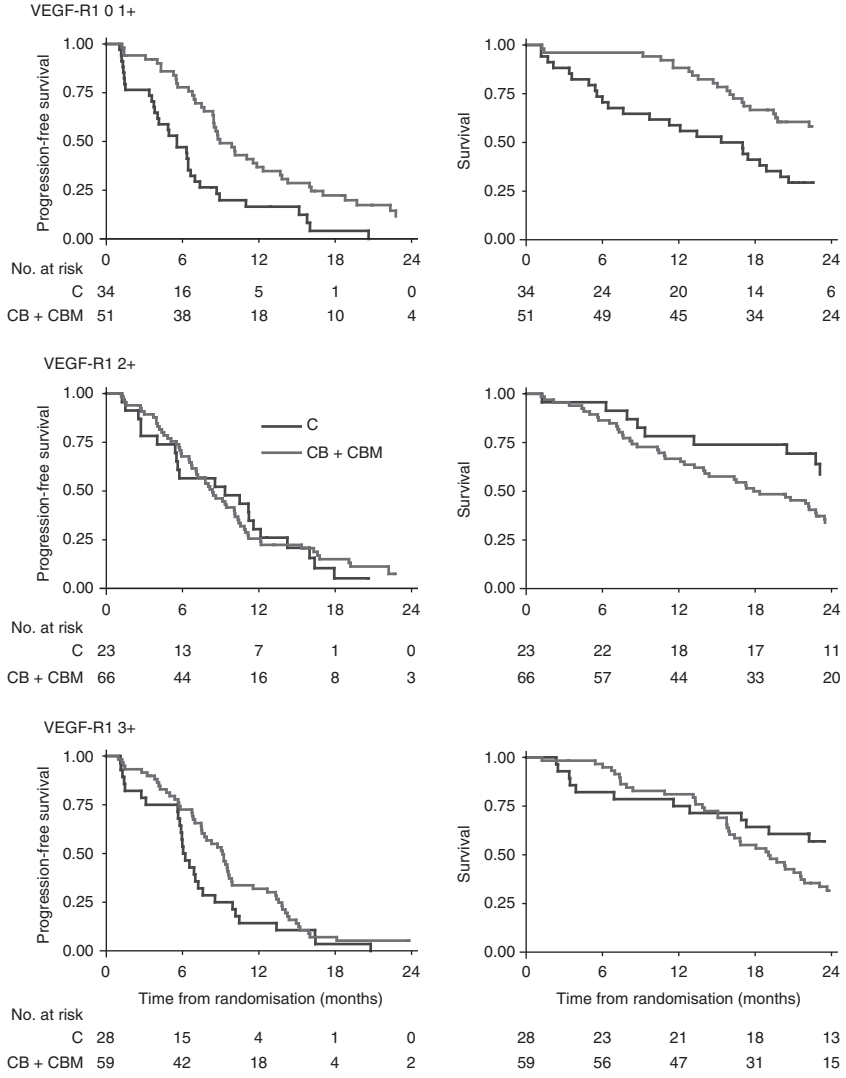

Figure 4. Kaplan–Meier curves for (**A**) PFS and (**B**) OS according to VEGF-R1 expression in analyses adjusted for multiple comparisons.

when using RECIST response criteria (Chun *et al*, 2009; Shindoh *et al*, 2012). We acknowledge that our results are also limited, because there might be potential interactions between mitomycin C with bevacizumab on PFS benefit. However, there was no significant interaction between VEGF-D and treatment (CB *vs* CBM) for PFS ($P=0.94$) and OS ($P=0.62$) (Supplementary Figure S2). Finally, this was a *post-hoc* analysis of multiple biomarkers of the MAX trial, and our findings may be related to a random effect.

Bevacizumab efficacy has no clinically useful predictive biomarker, such as *KRAS* mutation status, which is a definitive negative predictive biomarker for efficacy of epidermal growth factor receptor antibody therapy in advanced colorectal cancer (Lievre *et al*, 2006; Amado *et al*, 2008; De Roock *et al*, 2008; Van Cutsem *et al*, 2008; Bardelli and Siena, 2010; De Roock *et al*, 2010; Douillard *et al*, 2010; Rizzo *et al*, 2010; Van Cutsem *et al*, 2011; Bokemeyer *et al*, 2012). Various studies have examined the associations of potential biomarkers with bevacizumab efficacy. These markers include levels of baseline circulating endothelial cells, circulating angiogenesis-related cytokines such as interleukin-8 and PlGF 16 (Jayson *et al*, 2005; Dellapasqua *et al*, 2008; Willett *et al*, 2009; Kopetz *et al*, 2010), and SNPs in VEGF-A, VEGFR-1, and interleukin-8 (Schneider *et al*, 2008; Schultheis *et al*, 2008; Zhang *et al*, 2009; Jubb and Harris, 2010; Loupakis *et al*, 2011; Lambrechts *et al*, 2012; Collinson *et al*, 2013; Maru *et al*, 2013; Miles *et al*, 2013). Technical measurement difficulties and lack of validation in large randomised trials have limited their translation into routine clinical use (Furstenberger *et al*, 2005; Rowand *et al*, 2007; Mancuso *et al*, 2009; Maru *et al*, 2013). SNPs studies have produced conflicting findings and are compromised by multiple analyses that limit interpretation of apparently significant published *P*-values (Schneider *et al*, 2008; Schultheis *et al*, 2008; Zhang *et al*, 2009; Jubb and Harris, 2010; Lambrechts *et al*, 2012).

VEGF-A is thought to mediate angiogenesis through interaction with VEGFR-2, leading to an increase in blood vessel formation through changes in endothelial proliferation, cellular permeability, and cell migration (Kerbel, 2008). The VEGF family members VEGF-C and VEGF-D bind to VEGFR-3, leading to lymphangiogenesis, but can also bind to VEGFR-2, causing angiogenesis (Achen and Stacker, 1998; Rissanen *et al*, 2003). This interaction occurs after the removal of N- and C-terminal propeptides from the central VEGF homology domains and receptor flanking sites, generating mature forms of the protein (Stacker *et al*, 1999). These interactions led us to hypothesise that overexpression of either VEGF-C or VEGF-D could lead to resistance to bevacizumab.

The association of elevated VEGF-D levels with inferior efficacy of bevacizumab in this study is biologically plausible and is

supported by preclinical data demonstrating that VEGF-D has been shown to bind to VEGFR-2, triggering angiogenesis (Achen and Stacker, 1998; Stacker *et al*, 1999; Rissanen *et al*, 2003). Other studies have independently suggested a possible role for VEGF-D as a mediator of resistance to bevacizumab. Lieu *et al* (2013) showed that plasma VEGF-D levels increased upon tumour progression in patients with colorectal cancer receiving chemotherapy plus bevacizumab. Similarly, in the CALGB 80303 study, in patients with pancreatic cancer +, the subgroup with low plasma VEGF-D levels derived benefit from bevacizumab, while the main intention-to-treat population did not (Nixon *et al*, 2011). Unfortunately blood samples were not available from patients from either MAX or CAIRO-2 to assess the predictive role of circulating VEGF-D levels and validate these earlier studies.

Interpreting our results warrants caution, as only 32 patients with 0–1+ expression significantly benefited from bevacizumab treatment. The global test for interaction to account for multiple comparisons did not show statistical significance ($P = 0.22$) for PFS. In the independent population of patients in the CAIRO2 trial, VEGF-D tumour expression did not discriminate PFS or OS, although the 95% confidence intervals were wide. Unlike the MAX study, CAIRO2 could not adequately assess the predictive value of VEGF-D, as all patients in the control arm were treated with bevacizumab and chemotherapy. Yet if VEGF-D is a predictive biomarker for bevacizumab benefit as suggested by the results of the analysis in the MAX trial population, we would expect to see a clear difference in outcome in the CAIRO-2 population according to VEGF-D tumour expression. However, the patient population and the chemotherapy backbone were also different in the two trials and possibly accounted for the different outcomes.

In the MAX study, VEGFR-1 overexpression was also strongly associated with a lack of OS benefit from bevacizumab. VEGFR-1 overexpression, however, did not demonstrate a similar significant association with PFS. Two separate studies have found no association between VEGFR-1 overexpression and OS benefit from bevacizumab (Foernzler *et al*, 2010; Van Cutsem *et al*, 2011, 2012). The significance of the finding is therefore uncertain, and replication will be attempted in an appropriate secondary cohort. Given that angiogenesis is a complex phenomenon, there are several other biomarkers, including neuropilin-1 (Van Cutsem *et al*, 2012) and PlGF, that are worthy of further investigation using our tissue resource from MAX.

The process of identifying predictive biomarkers for bevacizumab and other targeted therapies is important for maximising benefits to patients while minimising cost and toxicity. Also, comprehensive evaluation of a relevant pathway could provide future therapeutic opportunities, because, if VEGF-D is validated as a mechanism of resistance to bevacizumab, therapeutic approaches that target VEGF-D may assist in overcoming resistance to bevacizumab.

In conclusion, our study demonstrates that VEGF-D tumour expression is a potential predictive biomarker for bevacizumab efficacy on PFS. Despite the biological plausibility associated with VEGF-D, the study is hypothesis-generating and further confirmation of its predictive value is still required.

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CONFLICT OF INTEREST

Dr Tebbutt has received honoraria and worked in a consulting role for Roche. Dr Price has been an uncompensated member of Roche advisory boards. The other authors declare no conflict of interest.

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