

Keywords: gastric cancer; angiogenesis; Angiopoietin-2; bevacizumab; liver metastasis; biomarker

Evaluation of Angiopoietin-2 as a biomarker in gastric cancer: results from the randomised phase III AVAGAST trial

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Background: In the phase III AVAGAST trial, the addition of bevacizumab to chemotherapy improved progression-free survival (PFS) but not overall survival (OS) in patients with advanced gastric cancer. We studied the role of Angiopoietin-2 (Ang-2), a key driver of tumour angiogenesis, metastasis and resistance to antiangiogenic treatment, as a biomarker.

Methods: Previously untreated, advanced gastric cancer patients were randomly assigned to receive bevacizumab ($n = 387$) or placebo ($n = 387$) in combination with chemotherapy. Plasma collected at baseline and at progression was analysed by ELISA. The role of Ang-2 as a prognostic and a predictive biomarker of bevacizumab efficacy was studied using a Cox proportional hazards model. Logistic regression analysis was applied for correlations with metastasis.

Results: Median baseline plasma Ang-2 levels were lower in Asian (2143 pg ml^{-1}) vs non-Asian patients (3193 pg ml^{-1}), $P < 0.0001$. Baseline plasma Ang-2 was identified as an independent prognostic marker for OS but did not predict bevacizumab efficacy alone or in combination with baseline VEGF. Baseline plasma Ang-2 correlated with the frequency of liver metastasis (LM) at any time: Odds ratio per 1000 pg ml^{-1} increase: 1.19; 95% CI 1.10–1.29; $P < 0.0001$ (non-Asians) and 1.37; 95% CI 1.13–1.64; $P = 0.0010$ (Asians).

Conclusions: Baseline plasma Ang-2 is a novel prognostic biomarker for OS in advanced gastric cancer strongly associated with LM. Differences in Ang-2 mediated vascular response may, in part, account for outcome differences between Asian and non-Asian patients; however, data have to be further validated. Ang-2 is a promising drug target in gastric cancer.

Gastric cancer is the second most frequent cause of cancer death in the world (Brenner *et al*, 2009; Jemal *et al*, 2011). There is substantial geographic variation in the incidence and mortality of gastric cancer, with the highest rates in East Asia and the lowest in

North America (Kamangar *et al*, 2006). Asian patients, however, appear to have increased survival rates compared with their Western counterparts (Bollschweiler *et al*, 1993). Nevertheless, survival rates are among the worst of any solid tumour despite

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Received 25 September 2015; revised 29 December 2015; accepted 20 January 2016; published online 31 March 2016

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modern surgery. Therefore, identifying additional predictive and prognostic markers is an important step to improving current treatment approaches and extending survival.

The global, randomised, double-blind phase III AVAGAST (BO20904/AVF4200g) trial was conducted to evaluate the benefit of combining the vascular endothelial growth factor (VEGF) targeting antibody bevacizumab with cisplatin plus fluoropyrimidine *vs* placebo plus chemotherapy as the first-line treatment for patients with advanced gastric cancer. Addition of bevacizumab significantly prolonged progression-free survival (PFS) compared with placebo (median, 6.7 *vs* 5.3 months; hazard ratio (HR), 0.80; $P=0.0037$) and increased overall response rate (46.0% *vs* 37.4%; $P=0.0315$). This was true both for Asian and non-Asian patients. However, the difference in overall survival (OS) – the primary study end point – did not reach statistical significance (12.1 *vs* 10.1 months; HR, 0.87; $P=0.1002$; Ohtsu *et al*, 2011). Yet, in subgroup analyses, a survival benefit from bevacizumab was observed in patients enrolled in North and Latin America (HR 0.63; 95% CI 0.43–0.94), whereas patients enrolled in Asia had no benefit (90% from Japan and Korea; HR 0.97; 95% CI 0.75–1.25) and European patients showed intermediate-risk reduction (HR 0.85; 95% CI 0.63–1.14). Similar observations were made for the chemotherapy only arm with a median OS of 12.1 months in Asian, 8.6 months in European and 6.8 months in Pan-American patients (Ohtsu *et al*, 2011). In part, a higher rate of later line chemotherapy exposure in Asian patients might have contributed to these findings. It has not yet been clearly established that biological mechanisms contribute to the outcome differences observed between Asian and non-Asian patients. Although comprehensive molecular characterisation of gastric cancer did not show any systematic differences in the distribution of four subtypes of gastric cancer between patients of East Asian and Western origin (Cancer Genome Atlas Research Network, 2014), gene expression signatures of tumour immunity distinguished Asian and non-Asian gastric adenocarcinomas (Lin *et al*, 2015).

The value of antiangiogenic therapy for patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma has recently been demonstrated in the phase III REGARD trial comparing the VEGF-receptor-2 antibody ramucirumab with placebo in second-line treatment. Median OS was increased from 3.8 to 5.2 months in the ramucirumab arm compared with the placebo group; HR 0.776; 95% CI 0.603–0.998; $P=0.047$. A similar survival benefit was observed when ramucirumab was combined with chemotherapy (Wilke *et al*, 2014). Despite intense efforts, however, predictive biomarkers indicating the efficacy of antiangiogenic treatment have not yet been developed and novel prognostic biomarkers are urgently needed (Murukesh *et al*, 2010; Hegde *et al*, 2013).

The Tie2 ligand Angiopoietin-2 (Ang2) mediates endothelial cell (EC) responsiveness to tumour-derived cytokines and has recently emerged as a promising target for second-generation antiangiogenic drug development that can be combined with established anti-VEGF/VEGFR therapies (Hashizume *et al*, 2010; Koh *et al*, 2010; Gerald *et al*, 2013; Srivastava *et al*, 2014). One of the earliest responses of activated ECs is to secrete Ang-2 to facilitate blood vessel destabilisation and induce angiogenesis (Gerald *et al*, 2013). The activity of Ang-2 as an opponent of VEGF inhibition has recently been investigated in preclinical tumour models. Specifically, upregulation of Ang-2 during anti-VEGF treatment was identified as a mechanism of acquired resistance (Daly *et al*, 2013; Rigamonti *et al*, 2014). In addition, Ang-2 has a central role in the vascular inflammatory response by recruiting bone marrow-derived myeloid cells (Scholz *et al*, 2011) that promote VEGF-independent tumour angiogenesis (Ferrara, 2010). More importantly, there is a growing body of evidence indicating that Ang-2 is centrally involved in the formation of tumour metastasis (Imanishi *et al*, 2007; Koh *et al*, 2010; Mazziere *et al*,

2011; Schulz *et al*, 2011; Albin and Noonan, 2012; Holopainen *et al*, 2012; Im *et al*, 2013; Rigamonti and De Palma, 2013; Srivastava *et al*, 2014). Ang-2 not only controls the recruitment of tumour-promoting metastasis-associated macrophages but also affects the angiogenic response to myeloid-derived proangiogenic cytokines, such as Bv8 (Srivastava *et al*, 2014), which results in anti-VEGF refractoriness. Ang-2 thereby contributes to tumour progression by linking the metastatic inflammasome and the angiogenic programme.

On this basis, Ang-2 represents a promising biomarker in the context of VEGF-directed antiangiogenic cancer treatment. Plasma Ang-2 levels are elevated in cancer patients and are associated with a worse prognosis in a number of malignancies (Park *et al*, 2007; Helfrich *et al*, 2009; Goede *et al*, 2010; Maffei *et al*, 2010; Llovet *et al*, 2012). With respect to gastric cancer, preoperative Ang-2 levels correlated with lymph node status in patients with early gastric cancer (Jo *et al*, 2009).

Here we report on the value of Ang-2 as a biomarker in gastric cancer patients enrolled in the AVAGAST trial.

MATERIALS AND METHODS

Study design. The study design of the AVAGAST trial (NCT00548548) has been described previously. In brief, patients with untreated, histologically confirmed, unresectable, locally advanced or metastatic adenocarcinoma of the stomach or gastro-oesophageal junction were assigned to bevacizumab ($n=387$) or placebo ($n=387$) in combination with cisplatin for six cycles plus capecitabine until disease progression or intolerable adverse effects occurred. In patients unable to take oral medications, fluorouracil was substituted for capecitabine. The protocol was approved at each participating site by an independent ethics committee or institutional review board. The trial was carried out in accordance with the declaration of Helsinki. All patients provided written informed consent before study entry.

Sample collection. The biomarker analysis was predefined in the study protocol. Baseline blood and tumour tissue (primary or recurrent) samples were collected from all randomly assigned patients. Blood samples (5 ml collected in EDTA) for plasma isolation were taken between randomisation and start of treatment. Samples were analysed using a commercially available ELISA Kit (Quantikine; R&D Systems GmbH, Wiesbaden, Germany) according to the instructions of the manufacturer. For the detection of VEGF-A, plasma samples were analysed at Roche Diagnostics (Penzberg, Germany) using IMPACT technology as previously described (Van Cutsem *et al*, 2012), which preferentially detects short VEGF isoforms.

Statistical analysis. The cutoff date for the primary efficacy analysis was 30 November 2009, when the predefined limit of 509 deaths was reached. Correlations between biomarkers and OS, defined as time between randomisation and death irrespective of cause, and PFS, defined as time between randomisation and first documented disease progression or death, was assessed. The median biomarker value at baseline was used to dichotomise patients into low or high biomarker groups. Time-to-event end points were analysed using Kaplan–Meier methods. The prognostic and predictive value of the biomarker was assessed using Cox proportional hazards models, including treatment group, biomarker group and interaction between treatment and biomarker as covariates in the model. Additional models that included stratification factors (i.e., geographic region, route of fluoropyrimidine treatment, disease status) as covariates were also evaluated. Logistic regression analysis was performed to investigate the correlation between liver metastasis and baseline Ang-2 levels and the odds ratio relating the presence of liver metastasis per

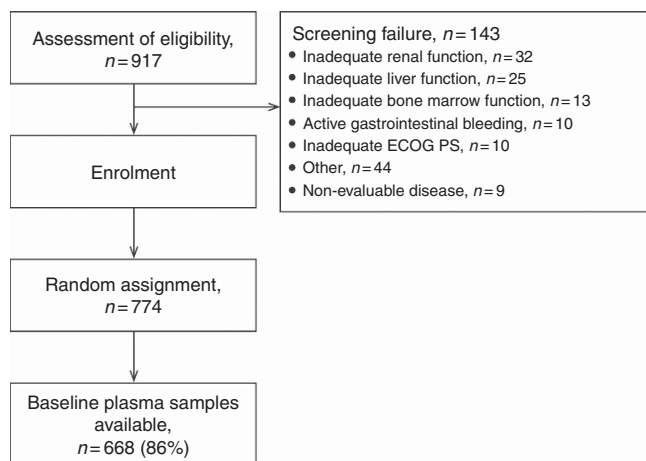


Figure 1. CONSORT diagram. ECOG = Eastern Cooperative Oncology Group; GI = gastro-intestinal; PS = performance status.

1000 pg ml^{-1} increase in baseline Ang-2 was calculated. Chi-square test was used to compare differences in the occurrence of liver metastasis during treatment for patients with high or low levels of the biomarker at baseline. All biomarker analyses were performed according to the REMARK criteria (McShane *et al*, 2005); for CONSORT diagram, see Figure 1.

RESULTS

Patients. From September 2007 to December 2008, 774 patients with advanced gastric cancer were enrolled in the AVAGST trial (Figure 1). Baseline plasma samples were available from 668 patients (86%). Patient characteristics of the biomarker populations were similar to those of the overall study population (data not shown).

Ang-2 biomarker levels at baseline. Demographic variables (age and sex) of the Ang-2 biomarker populations (high vs low Ang-2) were well balanced. There was a weak correlation between prior (neo)-adjuvant or adjuvant therapy and low Ang-2 levels at baseline ($P=0.0405$). Furthermore, there was a trend towards a decreased frequency of diffuse gastric cancer in the high Ang-2 cohort (44% vs 52%; $P=0.0528$). On the other hand, Ang-2-high patients presented with a significantly higher frequency of liver metastasis at baseline (42% vs 24%; $P>0.0001$). In addition, the frequency of patients with ≥ 2 metastatic sites (69% vs 60%; $P=0.02$) and the frequency of bone lesions (7% vs 2%; $P=0.0016$) was higher in the high Ang-2 cohort compared with the low Ang-2 cohort (Table 1). Because of the relation of Ang-2 with obesity and the metabolic syndrome (Silha *et al*, 2005), we studied the association of plasma Ang-2 with body mass index (BMI) at baseline. Indeed, patients with a BMI $> 25 \text{ kg m}^{-2}$ had higher Ang-2 levels than those with a BMI between 18.5 and 25 kg m^{-2} (2913.0 vs 2393.0 pg ml^{-1}). Interestingly, underweight/cachectic patients ($n=36$; BMI $< 18.5 \text{ kg m}^{-2}$) showed intermediate baseline plasma Ang-2 levels of 2643.0 pg ml^{-1} (Table 1).

Finally, baseline plasma Ang-2 levels were significantly higher in patients enrolled from non-Asian regions than in patients from the Asia-Pacific region (median Ang-2 2143 vs 3193 pg ml^{-1} , $P<0.0001$; Mann-Whitney *U*-test).

Ang-2 prognostic biomarker evaluation. Median OS in the entire population (Figure 2) was longer in patients with low compared with high Ang-2 levels (13.7 months vs 10.0 months, HR 0.77; 95% CI, 0.64 to 0.93; $P=0.0055$), and Ang-2 levels retained

independent prognostic value in a multivariate analysis, along with clinical characteristics (ECOG performance status, prior gastrectomy, liver metastasis and type of gastric cancer), HR 1.23; 95% CI 1.01–1.49; $P=0.0385$ (Table 2). However, no correlation between baseline Ang-2 and PFS (Figure 2) was found in both univariate and multivariate analyses; HR 1.07; 95% CI 0.90–1.27; $P=0.4566$. Additionally, separate analyses were carried out in the placebo arm and the bevacizumab arm, respectively, regarding PFS and OS according to Ang-2 biomarker levels high vs low. Low Ang-2 levels were associated with prolonged OS in both treatment arms analysed separately. Detailed data are given in Supplementary Table S1.

Ang-2 predictive biomarker evaluation. Biomarker analysis was performed for the entire available study cohort ($n=668$). Ang-2 baseline levels were not predictive of clinical outcome in response to bevacizumab. The HR for PFS in the low Ang-2 group was 0.79; 95% CI 0.62–0.99 vs 0.82 95% CI 0.65–1.03 in the high Ang-2 group. The HR for OS in the low Ang-2 group was 0.80; 95% CI 0.62–1.05 vs 0.87; 95% CI 0.67–0.1.13 in the high Ang-2 group with an interaction *P*-value of 0.74. In multiple Cox regression models, Ang-2 levels were not predictive of benefit from bevacizumab with respect to PFS or OS. In addition, subgroup analysis for each ethnic group (Asians vs non-Asians) using the Ang-2 medians for dichotomisation failed to demonstrate a predictive role for the response to bevacizumab (Supplementary Figure S1). Forest plots of HR for PFS and OS were analysed for subgroups with VEGF and Ang-2, respectively, either being high/high, high/low, low/high or low/low. These analyses demonstrated that VEGF was the predominant marker in predicting the response to bevacizumab in non-Asian patients and Ang-2 did not contribute significantly in this respect (Figure 3).

Ang-2 and liver metastasis. Baseline plasma Ang-2 levels were higher in patients with liver metastasis than in patients without liver metastasis (3175.5 pg ml^{-1} compared with 2374 pg ml^{-1} , $P<0.0001$; Table 1). The frequency of liver metastasis was higher in non-Asian than in Asian patients (39% vs 28%, Chi-Square; $P=0.003$) and correlated with baseline Ang-2 levels. In non-Asian patients, the frequency of liver metastasis in the high vs low Ang-2 group was 45% and 27%, respectively. In Asian patients, the frequency of liver metastasis at baseline in the high vs low Ang-2 group was 37% and 23%, respectively. Logistic regression analysis confirmed that Ang-2 levels were strongly correlated with the frequency of liver metastasis at any time (Figure 4). The odds ratio per 1000 pg ml^{-1} increase in baseline plasma Ang-2 was 1.19; 95% CI 1.10–1.29; $P<0.0001$ (non-Asian) and 1.37; 95% CI 1.13–1.64; $P=0.0010$ (Asian).

A total of 16 of the 445 patients without liver metastasis at baseline (3.6%) developed liver metastasis at progression and 44 of the 222 patients (19.8%) who initially presented with liver metastasis developed new metastatic lesions in the liver during treatment.

Ang-2 levels at progression. Given that Ang-2 is biologically involved in tumour progression and metastasis, we examined plasma samples at progression in 379 of the 668 patients (56.7%). Interestingly, in the entire cohort, there was no change in median Ang-2 levels (2567 vs 2521.0 pg ml^{-1} , NS) upon progression. In fact, Ang-2 levels (2217.0 pg ml^{-1} ; $n=183$) at progression were lower in bevacizumab-treated patients compared with patient receiving chemotherapy only (2891.0 pg ml^{-1} ; $n=196$). In patients ($n=16$) who were free of liver metastasis at baseline and who developed liver metastasis at progression, Ang-2 levels increased from 2476 to 2899 pg ml^{-1} and an even distribution regarding ethnicity as well as treatment arm was found (Supplementary Figure S2). In the presence of liver metastasis, bevacizumab treatment was associated with a marked decrease in

Table 1. Clinical characteristics by baseline Ang-2 biomarker

	Ang-2 (n = 668) median biomarker level	P-value	Ang-2 low level	Ang-2 high level	P-value
ECOG PS					
≥ 1	2707.0	0.0036	168 (50%)	191 (57%)	0.0877
0	2478.0		166 (50%)	143 (43%)	
Prior gastrectomy					
No	2568.0	0.6129	240 (72%)	241 (72%)	1.0000
Yes	2564.0		94 (28%)	93 (28%)	
Prior adjuvant/neoadjuvant chemotherapy					
No	2607.0	0.0133	301 (90%)	316 (95%)	0.0405
Yes	2125.0		33 (10%)	18 (5%)	
Age category, years					
40–65	2515.0	0.1379			
< 40	2485.5				
≥ 65	2608.0				
Body mass index, kg m⁻²					
18.5–25	2393.0	0.0032	239 (72%)	198 (59%)	0.0011
< 18.5	2643.0		36 (11%)	42 (13%)	
≥ 25	2913.0		58 (17%)	93 (28%)	
Sex					
Female	2638.5	0.5450	102 (31%)	114 (34%)	0.3629
Male	2503.0		232 (69%)	220 (66%)	
Liver metastasis					
No	2374.0	<0.0001	253 (76%)	192 (57%)	<0.0001
Yes	3175.5		81 (24%)	141 (42%)	
Number of metastatic sites at baseline					
≤ 1	2391.0	0.0014	133 (40%)	104 (31%)	0.0235
≥ 2	2707.0		201 (60%)	230 (69%)	
Bone metastasis at baseline					
No	2503.0	0.0004	327 (98%)	309 (93%)	0.0016
Yes	3532.0		7 (2%)	24 (7%)	
Type of gastric cancer					
Diffuse	2418.5	0.0360	174 (52%)	148 (44%)	0.0528
Intestinal	2662.0		120 (36%)	135 (40%)	
Mixed	2759.5		23 (7%)	29 (9%)	
Missing	2820.0		17 (5%)	22 (7%)	

Abbreviations: Ang-2 = Angiotensin-2; ECOG = Eastern Cooperative Oncology Group; PS = performance status. Plasma Ang-2 biomarker was dichotomised at the median to define high vs low Ang-2.

plasma Ang-2 levels at progression while in the chemotherapy-treated group there was a trend towards increased plasma Ang-2 levels at progression (Supplementary Table S2).

In summary, we found that (i) Ang-2 levels at baseline are higher in non-Asian compared with Asian gastric cancer patients; (ii) baseline plasma Ang-2 does not predict response to bevacizumab treatment; (iii) baseline plasma Ang-2 represents a novel prognostic biomarker in gastric cancer; and (iv) baseline Ang-2 levels strongly correlate with the frequency of liver metastasis across both ethnic groups.

These findings highlight differences between ethnic groups in gastric cancer patients related to tumour angiogenesis. To the best of our knowledge, these are the first data derived from a large clinical trial correlating plasma Ang-2 levels with metastasis in gastric cancer.

DISCUSSION

Within the biomarker programme of the AVAGAST trial, we studied the role of Ang-2 as a biomarker in advanced gastric cancer patients.

There is accumulating evidence that Asian and non-Asian gastric cancer patients differ in the course and prognosis of the disease (Gill *et al*, 2003; Kim *et al*, 2010). In our analyses,

baseline Ang-2 levels were significantly lower in Asian compared with non-Asian gastric cancer patients, and similar differences were recently reported regarding baseline VEGF levels (Van Cutsem *et al*, 2012). Of note, even in healthy individuals Ang-2 levels are lower in Asians vs non-Asians (Cho *et al*, 2011). From this background, it can be speculated that genetic and/or environmental factors exist, which might be related to differences in angiogenic response between non-Asian and Asian gastric cancer patients that might influence clinical outcome. Data on biological variables related to outcome differences, however, are controversial: comprehensive molecular characterisation of gastric cancer did not show any systematic differences in distribution of subtypes between patients of East Asian and Western origin (Cancer Genome Atlas Research Network, 2014). In contrast, gene expression signatures of tumour immunity distinguished Asian and non-Asian gastric adenocarcinomas (Lin *et al*, 2015). Our findings have to be validated in additional cohorts.

Despite the fact that extensive efforts have been made, no biomarker has been identified, predicting the efficacy of anti-angiogenic drug treatment. In the AVAGAST trial, baseline VEGF-A levels have been proposed to be predictive for the benefit to bevacizumab treatment specifically in the non-Asian study population (Van Cutsem *et al*, 2012). Despite the fact that

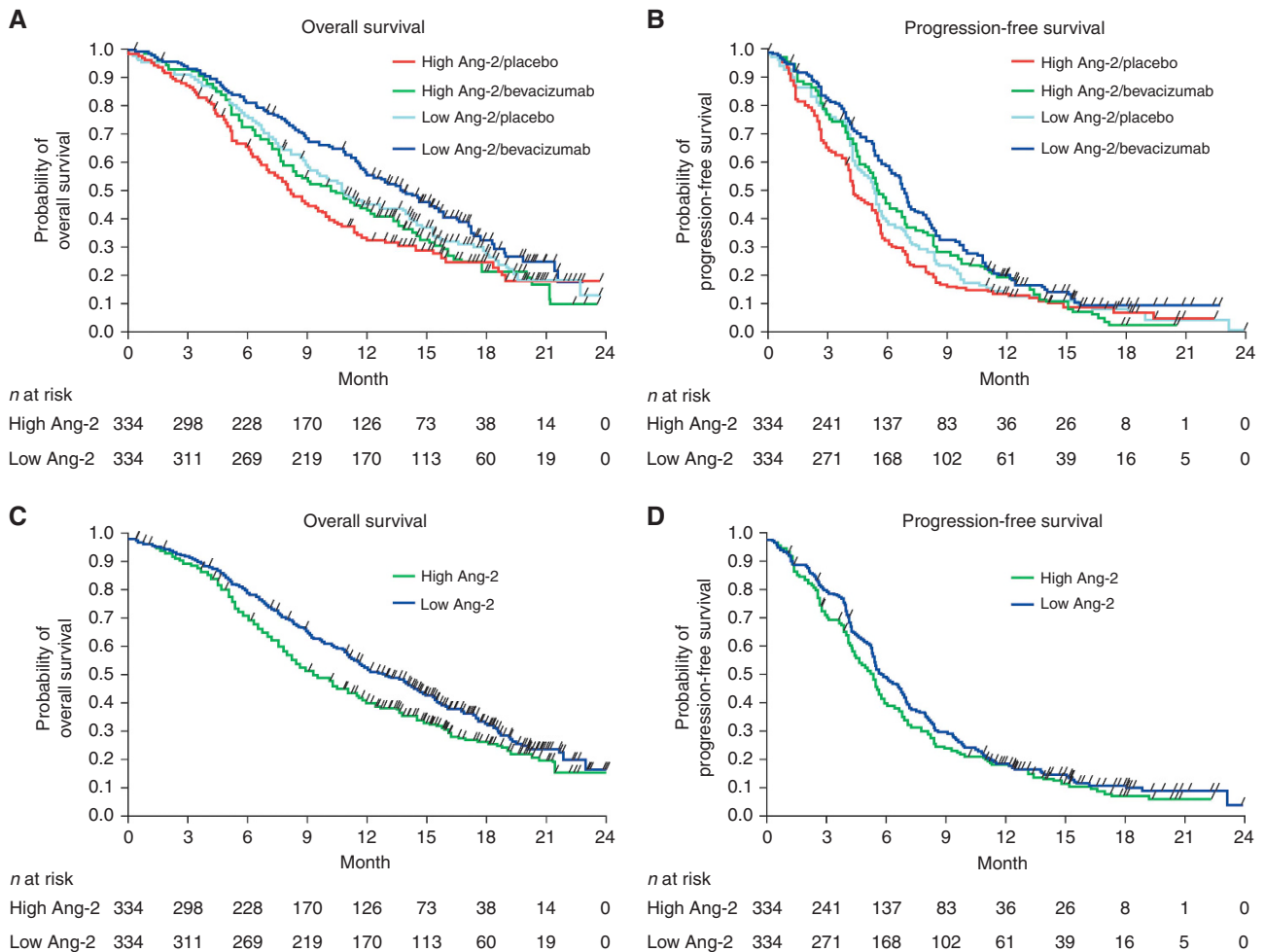


Figure 2. Overall survival and progression-free survival according to baseline Ang-2 levels. (A) Kaplan–Meier estimates of overall survival according to treatment arm and biomarker level. (B) Kaplan–Meier estimates of progression-free survival according to treatment arm and biomarker level (i.e., baseline plasma Ang-2 levels dichotomised by median value). (C) Kaplan–Meier curves of overall survival according to biomarker and (D) Kaplan–Meier curves of progression-free survival according to biomarker including both treatment arms (entire biomarker population).

Table 2. Multivariate analyses of all-patient cohorts to identify factors independently prognostic for OS in patients with advanced or metastatic gastric cancer

Effect/covariate included in the model	Hazard ratio	95% confidence interval for hazard ratio	P-value
Biomarker level (median cutoff)	1.23	1.01–1.49	0.0385
ECOG performance status (≥ 1 vs 0)	1.70	1.39–2.07	<0.0001
Prior gastrectomy (yes vs no)	0.60	0.47–0.77	<0.0001
Prior adjuvant/neoadjuvant chemotherapy (yes vs no)	1.07	0.07–1.63	0.7582
Age at randomisation (years)	1.00	0.99–1.00	0.2946
Sex (male vs female)	1.11	0.90–1.37	0.3240
Liver metastasis (yes vs no)	1.31	1.04–1.65	0.0198
Number of metastatic sites at baseline (≥ 2 vs ≤ 1)	1.13	0.91–1.41	0.2613
Bone metastasis at baseline (yes vs no)	1.42	0.94–2.19	0.0977
Type of gastric cancer (intestinal vs mixed)	0.72	0.53–0.96	0.0240
Type of gastric cancer (diffuse vs mixed)	1.01	0.76–1.34	0.9539

Abbreviations: Ang-2 = Angiopoietin-2; OS = overall survival.

preclinical data indicate an interaction between Ang-2 and VEGF that potentially influences the efficacy of VEGF-targeting drugs (Ferrara, 2010; Kim and Koh, 2011; Rigamonti *et al*, 2014), baseline Ang-2 levels were not predictive for OS or PFS in response to bevacizumab in our study. Moreover, combined VEGF/Ang-2 biomarker evaluation did not further improve the predictive value already demonstrated for VEGF (Van Cutsem *et al*, 2012).

Ang-2 was identified as a novel prognostic biomarker of survival in advanced gastric cancer as demonstrated by multivariate analysis. In line with our findings, baseline plasma or serum Ang-2 has previously been shown to be a prognostic biomarker of survival in other solid tumour entities, such as colorectal cancer, hepatocellular carcinoma, non-small cell lung cancer and melanoma (Park *et al*, 2007; Helfrich *et al*, 2009; Goede *et al*, 2010; Volkova *et al*, 2011; Llovet *et al*, 2012).

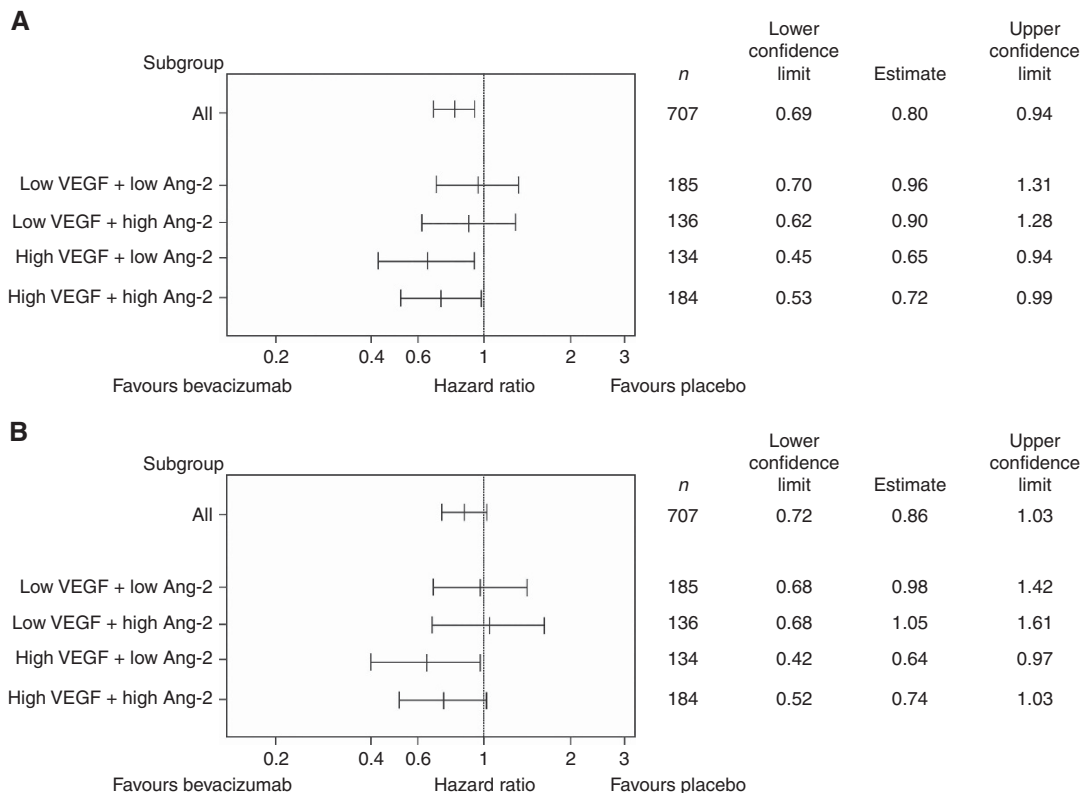


Figure 3. Combined VEGF/Ang-2 biomarker analysis. Forest plots of hazard ratios (bevacizumab plus chemotherapy vs placebo plus chemotherapy) for (A) progression-free survival by biomarker and (B) overall survival by biomarker (high VEGF/high Ang-2; high VEGF/low Ang-2; low VEGF/high Ang-2 and low VEGF/low Ang-2).

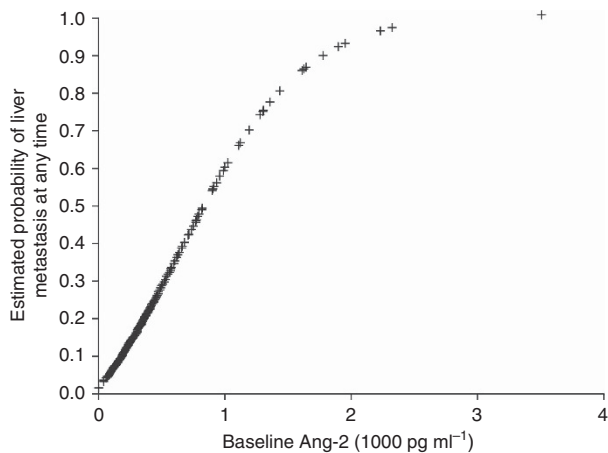


Figure 4. Logistic regression analysis: Baseline Ang-2 levels and frequency of liver metastasis at baseline.

In our study, the frequency of liver metastasis was strongly correlated with baseline Ang-2 levels across ethnic groups (Figure 4). It is tempting to speculate that this association might contribute to the reduced OS in gastric cancer patients with elevated Ang-2 levels. Interestingly, the presence of bone metastasis was also correlated with high pretherapeutic Ang-2 levels ($P = 0.0016$); however, in view of the overall low number of cases with bone metastasis, no further statistical analysis was performed (Table 1). Recently, a number of preclinical studies in patients with pancreatic ductal adenocarcinoma (Schulz *et al*, 2011), breast cancer (Sfiligoi *et al*, 2003) and early gastric cancer (Jo *et al*, 2009) have demonstrated that Ang-2 levels were correlated with lymph node metastasis, and preclinical studies indicate that Ang-2 is centrally involved in the formation of tumour metastasis

(Imanishi *et al*, 2007; Koh *et al*, 2010; Mazziere *et al*, 2011; Minami *et al*, 2013; Schulz *et al*, 2011; Albin and Noonan, 2012; Holopainen *et al*, 2012; Im *et al*, 2013; Rigamonti and De Palma, 2013; Srivastava *et al*, 2014). Although Ang-2 plasma levels in our study may reflect the extent of disease, the finding that plasma Ang-2 levels did not further increase at progression in the entire cohort at least points to possible site-specific differences with respect to liver metastasis. Although we cannot validate this hypothesis owing to the lack of availability of tumour tissue from primary tumour vs metastatic sites in our cohort, recent data from colorectal cancer patients clearly demonstrated increased Ang-2 protein levels in liver metastases compared with respective primary tumours and lung metastases (Kahlert *et al*, 2014).

Another question that cannot be addressed by the data presented here is the origin of Ang-2 expression that results in the plasma levels observed in gastric cancer patients. Ang-2 is preferentially expressed in ECs and subsequently stored in the Weibel-Pallade bodies. In line with this finding, a number of studies has demonstrated increased Ang-2 expression within the tumour stroma (Goede *et al*, 2010), while tumour cell-derived expression was limited to distinct tumour entities (Koga *et al*, 2001; Sfiligoi *et al*, 2003; Lee *et al*, 2006). In this respect, very recently, mechanisms have been identified that lead to an upregulation of Ang-2 expression in gastric cancer cells (Chen *et al*, 2015). Additionally, induction of Ang-2 from non-neoplastic vasculature related to vascular inflammatory response (Fiedler *et al*, 2006; Kim and Koh, 2011) might represent another source of plasma Ang-2. As an example, circulating Ang-2 levels are increased in overweight and obese individuals (Silha *et al*, 2005). In line with this, we found higher Ang-2 levels in patients with a BMI $> 25 \text{ kg m}^{-2}$ compared with those with a BMI between 18.5 and 25 kg m^{-2} ($2913.0 \text{ vs } 2393.0 \text{ pg ml}^{-1}$). In contrast, underweight/cachectic patients ($n = 36$; BMI $< 18.5 \text{ kg m}^{-2}$) showed intermediate baseline plasma Ang-2 levels of $2643.0 \text{ pg ml}^{-1}$, and it can be speculated that an

increased inflammatory response (Tsoli and Robertson, 2013) in cancer cachexia might contribute to this finding.

Finally, an analysis including the entire cohort revealed that Ang-2 levels at metastatic progression were unchanged. In the bevacizumab-treated group, however, Ang-2 levels were decreased at progression, compared with the chemotherapy only group (2217.0 vs 2891.0 pg ml⁻¹). This finding was specifically true for patients with liver metastasis at baseline (see Supplementary Table S2). Interestingly, data from a mouse RIP1-Tag2 model of pancreatic neuroendocrine tumours correlated upregulation of Ang-2 with resistance to anti-VEGF treatment. On the other hand, in a murine model of ovarian cancer, VEGF induced the expression of Ang-2 (Zhang *et al*, 2003). Thus inhibition of VEGF might indeed be related to a decrease in Ang-2 production.

Recently, trebananib demonstrated improved PFS in a phase III study (Monk *et al*, 2014) in women with recurrent ovarian cancer but later on failed to improve OS (Sheridan, 2015). The specific mechanism of trebananib targeting both Ang-1 and Ang-2 has been discussed as a possible reason for these negative results, given the role of Ang-1 in maintaining normal vasculature and its inhibitory effect on metastasis. In addition, the fact that Ang-2 is most important during early stages of tumour progression (Helfrich *et al*, 2009) may have had a role. Other drugs are currently in late-stage clinical development targeting Ang-2 alone or in combination with VEGF (Brown *et al*, 2010; Huang *et al*, 2011; Karlan *et al*, 2012; Kienast *et al*, 2013). The data presented here should encourage the initiation of clinical trials testing such compounds in the adjuvant and palliative setting in gastric cancer patients. Both the central role of Ang-2 in tumour angiogenesis, which represents an important and well-established target in advanced gastric cancer treatment, and recent findings linking the pro-tumorigenic activity of dopamine and cAMP-regulated phosphoprotein, Mr 32000 (DARPP-32), which is overexpressed in gastric cancer, with STAT3-mediated upregulation of Ang-2 expression in gastric cancer tumour cells (Chen *et al*, 2015) point towards a role of Ang-2 as a drug target in gastric cancer.

In summary, marked differences in baseline plasma Ang-2 levels exist between Asian and non-Asian gastric cancer patients. Ang-2 is not a predictive biomarker for bevacizumab efficiency in gastric cancer but has a role as an independent prognostic biomarker for survival and is strongly correlated with liver metastasis. Based on this, the biomarker might prove valuable for the stratification of patients with advanced gastric cancer before randomisation in clinical trials. Ang-2 represents a promising drug target in gastric cancer.

ACKNOWLEDGEMENTS

We thank Professor Dr F Lordick and Dr D Hasenclever for most helpful discussions.

CONFLICT OF INTEREST

Genentech has provided financial support to the University of Cologne (UT Hacker) to carry out this project. There are no other potential conflicts of interest.

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