European Journal of Cancer 127 (2020) 150–157

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Biomarker analyses of second-line ramucirumab in patients with advanced gastric cancer from RAINBOW, a global, randomized, double-blind, phase 3 study

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Received 30 May 2019; received in revised form 21 October 2019; accepted 27 October 2019

KEYWORDS
Ramucirumab;
Gastric cancer;
Gastroesophageal junction cancer;
Biomarker;
Predictive;
Prognostic

Abstract ***Background:*** The RAINBOW trial showed that second-line ramucirumab with paclitaxel prolongs overall survival (OS) and progression-free survival (PFS) compared with placebo plus paclitaxel for treatment of advanced gastric/gastroesophageal junction cancer. Plasma samples were collected from patients during the trial and tested to identify predictive and prognostic biomarkers.

Patients and methods: Circulating factors in plasma samples from mutually exclusive subsets of RAINBOW patients were assayed using: Intertek assays (24 markers, 380 samples, 57% of patients) and Lilly-developed assay (LDA) platform (5 markers, 257 samples, 39% of

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https://doi.org/10.1016/j.ejca.2019.10.026

patients). Time-trend plots were generated for each marker from the Intertek assays. Baseline patient data were dichotomized into low- and high-marker subgroups. Markers were analyzed for predictive effects using interaction models and for prognostic effects using main-effects models.

Results: The Intertek and LDA populations were representative of the full trial population. Plasma levels of VEGF-D and PlGF increased from baseline levels during treatment, then declined after treatment discontinued. Angiopoietin-2 exhibited a decrease during treatment, then increased after treatment discontinuation. No clear time trend was evident with the other markers. Analyses of baseline biomarker expression and its relationship with efficacy variables found no biomarker was predictive for efficacy outcomes, including VEGF-D. However, CRP, HGF, ICAM-3, IL-8, SAA, and VCAM-1 were identified as potential prognostic markers with low baseline levels corresponding to longer OS and PFS.

Conclusions: Pharmacodynamic and prognostic relationships were found from the exploratory biomarker analyses in RAINBOW; however, no predictive markers for ramucirumab in gastric cancer were identified in this trial.

1. Introduction

Worldwide cancer statistics record gastric/gastroesophageal junction (GEJ) cancer as a major cause of cancer-related deaths. About 1 million new cases of gastric cancer occurred in 2012, along with hundreds of thousands of new GEJ cancers, two-thirds with locally advanced or metastatic disease [1,2].

Patients receiving initial treatment with first-line platinum/fluoropyrimidine chemotherapy, with or without docetaxel or epirubicin, may initially respond to treatment but relapse over time [3]. Second-line chemotherapy with irinotecan or a taxane has shown a survival benefit over best supportive care in open-label randomized trials [4–6]. In the REGARD trial, single agent ramucirumab, a human IgG1 monoclonal antibody against the vascular endothelial growth factor receptor-2 (VEGFR-2), was the first biological agent that yielded a survival benefit when used as second-line treatment of metastatic gastric/GEJ cancer [7]. The RAINBOW trial paired second-line ramucirumab with paclitaxel for the treatment of advanced gastric/GEJ cancer [8]. Median overall survival (OS) was extended by 2.2 months (median 9.6 vs 7.4; hazard ratio [HR] = 0.807, $P = 0.017$), and progression-free survival (PFS) by 1.5 months (median 4.4 vs. 2.9; HR = 0.635, $P < 0.0001$), with improvement in tumor response rate (28% vs 16%, $P = 0.0001$) and disease control rate (80% vs 64%, $P < 0.0001$) as well.

A secondary objective of the RAINBOW trial was to examine biomarkers that may predict efficacy of ramucirumab. To this end, plasma samples were collected at baseline and intervals during the trial. The samples were assayed for biomarkers associated with angiogenesis since this process is targeted by ramucirumab and is important for tumor growth and metastasis. For example, high levels of vascular endothelial growth factor (VEGF)-A and VEGF-C are associated with poor prognosis and aggressive tumor growth and metastasis of gastric cancers [9–11]. Additional pro-angiogenic cytokines include VEGF-B, VEGF-D, and placental growth factor (PlGF) [12]. Biomarkers identified in other studies as relevant for cancer growth and progression were also assayed.

There are currently no consistently predictive biomarkers for selection of patients more likely to benefit from an antiangiogenic drug. Clinical studies of bevacizumab, an antibody that selectively binds the VEGF-A ligand and which is approved for treatment of several tumor types, indicate VEGF-A concentration may be prognostic; however, its utility as a predictive marker has been described as inconsistent and unlikely to be a strong predictive marker [13,14].

The analyses presented here sought to identify prognostic and predictive biomarkers to aid future treatment selection for patients with gastric/GEJ cancer.

2. Methods

2.1. Study design, sample collection and analysis

The design of the global, randomized, double-blind RAINBOW phase III trial (ClinicalTrials.gov NCT01170663) has been published [8] and is summarized in the appendix. Plasma was collected from all patients: prior to treatment initiation, prior to Cycle 2, Day 15 (4th ramucirumab/placebo infusion), prior to Cycle 4, Day 1 (7th ramucirumab/placebo infusion), and 30 days after discontinuing treatment. Beginning at study onset, available samples were assessed with Intertek assays (performed by Alta Intertek, San Diego, CA) that used quantitative sandwich electrochemiluminescent prototype kits. Later, Lilly-developed assays (LDAs) targeting key VEGF-family markers, became available, and patient samples were then assayed

exclusively with these assays (performed by PDD [Richmond, VA] and BioAgilytix [Durham, NC]). The LDAs were also quantitative sandwich electrochemiluminescent assays and were used exclusively in baseline plasma samples. VEGF-A was not assessed in either assay platform because plasma samples were collected in heparin tubes, and heparin has been found to interfere with the VEGF-A assay. Additional assay details are available in the online appendix.

2.2. Statistical analysis

The Intertek population consisted of all patients in the intent-to-treat (ITT) population with ≥1 Intertek biomarker value across all visits. The LDA population was defined as all patients in the ITT population with ≥1 LDA result at baseline.

To assess relationships between markers and clinical outcomes, most biomarkers were dichotomized at the median concentration observed for that marker, and the data separated into high and low groups and treated as binary variables. Markers were dichotomized at the lower limit of quantitation if ≥ 20% of the values were below the limit of quantitation. Biomarkers with >80% of the samples outside the range of quantitation were not analyzed.

The predictive effect of each biomarker on OS/PFS was performed using Cox's proportional hazards model with the following factors: treatment groups, biomarker (high/low), treatment-by-biomarker interaction, and with the stratification variables as covariates (geographic region, time to progression after first dose of first-line therapy [<6 months vs ≥6 months], and disease measurability [yes vs no]). The hazard ratio and its two-sided 95% confidence limits and p-value of testing the interaction of treatment and biomarkers were reported accordingly.

The relationship of biomarker VEGF-D with OS/PFS was evaluated in more depth using Subpopulation Treatment Effect Pattern Plot (STEPP) analysis, a graphical approach that constructs overlapping patient subpopulations with varying values of a characteristic, here VEGF-D. For this analysis, each subgroup contained ≥80 patients and overlapped with the previous subgroup by ≤60 patients at most. The HR of treatment effect was determined within each subgroup, and the results were plotted to illustrate how treatment effect changes across various VEGF-D levels.

This trial was not powered for biomarker analyses. No adjustments were made for multiplicity. The statistical analyses were conducted using SAS® software Version 9.1.3 or higher. Additional statistical methods are found in the appendix.

3. Results

Plasma samples from mutually exclusive subsets of RAINBOW trial patients were tested for circulating factors using the Intertek (380 samples, 57% of ITT patients) and LDA (257 samples, 39% of ITT patients) platforms (Table S1). Both the Intertek and LDA populations were non-random, consisting of only those patients whose baseline samples were provided during the portion of the trial when that assay platform was in use. There was a greater percentage of Asians in the Intertek population and a greater percentage of Whites in the LDA population, an imbalance likely due to the greater number of patients enrolled by Asian investigative sites early in the trial. Apart from race and region, the Intertek and LDA populations appeared to be fairly representative of the ITT population based on summaries of demographic and disease characteristics (Table S2). Hazard ratios for the assessment of treatment effects (regardless of biomarkers) were similar in the ITT and Intertek populations and in the ITT and LDA populations (Table S1), although the PFS HR and the tails of the Kaplan–Meier curves for the LDA population compared with the ITT population showed some differences (Fig. S1).

The Intertek assay platform tested for 24 biomarkers, with the number of available Intertek results varying by biomarker (range 103–373, or 15%–56% ITT; Table S1, Table 1). The LDA platform tested for five biomarkers; most of the available 257 samples were tested for each biomarker (range 252–257, or 38–39% ITT).

Intertek assays were used to characterize the change in plasma biomarker levels during the treatment period and 30 days after the treatment was discontinued. As shown in Fig. 1, both VEGF-D and placental growth factor (PlGF) increased from baseline levels during treatment with ramucirumab, VEGF-D by approximately 50% (Fig. 1A) and PlGF by approximately 900% (Fig. 1B). The median plasma level for both biomarkers declined after the treatment was discontinued. In contrast, median plasma levels of angiopoietin-2 exhibited a decrease during the treatment period in ramucirumab-treated patients, and then increased toward baseline when measured 30 days after treatment discontinuation (Fig. 1C). Analysis of plasma levels of the other biomarkers found no strong pattern in expression levels during the trial (Fig. S2).

Baseline Intertek assay results were analyzed for a predictive relationship between biomarker level and efficacy outcome (OS, PFS) using interaction models. Eighteen markers were treated as binary for correlations with clinical outcomes; six markers were not analyzed due to high proportion of samples beneath the level of quantitation (Table 1). For most biomarkers, there was no significant association between clinical outcome and interaction of treatment by marker, including those of the VEGF pathway (Table 1). There was a significant treatment-by-marker interaction for Hepatocyte Growth Factor (HGF) with PFS (unadjusted interaction P-value = 0.0366); however, the interaction p-value for OS was not significant (P = 0.3857). Similarly,

Table 1
Analysis of biomarker predictive relationships.

Marker Name	Cutpoint	High expression level (*N*)	Low expression level (*N*)	OS interaction *P*-value[a]	PFS interaction *P*-value[a]
Intertek Platform[b]					
ANG-1	LLOQ (3.0 ng/mL)	222	83	0.2304	0.9945
ANG-2	Median (6.5 ng/mL)	176	176	0.5965	0.7290
Soluble c-KIT	Median (35.5 ng/mL)	110	104	0.5568	0.3838
CRP	Median (4625 ng/mL)	166	165	0.1662	0.4581
HGF	LLOQ (257.0 pg/mL)	114	58	0.3857	0.0366
ICAM-3	LLOQ (24.2 ng/mL)	70	270	0.6154	0.9476
ICAM-1	Median (340 pg/mL)	176	173	0.7525	0.8958
IL-8	LLOQ (16.1 pg/mL)	102	249	0.7763	0.9752
IL-12	LLOQ (19.2 pg/mL)	141	170	0.6607	0.3191
P-selectin	Median (71.1 ng/mL)	170	170	0.1132	0.0650
PDGF-A	LLOQ (1.5 pg/mL)	81	22	0.4271	0.8808
PlGF	Median (21.2 pg/mL)	179	178	0.6693	0.3303
SAA	Median (5782.5 ng/mL)	167	167	0.2658	0.4837
VCAM-1	Median (560.1 ng/mL)	183	181	0.5615	0.8994
VEGF-C	LLOQ (261.8 pg/mL)	290	83	0.2723	0.9946
VEGF-D	LLOQ (656.1 pg/mL)	129	244	0.9165	0.9530
sVEGFR-1	Median (119.0 pg/mL)	184	183	0.6590	0.9864
sVEGFR-2	Median (11625.0 pg/mL)	186	185	0.5295	0.7852
LDA Platform					
VEGF-C	Median (88.7 pg/mL)	126	126	0.5270	0.6033
VEGF-D	Median (73 pg/mL)	132	125	0.4908	0.7626
sVEGFR-1	Median (211 pg/mL)	133	124	0.6285	0.5822
sVEGFR-2	Median (19.6 ng/mL)	127	127	0.5028	0.2134
sVEGFR-3	Median (103.8 ng/mL)	127	127	0.2956	0.8143

Abbreviations: ANG = angiopoietin; bFGF = basic fibroblast growth factor; CRP = C-reactive protein; HGF = hepatocyte growth factor; ICAM = intercellular adhesion molecule; IL = interleukin; LDA = Lilly-developed assay; LLOQ = lower limit of quantitation; *N* = number of patients; OS = overall survival; PDGF = platelet-derived growth factor; PFS = progression-free survival; PlGF = placenta growth factor; SAA = serum amyloid A; SDF = stromal cell derived factor; sNRP = soluble neuropilin; soluble cKIT = soluble tyrosine protein kinase kit; sVEGFR = soluble vascular endothelial growth factor receptor; VCAM = vascular cell adhesion molecule; VEGF = vascular endothelial growth factor.

Note: Among the four markers that were measured on both assay platforms (VEGF-C, VEGF-D, sVEGFR-1, and sVEGFR-2), differences in reagents and conditions between assay platforms likely contributed to differences in measured levels of these baseline markers between the two patient subpopulations (additional assay information is in the Online Appendix).

[a] *P*-value for testing the treatment-by-biomarker subgroup interaction obtained using a likelihood ratio test. *P*-value not adjusted for testing multiple biomarkers.

[b] For Intertek assays, patients were divided into high-expression or low-expression subgroups. bFGF, sNRP-1, SDF-1a, IL-4, Thrombomodulin, and E-selectin were not analyzed because greater than 80% of the samples were outside the range of quantitation.

baseline assay results from the LDAs were analyzed for correlations with OS and PFS. As with the Intertek assays, there were no significant treatment-by-marker interactions for any of the baseline LDA biomarker measures (Table 1).

Additional analysis was undertaken to further explore the relationship of VEGF-D plasma levels with clinical outcomes because a trend for greater ramucirumab efficacy with higher VEGF-D levels had recently been observed in metastatic colorectal cancer [15]. STEPP figures were created that show the point estimate for the treatment HR across the range of VEGF-D levels. The STEPP figures confirmed that OS (Fig. 2A and C) and PFS (Fig. 2B and D) do not trend with VEGF-D levels as measured with the Intertek platform (Fig. 2A and B) and LDA platform (Fig. 2C and D). OS and PFS HR remained relatively flat or did not consistently trend upward or downward as VEGF-D levels increased. A Cox regression analysis where all VEGF-D high/low cutoffs within its inter-quartile range were tested for their interaction effect with treatment showed that the smallest unadjusted interaction p-values for VEGF-D were 0.204 for OS and 0.469 for PFS as measured with the Intertek assay. Likewise, LDA measurements identified the smallest unadjusted interaction p-value for VEGF-D as 0.116 for OS and 0.0778 for PFS. The distributions of VEGF-D levels for the two platforms are shown in Table S3. With the Intertek assay, over half the patients (65.4%) had baseline VEGF-D levels below the limit of quantitation on the assay (LLOQ = 656.1 pg/mL). The median VEGF-D level using the LDA was 73 pg/mL.

In addition to investigating potential predictive relationships, analyses were performed to examine whether any biomarker was prognostic for better or worse outcome. As shown in Table 2, six of the biomarkers assayed, C-reactive protein (CRP), hepatocyte

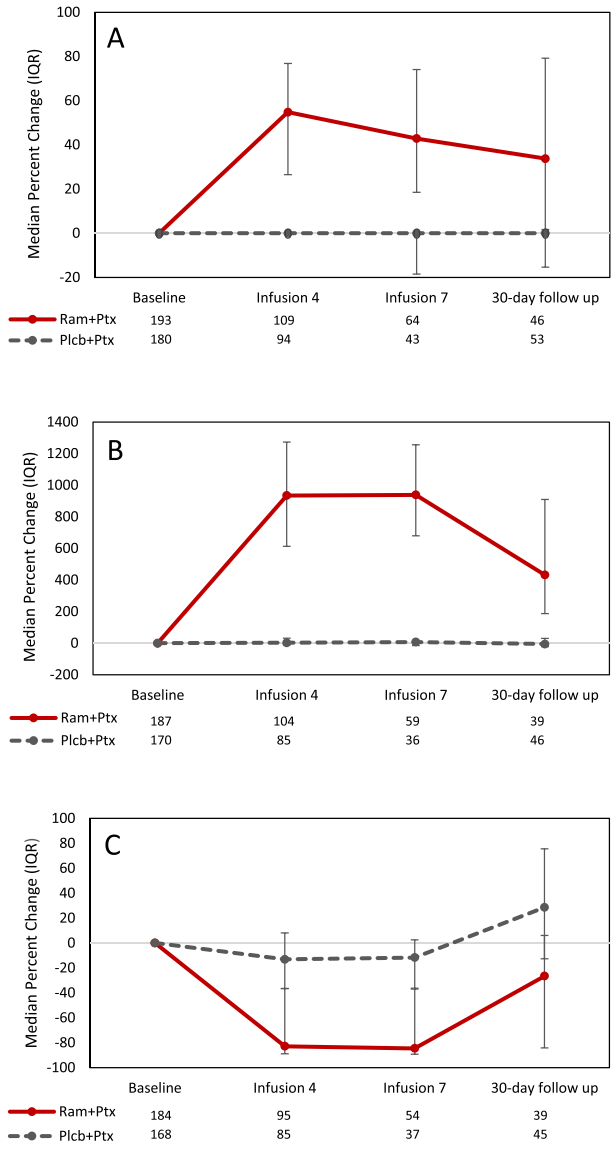

Fig. 1. Change in biomarker plasma concentration over time during treatment cycles. Biomarkers (A) VEGF-D, (B) placental growth factor (PlGF), and (C) angiopoietin 2 were measured in plasma at defined points in the clinical trial and plotted as median percent change (interquartile range). The number of patient samples at each time point is shown under each plot.

growth factor (HGF), intracellular adhesion molecule-3 (ICAM-3), interleukin-8 (IL-8), serum amyloid-A, and vascular cell adhesion molecule (VCAM-1) were identified as potential prognostic markers with low baseline levels corresponding to longer OS and PFS across the two treatment arms, using an alpha of 0.05. Analysis of data from the other biomarkers did not show a significant prognostic relationship. Marker–marker correlations showed that most of the six prognostic markers identified are highly correlated with each other (data not shown).

4. Discussion

Second-line ramucirumab-paclitaxel prolonged median OS and PFS over placebo-paclitaxel when administered to patients with advanced gastric/GEJ cancer in the RAINBOW trial. To identify biomarkers that can predict ramucirumab efficacy in patients with gastric/GEJ cancer, plasma samples from 637 patients (96% of the population) were analyzed using two different assay platforms referred to as Intertek and LDA. Of the 25 biomarkers analyzed, none of them were found to predict ramucirumab efficacy. While these analyses were limited in that they could not assay for VEGF-A, a key VEGFR2 ligand, due to heparin interference, the baseline concentration of VEGF-C, VEGF-D, sVEGFR-1, and sVEGFR-2 was analyzed in both assay platforms and neither platform found an association of biomarker concentration at baseline with efficacy outcome. STEPP analyses of the Intertek and LDA VEGF-D data confirmed that there is no consistent relationship between VEGF-D levels and ramucirumab efficacy in patients with second-line gastric cancer.

The lack of a predictive relationship for VEGF-D in patients with gastric cancer treated with ramucirumab differs from the results observed in the RAISE metastatic colorectal carcinoma trial in which ramucirumab-FOLFIRI was shown to improve survival over placebo-FOLFIRI. In the RAISE study, ramucirumab was suggested to be more efficacious (OS and PFS) among patients with higher baseline plasma VEGF-D levels (0.115 pg/mL cutoff) as determined by an exploratory research use-only assay, with efforts currently ongoing to develop a GMP-quality assay to confirm these findings [15]. The researchers hypothesized that patients with high levels of VEGF-D may have tumors that are particularly driven by the VEGF-D pathway and hence ramucirumab might be preferentially effective for treating metastatic colorectal carcinoma in those patients. Notably, patients participating in the RAISE clinical trial had all received first-line bevacizumab, so the high VEGF-D level could have developed as a mechanism of antiangiogenic resistance to the selective blocking of VEGF-A. In contrast, RAINBOW patients did not have prior antiangiogenics and may not have such a strong dependence on VEGF-D, among the various VEGFR ligands, such that VEGF-D would show a meaningful correlation. Supporting this hypothesis, the median VEGF-D level from heparinized plasma using the same VEGF-D assay among RAISE patients was higher than that of RAINBOW patients (135 pg/mL versus 73 pg/mL, respectively) [15].

Although VEGF-D plasma levels did not predict ramucirumab efficacy in RAINBOW, VEGF-D plasma levels did increase during ramucirumab treatment, falling back toward baseline after treatment discontinuation. Measurements at the same time points showed

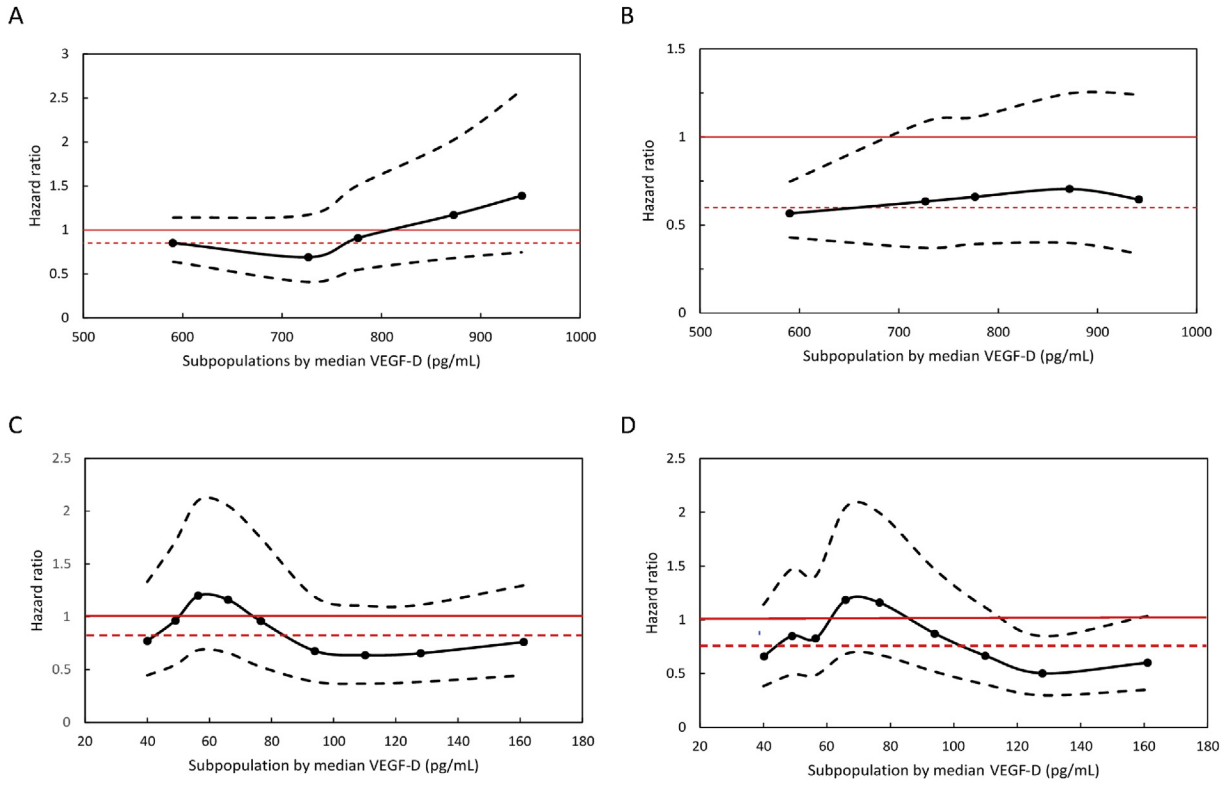

Fig. 2. OS and PFS STEPP figures examining treatment HR across a range of VEGF-D levels. STEPP figures were constructed with VEGF-D results (pg/ml) from Intertek (A, B) and LDA (C, D) platforms. OS HRs (A, C) and PFS HRs (B, D) are shown graphed with smoothing for STEPP populations using a sliding window with 80 patients/subgroup and 60 patients overlapping with the previous subgroup. Due to the large number of patients with values that were below the limit of quantitation (BLQ) for the Intertek platform (244 of 373 patients), the first point in the Intertek plots (A,B) is derived entirely from the BLQ results (rather than the window of 80 patients) and is imputed to 90% of the lower limit of quantitation (LLOQ). Because of the handling of the Intertek BLQ data, remaining data points for the Intertek figures do not include any overlapping data with the BLQ data point. For all four figures, the HR is plotted at the median VEGF-D level for that subgroup. The solid line represents an HR of 1.0; the dotted line represents the HR of the full population with results available for that marker.

patients receiving placebo had no change in VEGF-D plasma concentration. VEGF-D is believed to stimulate angiogenesis through VEGFR-2 and lymphangiogenesis through VEGFR-3 [16–18]. While it is not known why the VEGF-D levels measured in plasma increased in patients treated with ramucirumab, VEGF-D may be upregulated in response to the VEGFR-2 blockade.

Placental growth factor was also found to increase in plasma concentration among patients receiving ramucirumab treatment, with the plasma concentration dropping toward baseline after treatment discontinuation. Patients receiving placebo showed stable levels of PlGF during the trial. Evidence suggests that PlGF stimulates both vasculogenesis and angiogenesis through the VEGFR-1 receptor [16]. Although the mechanism of observed PlGF plasma concentration increase is uncertain, the VEGFR-2 blockade may upregulate the VEGFR-1 pathway leading to the increase in PlGF.

Angiopoietin-2 is the third biomarker found to fluctuate during ramucirumab treatment. Unlike VEGF-D and PlGF, plasma concentration of angiopoietin-2 decreased during ramucirumab treatment, moving back toward baseline levels after treatment discontinuation. During the same period, patients receiving placebo showed steady levels of angiopoietin-2. Angiopoietin-2 destabilizes vasculature, allowing sprouting angiogenesis to occur. Some evidence suggests that VEGF-A may upregulate angiopoietin-2 in tumors through interaction with VEGFR2 [19]. Decreases in angiopoietin-2 levels in the ramucirumab arm may be due to blockage of the VEGF upregulation of angiopoietin-2 due to ramucirumab binding.

The analyses reported here identified C-reactive protein, IL-8, serum amyloid-A, HGF, ICAM-3, and VCAM-1 as potential prognostic markers with low baseline plasma levels corresponding to longer OS and PFS across both treatment arms. High circulating levels of C-reactive protein, IL-8, and serum amyloid-A have been observed in other studies as a negative prognostic factor for gastric cancer [20–22]. Elevated levels of HGF mRNA has been observed in gastric tumors versus

Table 2
Analysis of potential prognostic markers[a].

				Overall Survival		Progression-free Survival	
	Cutpoint	High Level (*N*)	Low Level (*N*)	HR (95% CI)	Unadjusted *P* value	HR (95% CI)	Unadjusted *P* value
CRP	Median (4625 ng/mL)	166	165	2.1 (1.6, 2.7)	<0.0001	1.5 (1.2, 2.0)	0.0007
HGF	LLOQ (257.0 pg/mL)	114	58	1.9 (1.3, 2.7)	0.0007	1.8 (1.3, 2.6)	0.0009
ICAM-3	LLOQ (24.2 ng/mL)	70	270	1.4 (1.0, 1.8)	0.0377	1.4 (1.0, 1.8)	0.0382
IL-8	LLOQ (16.1 pg/mL)	102	249	1.5 (1.1, 1.9)	0.0039	1.3 (1.0, 1.7)	0.0401
SAA	Median (5782.5 ng/mL)	167	167	1.8 (1.4, 2.4)	<0.0001	1.3 (1.0, 1.7)	0.0420
VCAM-1	Median (560.1 ng/mL)	183	181	1.6 (1.3, 2.0)	0.0001	1.4 (1.1, 1.7)	0.0074

Abbreviations: CRP = C-reactive protein; HGF = hepatocyte growth factor; HR = hazard ratio; ICAM = intracellular adhesion molecule; IL = interleukin; SAA = serum amyloid A; VCAM = vascular cell adhesion molecule.

[a] Patient data were dichotomized into low- and high-marker subgroups using the cutpoints defined in Table 2. Hazard ratio (HR) compares high vs. low protein expression level groups using a main effects-only model that included treatment and the study stratification factors to control for additional factors that influence outcome differences.

normal gastric tissue where it has been hypothesized to play a role in tumor angiogenesis [23]. While circulating ICAM-1 has been identified as a possible prognostic factor for gastric cancer [24], we have not identified reports of soluble VCAM-1 and ICAM-3 as possible prognostic markers for gastric cancer. Angiopoietin-2 was also identified as a prognostic marker for the OS of gastric cancer patients by analysis of patient data from the first-line bevacizumab AVAGAST trial [25]; however, that association was not identified in our analyses of the RAINBOW trial data.

Despite multiple, approved anticancer treatments that target angiogenesis, there are no established, consistently predictive biomarkers to guide patient selection. The exploratory plasma analyses available from the RAINBOW study also did not identify a predictive biomarker for ramucirumab. While the analyses used well-characterized patient samples from a prospective, randomized trial and undertook longitudinal sampling over multiple time points, the analyses were limited in that they were not pre-planned and prospectively powered, and the assay platform was changed mid-trial. Furthermore, there was an imbalance in the proportion of Asians versus Whites in the two assay platforms, which may have impacted the results. Even with these limitations, analyses did identify several prognostic markers, lending credence to their role in the development of advanced cancer. Furthermore, pharmacodynamic trends with VEGF-D, PlGF, and angiopoietin-2 were observed, although the interpretation of these patterns remains to be elucidated. Additionally, although some evidence suggests that patients with mCRC exhibit greater ramucirumab efficacy (OS and PFS) when they have higher baseline plasma VEGF-D levels, this correlation was not observed among patients with advanced gastric/GEJ cancer. Given the lack of strong predictive relationships found for individual angiogenic factors to date, a more comprehensive approach that employs broad marker panels that are already validated for clinical use, may help identify biomarkers or signatures of biomarkers for anti-angiogenic therapies.

Funding

This work was supported by Eli Lilly and Company. D. Cunningham acknowledges support from NIHR Biomedical Research Centre at the Royal Marsden.

Role of the funding source

The study sponsor provided the study drug and worked with academic investigators to design the study and to collect, analyze and interpret the trial data.

Disclosure of potential conflict of interest

EVC reports research grants paid to institution from Amgen, Bayer, Boehringer Ingelheim, Celgene, Ipsen, Lilly, Roche, Merck Sharp & Dohme, Merck KGaA, Novartis, Roche, Servier and consultancy for Astrazeneca, Bayer, Bristol-Myers Squibb, Celgene, Lilly, Merck Sharp & Dohme, Merck KGaA, Novartis, Roche, Servier, outside the submitted work. KM reports grants and personal fees from Eli Lilly, during the conduct of the study; grants from MSD, Daiichi Sankyo, Shionogi, Kyowa Hakko Kirin, Gilead Sciences, Sanofi, Pfizer, and Merck Serono; personal fees from Chugai, Taiho, Takeda, Bayer, and from Eli Lilly, outside the submitted work. DC reports grants from 4SC, AstraZeneca, Bayer, Celgene, Clovis, Eli Lilly, Janssen, Medimmune, Merck, Merrimack and Sanofi, outside the submitted work. SA-B reports an advisory role with Merck, Roche, Celgene, Lilly, Nordic Pharma, Bristol-Myers Squibb and MSD Sharp & Dohme; is a speaker for Roche, Celgene, Lilly, Nordic Pharma, AIO gGmbH, MCI, Promedicis, and Forum für Medizinische Fortbildung; is CEO/Founder of IKF Klinische Krebsforschung GmbH; and has received research grants from Sanofi, Merck, Roche, Celgene, Vifor, Medac, Hospira, Lilly, German Cancer Aid (Krebshilfe), German Research Foundation, and the Federal Ministry of Education of Research. ZAW reports personal fees from Lilly, Merck, personal fees from BMS,

grants from Five Prime Therapeutics, outside the submitted work. AO reports grants and personal fees from BMS, and personal fees from Ono, Chugai, and Taiho, outside the submitted work. SRW, SM, DF, and RRH are Eli Lilly employees and own company stock. GB, AS, SC, JA, SCO report no conflicts of interest.

Acknowledgements

The authors thank the patients, investigators, and institutions involved in this study. They also thank Mary Dugan Wood for writing assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.10.026.

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