Biomarker analyses in REGARD gastric/GEJ carcinoma patients treated with VEGFR2-targeted antibody ramucirumab

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Background: Angiogenesis inhibition is an important strategy for cancer treatment. Ramucirumab, a human IgG1 monoclonal antibody that targets VEGF receptor 2 (VEGFR2), inhibits VEGF-A, -C, -D binding and endothelial cell proliferation. To attempt to identify prognostic and predictive biomarkers, retrospective analyses were used to assess tumour (HER2, VEGFR2) and serum (VEGF-C and -D, and soluble (s) VEGFR1 and 3) biomarkers in phase 3 REGARD patients with metastatic gastric/gastroesophageal junction carcinoma.

Methods: A total of 152 out of 355 (43%) patients randomised to ramucirumab or placebo had ⩾1 evaluable biomarker result using VEGFR2 immunohistochemistry or HER2, immunohistochemistry or FISH, of blinded baseline tumour tissue samples. Serum samples (32 patients, 9%) were assayed for VEGF-C and -D, and sVEGFR1 and 3.

Results: None of the biomarkers tested were associated with ramucirumab efficacy at a level of statistical significance. High VEGFR2 endothelial expression was associated with a non-significant prognostic trend toward shorter progression-free survival (high vs low HR = 1.65, 95% CI = 0.84,3.23). Treatment with ramucirumab was associated with a trend toward improved survival in both high (HR = 0.69, 95% CI = 0.38, 1.22) and low (HR = 0.73, 95% CI = 0.42, 1.26) VEGFR2 subgroups. The benefit associated with ramucirumab did not appear to differ by tumoural HER2 expression.

Conclusions: REGARD exploratory analyses did not identify a strong potentially predictive biomarker of ramucirumab efficacy; however, statistical power was limited.

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With an annual incidence of 1.4 million new cases worldwide and a mortality rate of 1.1 million (Ferlay *et al*, 2013), gastric and gastroesophageal junction (GEJ) cancer is often diagnosed at an advanced stage and is associated with a poor prognosis. Regimens of cytotoxic chemotherapy commonly used as first-line therapy for advanced gastric adenocarcinoma result in median survival ranging from 8 to 10 months (Wagner *et al*, 2010). Historically, there have been limited second-line treatment options.

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2)-mediated signalling and angiogenesis appear to have an important role in gastric cancer pathogenesis (Lieto *et al*, 2008; Murukesh *et al*, 2010; Suzuki *et al*, 2010). Ramucirumab is a human IgG1 monoclonal antibody that binds to VEGFR2 on blood vessel endothelial cells, thereby inhibiting VEGF ligand binding and receptor signalling, and limiting VEGF-induced angiogenesis and migration of endothelial cells, and slowing tumour growth (Spratlin *et al*, 2010). Given the absence of approved second-line therapies for gastric adenocarcinoma and the need to identify new therapies, we conducted a placebo-controlled, double-blind, phase 3 trial (REGARD) to evaluate the safety and efficacy of ramucirumab in patients with advanced gastric/GEJ adenocarcinoma who experienced disease progression after first-line platinum- or fluoropyrimidine-containing chemotherapy (Fuchs *et al*, 2014). In this trial, second-line ramucirumab significantly prolonged overall survival (OS) (median OS: 5.2 *vs* 3.8 months for ramucirumab and placebo, respectively; hazard ratio (HR) 0.776, 95% confidence interval (CI) 0.603–0.998, $P = 0.047$). Treatment with ramucirumab also significantly prolonged progression-free survival (PFS; HR, 0.483; 95% CI, 0.376–0.620; $P < 0.0001$). Ramucirumab appeared to be well tolerated in this patient population, with similar rates observed for most adverse events between the ramucirumab and placebo groups.

Identification of prognostic and predictive biomarkers to better select treatment options for patients is one of the principal goals of translational research (TR). To date, there are no validated predictive biomarkers for anti-angiogenic agents; however, study of VEGF-A and VEGFR expression in resected tumour specimens has indicated that elevated levels of some VEGF ligands and receptors are associated with shorter survival (Fondevila *et al*, 2004; Ozdemir *et al*, 2006; Liu *et al*, 2007; Osinsky *et al*, 2011), although other reports disputed this association (Lee *et al*, 2009; Bais *et al*, 2014). Given these prior data, analysis of factors related to ramucirumab mechanism of action was considered to represent an appropriate focus for REGARD biomarker analysis. We chose to explore candidate biomarkers in serum (soluble (s) VEGFR1, 3; VEGF-C, -D) and tumour biopsy (VEGFR2) for their prognostic value and potential predictive value for ramucirumab efficacy. On the basis of prior reports, we anticipated that higher baseline expression levels of VEGF-C, -D would be associated with poorer survival (Jüttner, *et al*, 2006; Cao *et al*, 2014). Conversely, we anticipated higher levels of sVEGFR1 and 3 would be associated with better survival due to their postulated function of limiting VEGF bioavailability (Al-Husein *et al*, 2012). As ramucirumab acts to limit VEGF binding to the membrane receptor, we were interested in assessing whether ramucirumab treatment would have its biggest effect in patients with low concentrations of sVEGFR1 and 3 and high serum VEGF concentration. Human epidermal growth receptor 2 (HER2) was also examined, as HER2-positive gastric cancer now represents an important disease subset that demonstrates sensitivity to trastuzumab, a HER2-blocking antibody (Bang *et al*, 2010). Although the VEGFR and HER2 pathways differ in receptor-associated signal transduction cascades, both result in endothelial cell proliferation. Therefore, we anticipated that patients with both HER2-positive and HER2-negative gastric cancer would likely respond to ramucirumab treatment.

MATERIALS AND METHODS

Study design and patients. REGARD study design and conduct has been reported earlier (Fuchs *et al*, 2014). Briefly, this global, randomised, double-blind, placebo-controlled phase 3 trial was conducted at 119 sites in 29 countries. Key eligibility requirements included metastatic or nonresectable, locally advanced gastric or GEJ adenocarcinoma; disease progression within 4 months of the final first-line platinum- or fluoropyrimidine-containing treatment, or within six months of the last dose of platinum or fluoropyrimidine-containing adjuvant treatment; measurable disease (defined by Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0; Therasse *et al*, 2000) or nonmeasurable evaluable disease; and an Eastern Cooperative Oncology Group (ECOG) performance status score of 0 or 1 (Oken *et al*, 1982). Exclusion criteria included any grade 3 or higher gastrointestinal bleeding (National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE, version 4.02) within 3 months before randomisation, any arterial thromboembolic event within 6 months prior to randomisation, or poorly controlled hypertension.

Randomisation proceeded as described previously (2 : 1 ratio) (Fuchs *et al*, 2014), with stratification by location of the primary tumour, geographic region, and degree of weight loss during the preceding 3 months. Patients received either intravenous ramucirumab (8 mg kg^{-1}) or placebo intravenously once every 2 weeks. Treatment continued until disease progression, unacceptable toxicity, or patient/physician decision.

Collection of pre-treatment tumour samples and baseline/post-baseline serum samples was optional in the REGARD study.

VEGFR2 and HER2 detection and scoring. Using a well-characterised, commercially available antibody and a specific, selective, and sensitive immunohistochemistry (IHC) assay, VEGFR2 protein expression was visualised in 5-micron sections prepared from formalin-fixed paraffin-embedded (FFPE) patient tumour specimens and quantified as previously described (Holzer *et al*, 2013). HER2 protein expression was also detected by IHC, and gene copy number was detected by fluorescence *in situ* hybridisation (FISH) in FFPE tissue specimens (5-micron sections). *HER2* FISH was analysed with the PathVysion HER2 DNA Probe Kit per manufacturer's instructions (Abbott Molecular, Des Plaines, IL, USA). HER2 IHC was determined with the HercepTest per manufacturer's instructions (Dako, Carpinteria, CA, USA).

All biomarker assay analyses were undertaken blinded. For VEGFR2 protein expression, the localisation of the staining was separately scored using the H-score in tumour vessels, tumour cell cytoplasm, and tumour nuclei, and was scored as an ordinal variable (reported as 0, 1 +, 2 +, or 3 +) for tumour cytoplasmic membrane.

HER2 positivity was scored using two sets of criteria: the ToGA trial eligibility criteria (FISH-positive or IHC3 +), and the more stringent criteria reported as a subgroup from ToGA (FISH-positive and IHC2 +; or IHC3 +; Bang *et al*, 2010).

Detection of VEGF and sVEGFR in serum. Mesoscale Diagnostics (MSD; Rockville, MD, USA) electrochemiluminescence (ECL) immunoassays were used for the detection of VEGF-C, VEGF-D, sVEGFR1, and sVEGFR3 in serum. Streptavidin MSD ELISA plates were washed with 1 × Tris-buffered saline containing 10 mM, Tris (pH 7.40), 150 mM NaCl, and 0.1% Tween (TBST) then blocked with 1 × TBST containing 1% bovine serum albumin (BSA). Following washes, 50 μl of 5 μg ml^{-1} biotin-labelled mouse VEGF-D antibody (Eli Lilly and Company, Indianapolis, IN, USA), biotin-labelled mouse monoclonal VEGF-C antibody (Novus Biologicals, Cat# NBP1–18626), or biotin-labelled VEGFR3

antibody (Eli Lilly and Company) was added to respective plates and incubated for 1 h with gentle shaking to allow binding. Next, VEGF-D (R&D Systems, Minneapolis, MN, USA), VEGF-C (PeproTech, Rock Hill, NJ, USA, Cat# 100-20C), or sVEGFR3 standards (Eli Lilly and Company) at varying concentrations were diluted in assay buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 1% Triton X-100; 5 mM EDTA; 5 mM EGTA) containing 1% BSA and supplemented with 100 μg ml^{-1} Heterophilic Blocking Reagent 1 (Scantibodies, Santee, CA, USA) and added to the wells of respective plates to generate a standard calibration curve. Serum samples were diluted and added to the plates in the same assay buffer. All samples, calibrators, and controls were incubated at room temperature for 2 h then the plates were washed with TBST, and 50 μl of appropriate 1 μg ml^{-1} ruthenium-labelled detection antibodies was added to the wells and allowed to incubate for 1 h at room temperature. Following a final wash step, 150 μl of 2 $\times$ MSD read buffer was added, and the wells were read on a MSD SECTOR Imager 6000 reader (Rockville, MD, USA), which recorded ruthenium ECL.

Similar methodologies were planned for measurement of VEGF-A, but were not carried out as it was found that platelet-derived VEGF-A in serum causes high variability among patient samples.

Statistical analysis. VEGFR2 staining levels were summarised by assessment type (e.g., neoplastic vessels, tumour cytoplasmic membrane, and so on). For those assessment types where there was a sufficient distribution of staining values, marker results were categorised as low or high using the median H-score value as the cutoff, or by using H-score $= 0$ *vs* > 0. Cox models were used to test the relationship of OS and PFS with biomarkers in a model that included treatment, marker level (high *vs* low), treatment by marker interaction, and the trial stratification factors

Table 1. Comparison of demographics, baseline disease characteristics, and efficacy outcomes in ITT and tissue TR populations

Variable, n (%)	ITT population		Tissue TR population	
	Ramucirumab (N = 238)	Placebo (N = 117)	Ramucirumab (N = 100)	Placebo (N = 52)
Race				
White	181 (76.1)	91 (77.8)	89 (89.0)	46 (88.5)
Asian	39 (16.4)	17 (14.5)	3 (3.0)	1 (1.9)
Black	4 (1.7)	2 (1.7)	3 (3.0)	1 (1.9)
Other	14 (5.9)	7 (6.0)	5 (5.0)	4 (7.7)
Geographic region				
North America, Europe, Australia, New Zealand	165 (69.3)	80 (68.4)	80 (80.0)	41 (78.8)
South and Central America, India, South Africa, Jordan, Egypt, Saudi Arabia, Lebanon	55 (23.1)	29 (24.8)	19 (19.0)	11 (21.2)
Asia	18 (7.6)	8 (6.8)	1 (1.0)	0 (0)
Age group				
<65	156 (65.5)	71 (60.7)	57 (57.0)	32 (61.5)
Sex				
Female	69 (29.0)	38 (32.5)	29 (29.0)	21 (40.4)
ECOG performance status				
0	67 (28.2)	31 (26.5)	26 (26.0)	11 (21.2)
1	171 (71.8)	85 (72.6)	74 (74.0)	41 (78.8)
2[a]	0	1 (0.9)	0	0
Location of primary tumour (IVRS)				
Gastric	178 (74.8)	87 (74.4)	71 (71.0)	41 (78.8)
Gastroesophageal junction	60 (25.2)	30 (25.6)	29 (29.0)	11 (21.2)
Histologic type				
Diffuse	96 (40.3)	44 (37.6)	41 (41.0)	21 (40.4)
Intestinal	52 (21.8)	35 (29.9)	27 (27.0)	15 (28.8)
Undetermined/unavailable	90 (37.8)	38 (32.5)	32 (32.0)	16 (30.8)
Progression-free interval on prior first-line therapy[b]				
<6 months	154 (65.5)	83 (70.9)	61 (61.6)	36 (69.2)
⩾6 months	81 (34.5)	34 (29.1)	38 (38.4)	16 (30.8)
Prior treatment with trastuzumab				
No	232 (97.5)	117 (100)	96 (96.0)	52 (100)
Median OS (months) (95% CI)	5.2 (4.4–5.8)	3.8 (2.8–4.7)	5.8 (5.2–7.4)	3.9 (2.5–5.8)
OS HR (95% CI)	HR = 0.776 (0.603–0.998)[c] P = 0.0473[d]		HR = 0.736 (0.497–1.091)[c] P = 0.1261[d]	
Median PFS (months) (95% CI)	2.1 (1.5–2.7)	1.4 (1.3–1.4)	2.7 (1.7–2.9)	1.3 (1.2–1.5)
PFS HR (95% CI)	HR = 0.483 (0.376–0.620)[c] P<0.0001[d]		HR = 0.495 (0.334–0.732)[c] P = 0.0004[d]	

Abbreviations: HR = hazard ratio; ITT = intent-to-treat; N = total number of patients in the corresponding arm and population; ECOG = Eastern Cooperative Oncology Group; n = the number of patients in the specified category; OS = overall survival; CI = confidence interval; PFS = progression-free survival; TR = translational research, with the TR population defined as the set of ITT patients with available tissue samples.

[a] Patients with a PS of 2 were a protocol violation.

[b] Defined as the time from start of first-line treatment to disease progression on first-line. Data are not available for 4 ramucirumab arm patients, of which 3 were in the ITT population, and 1 was in the TR population.

[c] HR and 95% CI obtained using a stratified Cox-proportional hazards model. One patient from the geographical region of Asia was excluded from the stratified analyses in the tissue population due to small sample size in this stratification factor level.

[d] Log-rank P-value (two-sided). P-value is not adjusted for multiple testing. One patient from the geographical region of Asia was excluded from the stratified analyses in the tissue population due to small sample size in this stratification factor level.

(location of primary tumour, weight loss over the prior 3 months, and geographical region) as covariates. Kolmogorov-type supremum tests were used to assess proportional hazard assumptions (Lin et al, 1993). To further explore the data, the same model was applied using marker cutoffs from the 25th to 75th percentiles of the range of observed marker values. Kaplan–Meier plots were used for visualisation.

Owing to the low number of HER2-positive patients, the relationship between clinical outcomes with HER2 status and treatment was assessed using bean plots (vertical scatter plots of individual efficacy outcomes by the marker-by-treatment categories).

Assessment of the relationship between markers in serum samples (VEGF-C, VEGF-D, sVEGFR1, and sVEGFR3) with outcomes was performed by scatter plot due to the small number of available samples.

No statistical corrections for multiple testing were performed.

RESULTS

Of the 355 randomised patients (2 : 1, 238 to ramucirumab and 117 to placebo) comprising the intent-to-treat (ITT) population in the REGARD trial, 152 patients (43% of the ITT population) had at least one evaluable IHC/FISH tissue biomarker result, comprising the tissue TR population. The TR population for serum analysis was 32 (9% of ITT). All samples analysed were collected before treatment initiation. The tissue population was fairly representative of the overall ITT study population with regard to efficacy outcomes, demographics, and baseline disease characteristics (Table 1).

VEGFR2 protein expression was assessed in tumour tissue from 147 patients (41.4% of the ITT population) using IHC; 5 patients in the tissue TR population did not have VEGFR2 IHC results. VEGFR2 protein expression was primarily localised to the vascular vs the non-vascular tumour cells. VEGFR2 staining in the tumour nuclei, cytoplasm, and cell membrane was minimal. The H-score for tumour nuclei was >0 in 9.7% (14/144) of samples; for cytoplasm, 22.9% (33/144) of samples had an H-score of >0. Similarly, only 11/144 (7.6%) of samples showed cytoplasmic membrane staining >0. The number of positive samples based on tumour cell staining was too small for correlative analyses with PFS or OS.

VEGFR2-positive staining in tumour blood vessels (H-score >0) was observed in 86.7% of samples with a range of values of 0–240, and H-score 25–75th percentiles of 10–80. The mean and median H-scores and their distributions were similar in the ramucirumab and placebo treatment arms (mean (s.d.): 49.8 (49.5) vs 45.5 (44.5); median: 35 vs 25, ramucirumab vs placebo, respectively).

The patients were divided into subgroups for correlative analyses of 'high' and 'low' VEGFR2 expression based on the median tumour vessel H-score (where the 'high' subgroup includes patients with an H-score greater than or equal to the median observed H-score across this trial, and the 'low' subgroup includes patients with an H-score less than the median observed value). To assess the potential prognostic value of VEGFR2 for OS (Figure 1A) and PFS (Figure 1B), the subgroups with high vs low VEGFR2 levels within the placebo arm were compared. The HR for PFS was 1.65 (95% CI 0.84–3.23, $P = 0.1821$), suggesting high VEGFR2 levels might be associated with earlier progression in the absence of treatment. However this finding was not statistically significant. Further data are needed to support an association of high VEGFR2 levels with a poorer prognosis.

The potential predictive value of VEGFR2 protein was also assessed by modelling both treatment arms. In both high and low VEGFR2 protein-staining subgroups, there was a trend toward

improved OS and PFS in the ramucirumab arm compared with the placebo arm, consistent with the results in the overall trial ITT population. The effect associated with ramucirumab appeared more pronounced in patients with high VEGFR2 expression (OS HR = 0.69, 95% CI = 0.38, 1.22; PFS HR = 0.35, 95% CI = 0.20, 0.59). However, there was evidence against the proportional hazards assumption ($P = 0.005$) for OS comparison between treatment arms in the high VEGFR2 subgroup, and statistical power to characterise the interactions between VEGFR2 expression and treatment with ramucirumab was limited for all analyses (Figure 2A–D, Table 2). To further explore the data, similar analyses were performed using other cutpoints to divide the patients into high and low VEGFR2-expressing subgroups: H-score of 0 vs >0 and the 25th to the 75th percentile of the VEGFR2 H-score values observed (data on file). For both PFS and OS, an HR <1 in both the high and low VEGFR2 protein expression subgroups suggested that patients treated with ramucirumab generally had benefit over the patients treated on the placebo arm, regardless of the cut-point examined. Together, these results suggest that all VEGFR2 IHC-defined subgroups of patients are likely to benefit from ramucirumab treatment; however, because of
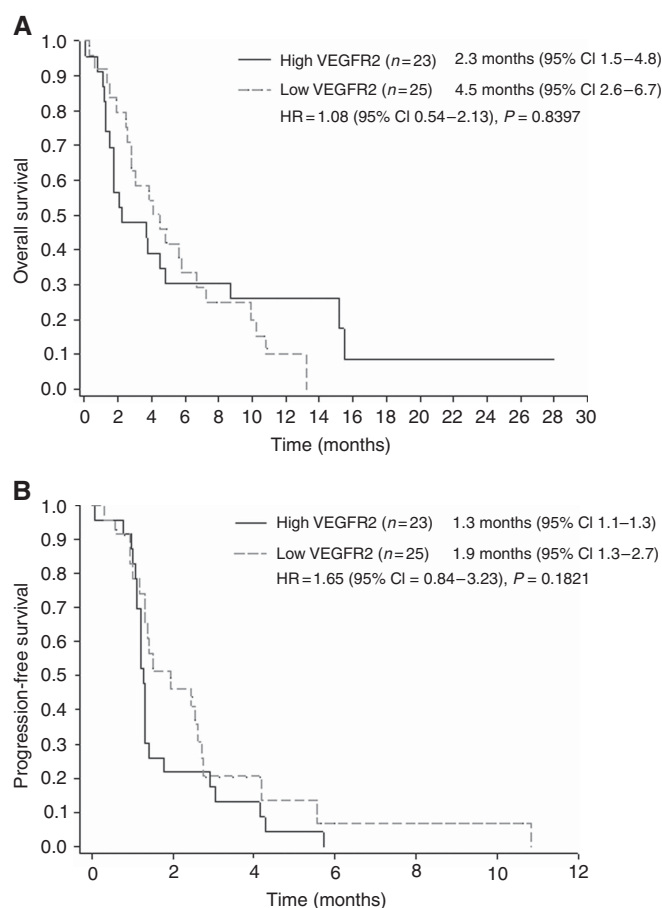

Figure 1. Kaplan–Meier plot of OS and PFS by high and low VEGFR2 groups in the placebo arm. Patients from the placebo arm with VEGFR2 IHC results from neoplastic vessels were dichotomised into the high and low subgroups, based on their individual VEGFR2 levels relative to the median level for the population (all patients across both arms with available results) (H-score median = 35). Kaplan–Meier plots for (A) OS and (B) PFS are shown. Inset tables give median survival times (months) and 95% CIs. Abbreviations: CI, confidence interval; HR, hazard ratio; n, number of patients; VEGFR2, vascular endothelial growth factor receptor 2.
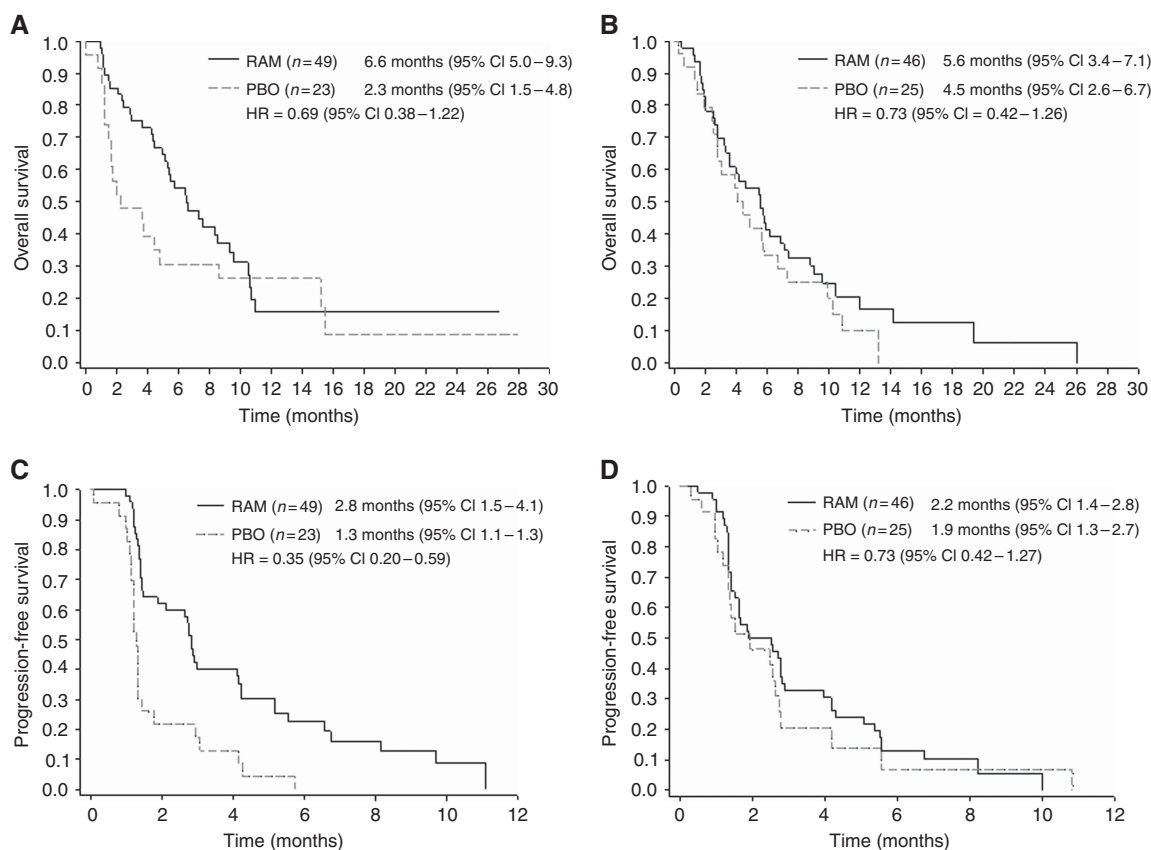
Figure 2. Kaplan–Meier plot of OS and PFS by treatment arm in the VEGFR2 high and low groups. Patients with VEGFR2 IHC results from neoplastic vessels were dichotomised into the high and low subgroups, based on their individual VEGFR2 levels relative to the median level for the population (H-score median = 35). Kaplan–Meier plots for OS in patients in the (**A**) high VEGFR2 group (H-score ⩾ 35) and (**B**) low VEGFR2 group (H-score < 35), and for PFS in the (**C**) high VEGFR2 group and (**D**) low VEGFR2 group are shown. Inset tables give median survival times (months) and 95% CIs. Abbreviations: CI, confidence interval; HR, hazard ratio; n, number of patients; PBO, placebo; RAM, ramucirumab; VEGFR2, vascular endothelial growth factor receptor 2.

limited sample size, these results should be interpreted with caution.

Assessment of tumour HER2 expression by IHC and FISH testing was performed on the tissue samples, with at least one result obtained from 147 of the 152 patients in the TR population. (The five patients without HER2 results are a different subset than those without VEGFR2 results.) HER2 positivity, as characterised by either FISH-positive or IHC3 +, was observed in 21 (14%) patients. Scatter plots comparing OS and PFS by treatment arm and HER2 status (Figure 3A and B) indicate no apparent lack of benefit of ramucirumab in patients with HER2-positive tumours, or conversely, among patients with HER2-negative tumours. However, there was some indication, albeit very limited by sample size in these subgroups, of ramucirumab benefit compared with the placebo arm in both HER2-positive and -negative tumours.

HER2 positivity was defined by more rigorous criteria: both FISH-positive and IHC2 +, or IHC3 +, was observed in 12 (8%) patient specimens, 3 in the placebo arm and 9 in the ramucirumab arm. This sample size is too small to draw any conclusions; however, as with the less-stringent HER2-positive criteria, there was no apparent lack of benefit for the HER2-positive patients (data not shown).

Measurement of circulating VEGF-C, VEGF-D, VEGFR1, and VEGFR3 was carried out in pre-treatment patient serum samples. Sample collection for biomarkers was initially performed only at specific sites, but then later added to other sites. Therefore, the population of patients with serum samples is limited (9.0% of ITT population) and did not tightly mirror the ITT population for demographics or efficacy outcomes. Scatter plots were used to

summarise the relationship between analyte expression and efficacy outcomes (OS and PFS; Figure 4A–H). In each case, no apparent relationship between serum protein quantity and treatment efficacy was observed.

DISCUSSION

Patient serum and tumour samples obtained during the REGARD metastatic gastric/GEJ phase 3 clinical trial were analysed for correlation of biomarker expression and ramucirumab-associated efficacy. The analyses focused on markers thought likely to be associated with ramucirumab efficacy, such as VEGF ligands and receptors, and gastric cancer biology. As REGARD was a monotherapy study, any differential effect of biomarker level on treatment efficacy could be attributed to ramucirumab.

The present exploratory research in the cohort of patients with available tissue and serum specimens failed to identify a candidate biomarker(s) that would facilitate selection of patients more likely to benefit from ramucirumab. Data did indicate that the benefit of ramucirumab may be more pronounced in patients with high VEGFR2 expression. This result further advances the hypothesis that the VEGF/VEGFR2 pathway is integral to tumour growth and targeting angiogenesis is a promising strategy. Importantly, the REGARD trial was not powered for this retrospective analysis, multiple markers were explored, and thus these findings should be explored in further prospective studies.

The IHC results confirmed the minimal expression of VEGFR2 in gastric tumour cells and the substantial expression of VEGFR2

Table 2. Overall and progression-free survival in VEGFR2 high and low groups as defined by VEGFR2 median and zero H-score cutpoints

	High VEGFR2		Low VEGFR2	
	Ramucirumab	Placebo	Ramucirumab	Placebo
Median H-score = 35				
N	49	23	46	25
Median OS (months; 95% CI)	6.6 (5.0, 9.3)	2.3 (1.5, 4.8)	5.6 (3.4, 7.1)	4.5 (2.6, 6.7)
OS treatment HR (95% CI)	0.69 (0.38, 1.22)		0.73 (0.42, 1.26)	
Median PFS (months; 95% CI)	2.8 (1.5, 4.1)	1.3 (1.1, 1.3)	2.2 (1.4, 2.8)	1.9 (1.3, 2.7)
PFS treatment HR (95% CI)	0.35 (0.20, 0.59)		0.73 (0.42, 1.27)	
H-score = 0				
N	84	40	11	8
Median OS (months; 95% CI)	5.8 (5.0, 7.3)	3.7 (1.8, 5.7)	5.6 (1.3, —)	4.0 (0.3, 13.2)
OS treatment HR (95% CI)	0.71 (0.46, 1.10)		0.67 (0.24, 1.87)	
Median PFS (months; 95% CI)	2.8 (1.6, 2.9)	1.3 (1.2, 1.9)	1.9 (1.3, 4.3)	1.4 (0.3, 5.6)
PFS treatment HR (95% CI)	0.49 (0.32, 0.75)		0.67 (0.25, 1.78)	

Abbreviations: CI = confidence interval; HR = hazard ratio; N = number of patient samples in each subgroup; OS = overall survival, PFS = progression-free survival; VEGFR2 = vascular endothelial growth factor receptor 2.

in neoplastic tumour vascular endothelium reported earlier by others (Smith et al, 2010). This observation is consistent with the presumed mechanism of action of ramucirumab; that is, targeting the VEGFR2 expressed on the vascular endothelium. The present data suggest an association between high VEGFR2 levels and worse prognosis (in particular PFS) in the placebo arm in this study. Importantly, the benefit of ramucirumab appears to be more pronounced in this same subgroup of patients with high VEGFR2 expression that is otherwise characterised by poor prognosis. Another recent study examining the VEGF/VEGFR pathway also observed a link between survival and differences in levels of these factors. In this case, Ock et al (2015) observed worse OS was correlated with a high VEGF/sVEGFR2 ratio as measured in the pre-treatment serum of gastric cancer patients.

Further evaluation is also required to define other biomarkers of angiogenesis and endothelial cell biology that may predict ramucirumab efficacy. Tumour blood vessels are known to be abnormal compared to non-tumour blood vessels and characterised by disorganised architecture, abnormal barrier function, and structural abnormalities of the basement membrane (Dvorak, 2003). However, whereas it is established that aberrant signalling occurs in the tumour, it remains to be determined whether the endothelial cells in tumour blood vessels are also characterised by specific gene mutations or aberrant RNA or protein expression.

Although conclusions are limited by small sample numbers, assessment of HER2 expression by IHC and FISH testing found no trend of an association between HER2 positivity and efficacy of ramucirumab; however, the expected low number of HER2-positive specimens was observed. HER2 positivity, defined by a patient sample that was either FISH-positive or IHC3 + , was observed in 21 (14%) patients. This percentage of HER2-positive patients is within the range of the overall incidence in gastric cancer reported in recent retrospective analyses of surgical specimens in advanced gastric cancer (10–17.4%; Ieni et al, 2013; Sheng et al, 2013; Betts et al, 2014).

Recently, whole-genome sequencing, comprehensive molecular profiling, and analysis of somatic copy-number alterations have been used to identify new driver mutations/alterations in gastric cancer tumour cells, although not the endothelium of tumour blood vessels supplying these tumours. These studies highlighted the molecular complexity of gastric cancer cells, but still leave many unanswered questions regarding the roles of the microenvironment and tumour vasculature (Lawrence et al, 2014; Lee et al, 2014; Wang et al, 2014). Notably, The Cancer Genome Atlas (TCGA) analysis of gastric cancer did identify VEGF
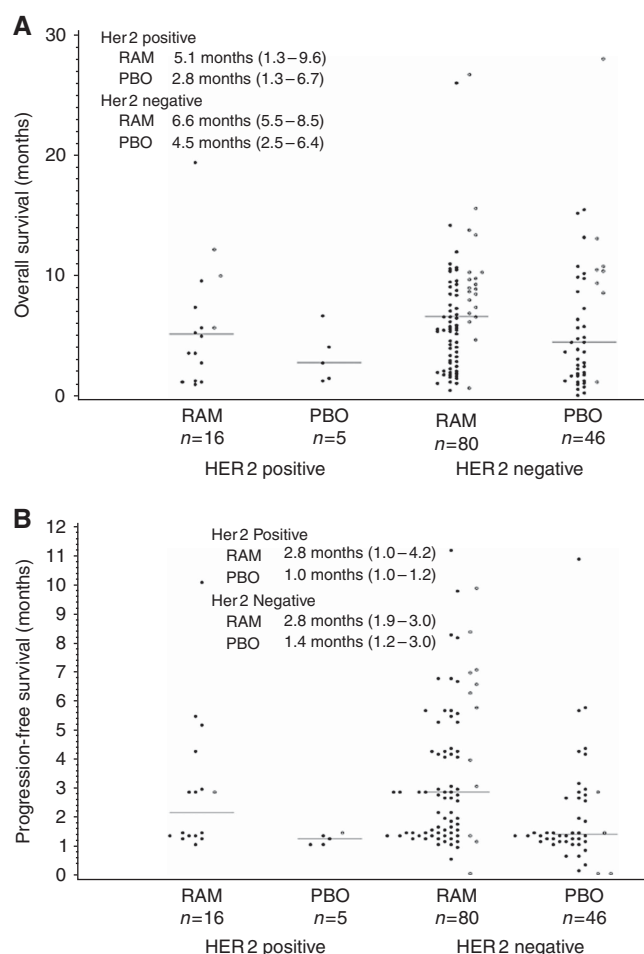

Figure 3. OS plot and PFS survival plot by HER2 positivity and treatment arm. (A) OS times and (B) PFS times are shown for patients grouped by HER2 classification and treatment arm. Open circles indicate patients with censored OS/PFS times and solid dots indicate patients with uncensored times. Horizontal lines and inset table show Kaplan–Meier median survival times (months) and 95% CIs. Abbreviations: HER2, human epidermal growth factor receptor 2; n, number of patients; OS, overall survival; PBO, placebo; PFS, progression-free survival; RAM, ramucirumab.
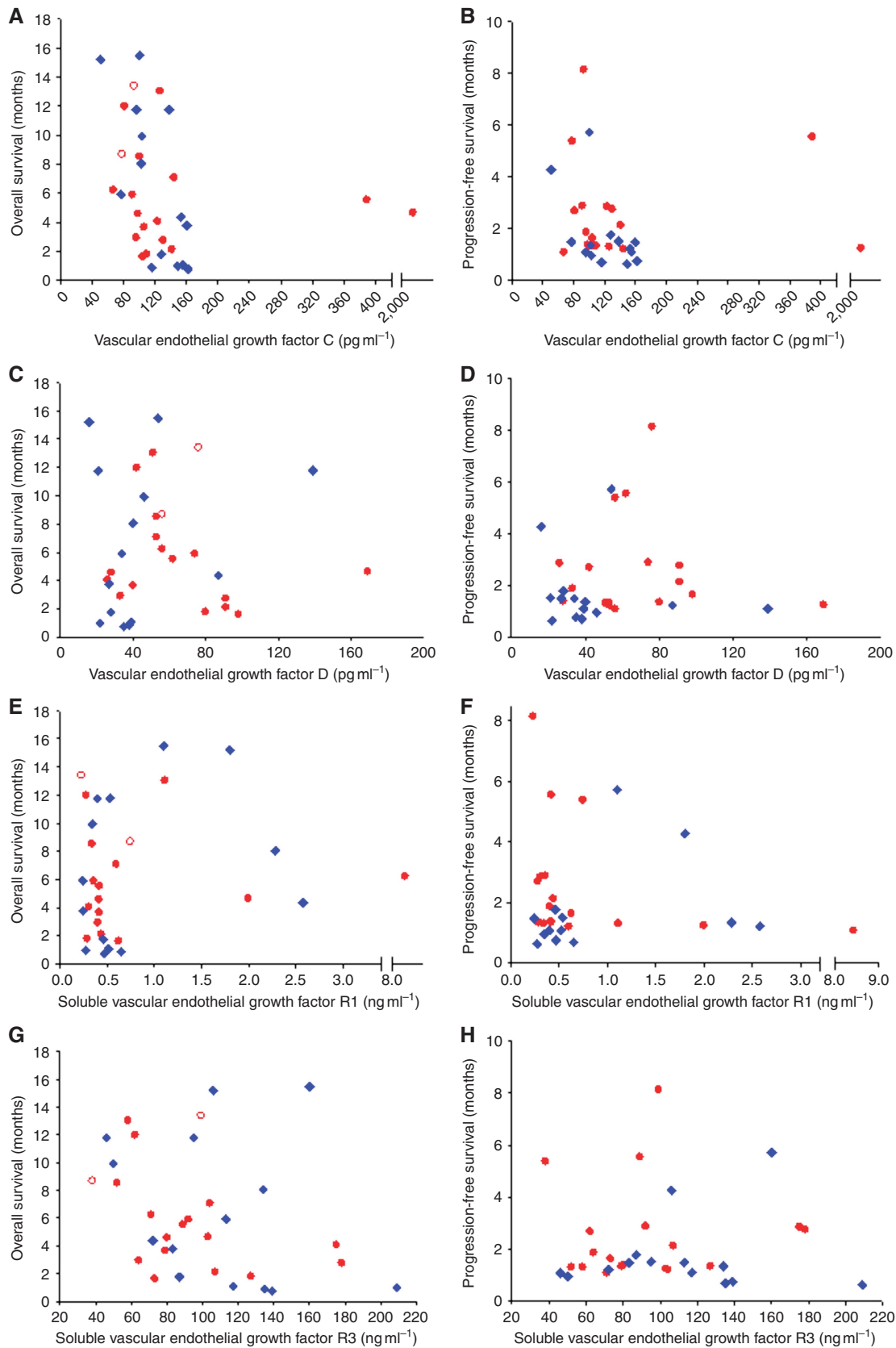
Figure 4. Scatter plots of OS and PFS *vs* biomarkers VEGF-C, VEGF-D, VEGFR1, and VEGFR3. OS values were plotted for each patient by serum concentration of (**A**) VEGF-C, (**C**) VEGF-D, (**E**) VEGFR1, and (**G**) VEGFR3. PFS was similarly plotted for each patient by serum concentration of (**B**) VEGF-C, (**D**) VEGF-D, and (**F**) VEGFR1, and (**H**) VEGFR3. Data from patients treated with ramucirumab are shown with a red circle (*n* = 18, except VEGFR1 where *n* = 17); data from patients treated with placebo are indicated by a blue diamond (*n* = 14). Censored observations are indicated with open symbols. The *x*-axis is discontinuous for plots **A**, **B**, **E**, and **F**.

amplifications in a subset of tumours (Lawrence *et al*, 2014). Other approaches using *in vitro* transcription factor activity profiling and integrative genomics resulted in the identification of three categories of factors/pathways involved: highly activated signalling pathways resulting from mutations; constitutively activated stress responsive pathways; and consistently downregulated nuclear receptor responsive factors. This functional profiling illustrates the complexity of gastric cancer biology and might help in discriminating therapeutic targets and signalling interactions for future studies (Periasamy *et al*, 2014). Thus, despite the rapidly evolving knowledge on gastric cancer cell biology, the same progress has not been made in the investigation of aberrant signalling in the vasculature. Therefore, limited information on predictive vessel biomarkers exists that could impact patient selection for treatments targeted at VEGFR2.

The present retrospective analyses have limitations. First, with the exception of HER2, the biomarkers that were examined were limited to the VEGF/VEGFR pathway. A broader examination of angiogenic factors would be beneficial, especially in light of evidence suggesting PDGF-BB (and perhaps other isoforms) may cause resistance to anti-VEGF therapy (Al-Husein *et al*, 2012). VEGFR2 expression by IHC is an exploratory biomarker and some analytical and interpretive matters remain to be resolved. It also remains to be determined whether there are differences in VEGFR2 expression between primary tumour and metastases, and whether VEGFR2 expression changes with time. Samples were collected in only a subset of patients leading to a potential selection bias and limiting the power of statistical analyses. The efficacy outcomes found in the present analyses will be re-examined in other recent phase 3 ramucirumab studies (REVEL, RAINBOW and RAISE; Garon *et al*, 2014; Wilke *et al*, 2014; Tabernero *et al*, 2015) that had greater participation in sample collection, and thus have the potential of generating a more robust data set. In addition, upcoming gastric cancer clinical trials involving ramucirumab treatment combinations and dosing schedules may yield more information about relationships between biomarker expression and efficacy outcomes.

To date, no reproducible predictive blood or tissue biomarkers have been identified despite years of extensive biomarker research across multiple solid tumour types in patients treated with anti-angiogenic agents (Shojaei, 2012). Although the present retrospective exploratory analysis of VEGFR2 and HER2 expression in available tumour tissue samples from the REGARD phase 3 trial also did not reveal a biomarker identifying patients who do and do not benefit from ramucirumab in metastatic gastric and GEJ adenocarcinoma, the benefit of this drug could be more pronounced in patients with high VEGFR2 expression. Further studies are needed to investigate the predictive potential of high VEGFR2 expression in patients with advanced gastric cancer treated with ramucirumab.

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CONFLICT OF INTEREST

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