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Original Article

Early increase of plasma soluble VEGFR-2 is associated with clinical benefit from second-line treatment of paclitaxel and ramucirumab in advanced gastric cancer

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Abstract: Ramucirumab plus paclitaxel is considered the standard of care in the second-line treatment of gastric carcinoma (GC). The aim of this study was to evaluate plasma vascular endothelial growth factor-A (VEGF-A), VEGF-D, and circulating soluble VEGF receptor-2 (sVEGFR-2) as possible markers of resistance or response to ramucirumab administered with paclitaxel in pretreated metastatic GC patients. Plasma samples were collected at different time points (on days 1 and 15 of the first 3 cycles, at best radiologic response and at disease progression). VEGF-A, VEGF-D and sVEGFR-2 were analysed by ELISA. Correlations of biomarker baseline levels or dynamic changes with outcome measures were assessed. Progression-free survival (PFS) was the primary endpoint of the study. Forty-one patients were enrolled. VEGF-A and VEGF-D, but not sVEGFR-2, values significantly increased during treatment compared to baseline ($P < 0.001$). A positive correlation between VEGF-A and sVEGFR-2 at cycle 2 was found ($P=0.045$). At univariate analysis, higher baseline levels of VEGF-A were associated with worse OS ($P=0.015$). Early increase of sVEGFR-2 levels after the first treatment cycle was the only factor associated with longer PFS (6.6 vs. 3.6 months, $P=0.049$) and OS (18.6 vs. 5.2 months, $P=0.008$). Significance of sVEGFR-2 early increase was retained at multivariate analysis for OS (HR 0.32; 95% CI 0.12-0.91; $P=0.032$). The reported results confirmed the prognostic role of baseline VEGF-A and, with the limitations of the limited sample size and the lack of a control arm, suggested that the early increase of sVEGFR-2 after 1 cycle of treatment could be a potential predictive biomarker of benefit from second-line ramucirumab plus paclitaxel in GC.

Keywords: Angiogenesis, biomarkers, gastric cancer, ramucirumab, sVEGFR-2, VEGF-A, VEGF-D

Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed tumour and the fourth leading cause of cancer-related death worldwide [1]. Metastatic GC is characterized by poor prognosis and systemic chemotherapy remains the mainstay of treatment [2].

Angiogenesis, especially vascular endothelial growth factor (VEGF) receptor 2 (VEGFR-2)-triggered new vessel formation, is an important hallmark in the metastatic spreading of tumours [3] and has nowadays entered the clinical scenario as a key therapeutic target [4, 5]. Ramucirumab [IMC-1121B (LY3009806)] is a human IgG-1 monoclonal antibody that targets the extracellular domain of VEGFR-2 with high affinity and prevents the binding of the agonist ligands, resulting in the inhibition of endothelial cells proliferation and migration [6, 7].

In metastatic GC two randomized phase III trials demonstrated a significant survival benefit for ramucirumab either alone (over placebo) [8] or in combination with paclitaxel (over single-agent paclitaxel) [9] as second-line therapy. Nonetheless, looking at the results of registra-

tive trials, only a minority of patients seems to achieve long-term benefit from ramucirumab, whereas about 50% of the cases show primary resistance to antiangiogenic treatment [8, 9]. Many efforts have been made to understand how to optimally select patients, in order to optimize the risk-to-benefit ratio of this agent [10, 11]. However, up to date no reliable predictive biomarkers for ramucirumab efficacy have been identified and validated in GC.

In this scenario, our investigation aims to evaluate relevant VEGF family members, such as VEGF-A and VEGF-D, and circulating soluble VEGFR-2 (sVEGFR-2) as putative markers of resistance or response to ramucirumab administered with paclitaxel in pre-treated metastatic GC patients. Particularly, we explored whether baseline values or dynamic changes during treatment could help in the identification of patients with higher chances of benefit from antiangiogenic therapy.

Materials and methods

Study population and procedures

PREDICTOR (Prospective study aiming at identifying and validating the predictive role of circulating angiogenic factors in patients with metastatic gastric carcinoma treated with second-line paclitaxel and ramucirumab) is a translational prospective study assessing the putative predictive role of different angiogenic mediators evaluable in peripheral blood samples in patients with advanced gastroesophageal carcinoma receiving standard second-line treatment. The first phase of the study (identification phase) is focused on the identification of the most promising biomarker to be subsequently validated in an independent patient cohort in the second phase (validation phase). Here we report the result of the identification phase.

We enrolled patients affected by metastatic adenocarcinoma of the gastroesophageal junction or the stomach who started second-line therapy with paclitaxel and ramucirumab at Azienda Ospedaliero-Universitaria Pisana. Patients who experienced disease progression after first-line chemotherapy with a platinum compound and a fluoropyrimidine (in association with trastuzumab when indicated) were eligible. Patient progressed during or within 6 months after completion of (neo-)adjuvant chemotherapy were also included. Previous treatment with a taxane was not allowed. Patients with life expectancy of less than 3 months according to investigator's judgement were excluded.

All patients received ramucirumab 8 mg/kg i.v. on days 1 and 15 in combination with paclitaxel 80 mg/m^2 i.v. on days 1, 8 and 15 of a 28-day cycle. Treatment was continued until disease progression, unacceptable toxicity, or patient refusal. Maintenance with single-agent ramucirumab was allowed in case of interruption of paclitaxel due to toxicity or after 6 months of treatment. Objective response was evaluated every 10-12 weeks according to RECIST 1.1 criteria [12]. Evaluating radiologist was blinded to the results of biomarker analyses.

The patients provided written informed consent before treatment administration and blood samples collection. The study protocol was approved by the Ethics Committee of the Area Vasta Nord Ovest (n. 20550).

Sample collection and biomarker analysis

Venous blood withdrawals were performed using tubes containing EDTA on days 1 (d1) and 15 (d15) of the first 3 cycles (c1 to c3) before treatment administration. Blood samples were immediately centrifuged at 4°C and plasma fractions were divided in five equal aliquots, frozen and stored at -80°C until assayed. Plasma samples were analyzed by immunoenzymatic assays for total concentration of VEGF-A, VEGF-D and sVEGFR-2 with the ELISA assay Quantikine (DVE00, DVED00 and DVR200, respectively; R&D Systems, Minneapolis, MN, USA). The experimental procedures were carried out according to the ELISA kit protocol, which was standardized and validated by the manufacturer. Optical density was determined using a Multiskan Spectrum microplate reader (Thermo Labsystems, Milan, Italy) set to 450 nm (with a wavelength correction of 540 nm). The results were expressed as picograms (pg) of VEGF-A, VEGF-D, and sVEGFR-2 per milliliter (ml) of plasma.

Statistical considerations

Estimations of time-to-event curves were generated with the Kaplan-Meier method. Overall

Table 1. Patient characteristics

Characteristics	N=41	%
Age years, median (range)	64	(41-80)
Gender		
Male	32	78.1
Female	9	22.0
ECOG performance status		
0	10	24.4
1	31	75.6
Primary tumour site		
Stomach	23	56.1
Cardia	15	36.6
Gastroesophageal junction	3	7.3
Previous gastrectomy		
Yes	22	53.6
No	19	46.4
HER-2 status		
Positive	13	31.7
Negative	28	68.3
Previous (neo-)adjuvant therapy		
Yes	12	29.3
No	29	70.7
Number of metastatic sites		
1	18	43.9
2	17	41.5
3	4	9.8
4	2	4.8
Sites of metastasis		
Peritoneum	24	58.5
Liver	16	39.0
Lung	9	22.0

Abbreviations: N, number; ECOG, Eastern Cooperative Oncology Group; HER-2, Human epidermal growth factor receptor-2.

survival (OS) was defined as the time from the first day of treatment until the day of death. Progression-free survival (PFS) was defined as the time from the first day of treatment until the day of disease progression or death. Patients alive at the time of analyses were censored at the date of their last follow-up visit, whereas those without disease progression were censored at the time of the last radiologic assessment.

Correlation of the baseline values and relative dynamic changes (calculated as the difference between the concentrations after 1 or 2 cycles of treatment and the baseline concentrations, *i.e.* Δ=[c2d1] - [c1d1] or Δ=[c3d1] - [c1d1]) of the evaluated pharmacodynamic markers with PFS was the primary endpoint. Response rate (RR) and OS represented secondary endpoints. We used log-rank test to compare OS and PFS, setting significance at $P < 0.05$ for a two-sided test. The hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated by a stratified Cox proportional hazards model. The association between biomarker levels and RECIST response was evaluated by chi-square test (two sided $P < 0.05$ for significance).

For both PFS and OS we initially performed a univariate assessment of the prognostic effect of each explored determinant, then a multivariate analysis was carried out using a stepwise Cox proportional hazards regression modelling and setting statistical significance at $P < 0.05$.

Linear correlations between VEGF-A and VEGF-D and between VEGF-A and sVEGFR-2 were explored through Pearson's correlation coefficient, while biomarker levels at different time points were compared with two-sided Wilcoxon non-parametric test.

Statistical analyses were carried out using statistical software packages SPSS 20.0 (IBM, Chicago, IL, USA) and Graphpad v8.0 (Inc., San Diego, CA, USA). The study is registered on ClinicalTrials.gov (NCT05301465). Due to its exploratory nature, no formal statistical hypothesis was made for the definition of the sample size in the identification phase of PREDICTOR.

Results

Patient characteristics, activity and toxicity

Forty-one patients were enrolled in the identification phase. Characteristics are summarized in **Table 1**.

With a median follow-up of 16.1 months, an average of 6 cycles per patient was administered. Toxicities were consistent with the known safety profile of the combination (**Table 2**). RR was 26.8% and disease control rate (DCR, calucated as the sum of objective responses and disease stabilizations) was 63.4%. At the time of analysis, 85% of patients had progressed with a median PFS of 5.6 months, while 65% of patients had died with a median OS of 15.1 months. At univariate analysis no significant correlation was found between any of the clinical factors tested and PFS or OS (**Table 3**).

Table 2. Maximum per-patient toxicity according to CTCAE, version 4.0

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
Hypertension	-	2 (4.8%)	-	-
Neutropenia	10 (24.4%)	-	5 (12.2%)	1 (2.4%)
Anemia	10 (24.4%)	4 (9.7%)	2 (4.8%)	-
Thrombocytopenia	3 (7.3%)	2 (4.8%)	-	-
Neurotoxicity	14 (34.2%)	11 (26.8%)	-	-
Asthenia	13 (31.7%)	10 (24.4%)	1 (2.4%)	-
Mucositis	13 (31.7%)	2 (4.8%)	-	-
Diarrhea	11 (26.8%)	1 (2.4%)	-	-
Nausea/vomiting	10 (24.4%)	-	1 (2.4%)	-
Anorexia	6 (14.6%)			
Cutaneous toxicity	5 (12.2%)	-	-	-

Data are presented as number of patients (percent).

Baseline samples analyses

All patients were evaluable with respect to baseline VEGF-A, VEGF-D and sVEGFR-2 values.

At univariate analysis, pre-treatment levels of VEGF-A lower than the median value of 28.90 pg/ml were associated with longer OS (median: 19.5 *vs.* 6.0 months; HR 2.78, 95% CI 1.15-6.77; P=0.015) (**Figure 1**). On the contrary, VEGF-D and sVEGFR-2 values (categorized according to the median baseline values) were associated neither with PFS nor with OS (**Table 4**).

No association was reported between baseline levels of VEGF-A, VEGF-D and sVEGFR-2 and RECIST response (all P > 0.05) (data not shown).

Longitudinal samples analyses

At c1d15 32 patients were evaluable for biomarker assays (c2d1: n=28; c2d15: n=31; c3d1: n=27; c3d15: n=17; at PD: n=25).

When compared to baseline values, both VEGF-A and VEGF-D significantly increased from c1d1 to each time point, exception made for the variation of VEGF-D at PD (**Figure 2A** and **2D**). No significant increase is evident between the levels recorded at the time of objective radiologic response and PD for both factors (**Figure 2B** and **2E**). Among refractory patients (*i.e.* experiencing PD as best response during treatment), VEGF-A (**Figure 2C**) but not VEGF-D (**Figure 2F**) significantly increased during treatment. On the contrary, no significant variations of sVEGFR-2 levels occurred throughout treatment, both in the overall population (**Figure 2G**) as well as in the separated subgroups with primarily sensitive (**Figure 2H**) or refractory (**Figure 2I**) disease.

As an exploratory analysis, we investigated the variations of the three determinants stratifying the study population according to the median PFS value (*i.e.* ≥ 5.6 or < 5.6 months): results did not change, the only exception being the loss of significant increase of VEGF-A and VEGF-D from baseline to progression in the population with PFS ≥5.6 months (data not shown).

Notably, with regard to the primary endpoint of PFS, early increase in sVEGFR-2 from baseline to c2d1 was associated with significantly longer PFS (median: 6.6 *vs.* 3.6 months; HR 0.38, 95% CI 0.15-0.99, P=0.049; **Figure 3A**). Moreover, OS was also longer among patients experiencing an increase in sVEGFR-2 at c2d1 (median: 18.7 *vs.* 5.2 months; HR 0.32, 95% CI 0.13-0.80, P=0.008; **Figure 3B**). Tested against VEGF-A baseline levels, sVEGFR-2 early increase was the only parameter retaining significance at multivariate regression for OS (HR 0.32, 95% CI 0.12-0.91, P=0.032; **Table 4**). Changes from baseline to c2d1 or c3d1 for VEGF-A and VEGF-D, as well as difference from baseline to c3d1 for sVEGFR-2, were not associated with PFS and OS (all P > 0.05) (**Table 4**).

Linear correlation between VEGF-A and VEGF-D and between VEGF-A and sVEGFR-2 was evaluated at each time point. Interestingly, a significantly positive correlation was observed between VEGFR-2 levels and VEGF-A at c2d1 (Pearson's correlation coefficient r=0.34, P= 0.045), as shown in **Figure 4**. Conversely, no significant correlations were found between VEGF-A and VEGF-D or sVEGFR-2 at the other time points (data not shown).

Discussion

Our study investigated the role of three angiogenesis-related mediators, such as VEGF-A,

Table 3. Impact of clinical and pathologic features on PFS and OS (univariate analysis)

	PFS		OS	
	HR (95% CI)	p	HR (95% CI)	p
ECOG performance status				
0 vs. 1	0.47 (0.23-0.96)	0.068	0.79 (0.40-1.66)	0.173
Sites of metastases, yes vs. no				
Liver	0.79 (0.40-1.66)	0.508	1.45 (0.46-3.25)	0.333
Lung	0.95 (0.41-2.21)	0.975	0.94 (0.35-2.54)	0.908
Peritoneum	1.09 (0-51-2.02)	0.918	1.75 (0.82-3.75)	0.176
Number of metastatic sites				
1-2 vs. 3-4	0.87 (0.41-1.86)	0.713	0.64 (0.27-1.55)	0.274
First-line PFS				
≥ 6 vs. < 6 months	0.91 (0.47-1.77)	0.808	0.54 (0.25-1.17)	0.584
First-line response				
PR vs. other	0.91 (0.39-2.13)	0.877	1.12 (0.47-2.71)	0.796
HER-2 status				
positive vs. negative	0.70 (0.36-1.39)	0.320	0.73 (0.34-1.61)	0.456
Signet ring cell histology				
yes *vs.* no	1.03 (0.43-2.47)	0.904	1.54 (0.50-4.74)	0.368

Abbreviations: ECOG, Eastern Cooperative Oncology Group; HR, Hazard ratio (95% CI, 95% confidence interval); OS, Overall survival; P, *p*-value; PFS, Progression-free survival; PR, Partial response.

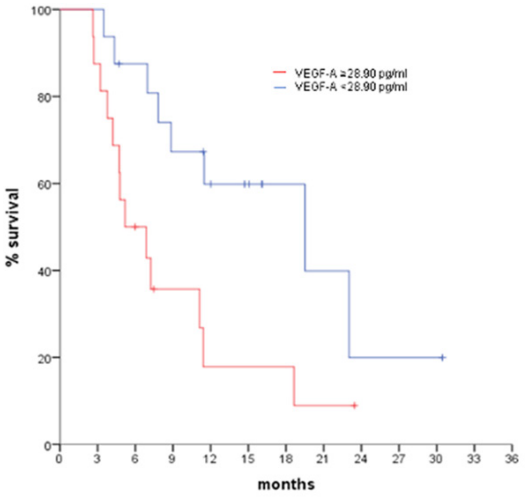

Figure 1. Correlation between baseline VEGF-A levels and overall survival.

VEGF-D and sVEGFR-2, as potential prognostic and predictive biomarkers in metastatic GC treated with second-line paclitaxel plus ramucirumab. We reported an association between higher baseline levels of VEGF-A and shorter OS. More intriguingly, we identified an association between an increase in sVEGFR-2 levels after 1 cycle and prolonged PFS and OS.

The correlation observed between higher circulating baseline VEGF-A and negative prognosis is consistent with literature data [13-15]. Evidence on its negative prognostic role already came from studies evaluating VEGF-A tumour tissue expression [16-18]. Consistency of the data across different studies, together with the limited clinical results reported in unselected populations with antiangiogenic agents [15], confirms the role of angiogenesis in mediating GC aggressiveness and strengthens the need for a personalized antiangiogenic approach in this disease.

Despite its increasing values during therapy at each scheduled time point, already reported in preclinical studies [19], no significant trend was observed for VEGF-A between best radiologic response and PD, suggesting the involvement of alternative transduction pathways mediating acquired resistance to ramucirumab [20, 21]. Indeed, it has been reported an increase in placental growth factor (PlGF) during treatment with ramucirumab and this could represent one of such mechanisms of escape from VEGFR-2 blockade [11].

Up to date, few clinical data about the role of sVEGFR-2 in metastatic GC have been published [22]. Studies conducted on murine mod-

Table 4. Impact of biologic circulating markers on PFS and OS (univariate and multivariate analyses)

	PFS				OS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
VEGF-A								
≥ *vs.* < median	1.37 (0.64-2.94)	0.391	-	-	2.78 (1.15-6.77)	0.015	2.11 (0.79-5.75)	0.136
Δ=[c2d1] - [c1d1] > *vs.* < 0	1.01 (0.29-3.45)	0.987	-	-	0.44 (0.09-2.14)	0.308	-	-
Δ=[c3d1] - [c1d1] > *vs.* < 0	1.01 (0.23-4.45)	0.985	-	-	0.95 (0.20-4.45)	0.943	-	-
VEGF-D								
≥ *vs.* < median	0.55 (0.26-1.19)	0.113	-	-	0.82 (0.35-1.93)	0.645	-	-
Δ=[c2d1] - [c1d1] > *vs.* < 0	0.65 (0.22-1.86)	0.419	-	-	0.93 (0.33-2.64)	0.895	-	-
Δ=[c3d1] - [c1d1] > *vs.* < 0	1.77 (0.54-5.84)	0.344	-	-	0.94 (1.19-4.38)	0.933	-	-
sVEGFR-2								
≥ *vs.* < median	1.15 (0.54-2.46)	0.704	-	-	1.54 (0.65-3.63)	0.321	-	-
Δ=[c2d1] - [c1d1] > *vs.* < 0	0.38 (0.15-0.99)	0.049	0.38 (0.15-0.99)	0.049	0.32 (0.13-0.80)	0.008	0.32 (0.12-0.91)	0.032
Δ=[c3d1] - [c1d1] > *vs.* < 0	1.18 (0.43-3.28)	0.745	-	-	1.37 (0.46-4.03)	0.568	-	-

Abbreviations: Δ=[c2d1] - [c1d1], difference between concentration at day 1 of cycle 2 and day 1 of cycle 1; Δ=[c3d1] - [c1d1], difference between concentration at day 1 of cycle 3 and day 1 of cycle 1; HR, Hazard ratio (95% CI, 95% confidence interval); OS, Overall survival; P, *p*-value; PFS, Progression-free survival.

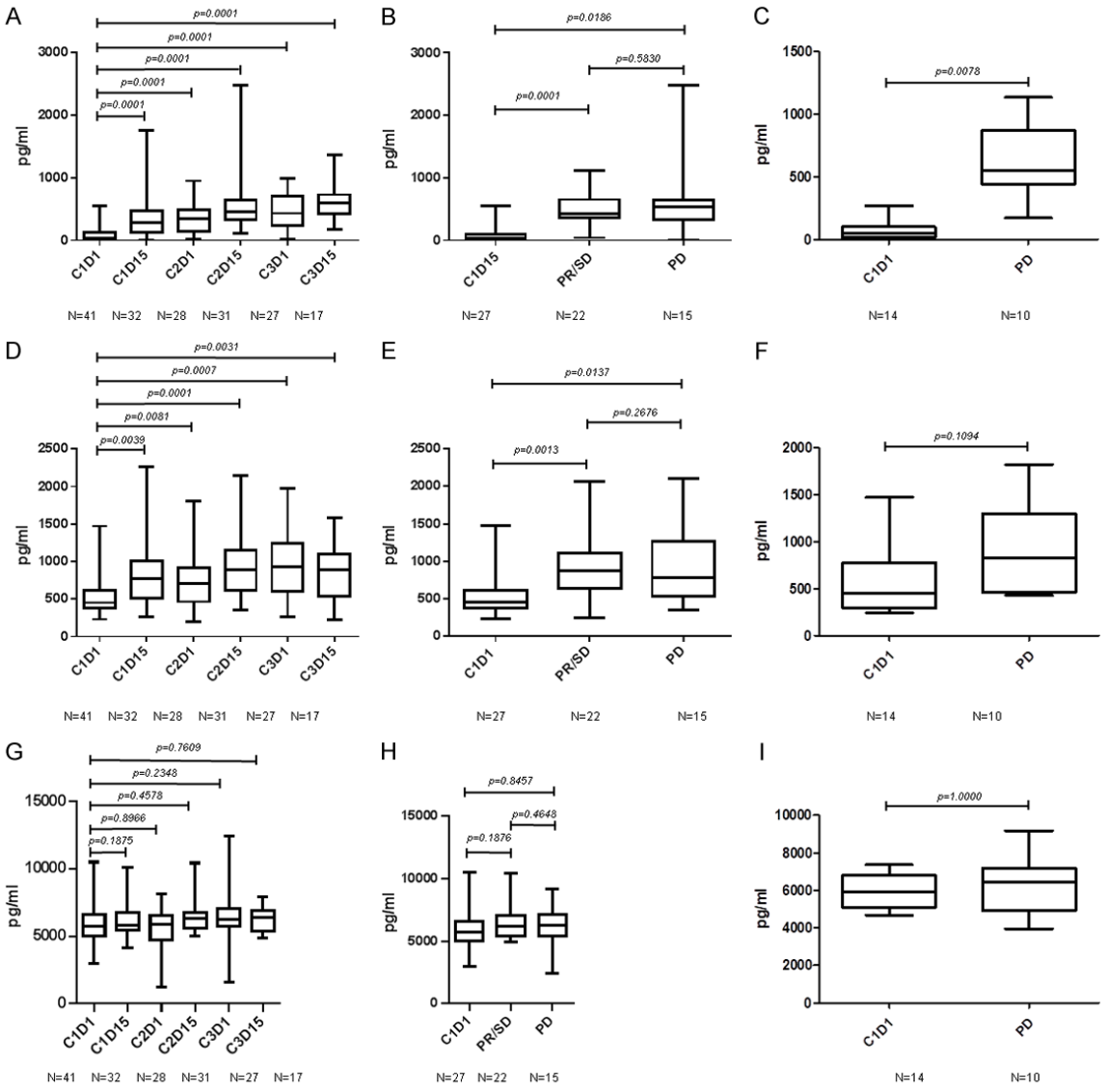

Figure 2. VEGF-A, VEGF-D and sVEGFR-2 during treatment. A. VEGF-A levels during treatment; B. VEGF-A levels during treatment among patients experiencing disease control, *i.e.* partial response (PR) or disease stabilization (SD); C. VEGF-A levels during treatment among patients experiencing early progressive disease (PD); D. VEGF-D levels during treatment; E. VEGF-D levels during treatment among patients experiencing disease control, i.e. partial response (PR) or disease stabilization (SD); F. VEGF-D levels during treatment among patients experiencing early progressive disease (PD); G. sVEGFR-2 levels during treatment; H. s-VEGFR2 levels during treatment among patients experiencing disease control, *i.e.* partial response (PR) or disease stabilization (SD); I. sVEGFR-2 levels during treatment among patients experiencing early progressive disease (PD).

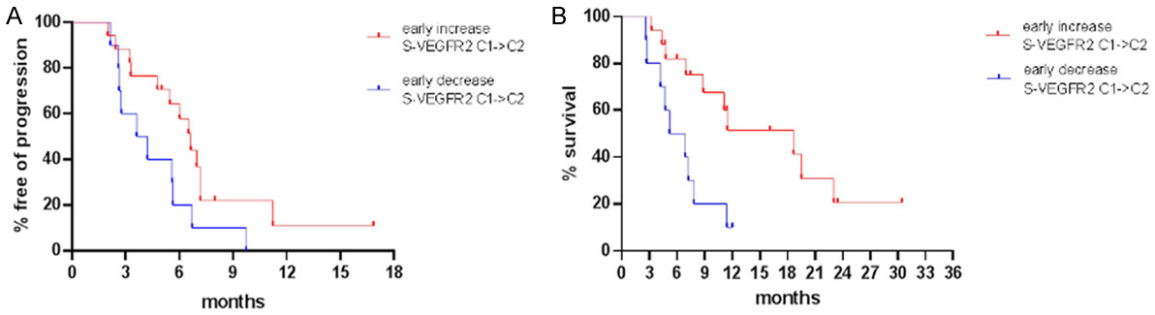

Figure 3. Correlation between early increase of sVEGFR2 and survival. **A. Progression-free survival; B. Overall survival.**

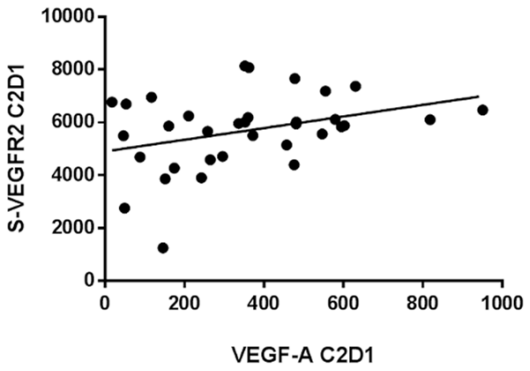

Figure 4. Scatterplot depicting correlation between VEGF-A and sVEGFR-2 levels at day 1 of cycle 2. Abbreviations: C2D1, cycle 2 day 1.

els of metastatic colorectal cancers had pointed out that, after the administration of the anti-VEGFR-2 antibody, a massive endothelial cells apoptosis occurs. Notably, this phenomenon anticipates cancer cells apoptosis [23, 24]. Limited preclinical data showed that sVEGFR-2 possibly takes origin from cancer cells [23], whereas more convincing evidence from Ebos et al. [25, 26] and translational analyses of REGARD [10] suggested a predominant endothelial origin of sVEGFR-2, finding higher levels in blood vessels rather than on tumour cells. The positive correlation we reported between VEGF-A and sVEGFR-2 at c2d1 contributes to support the role of endothelial cells in mediating sVEGFR2 dynamics. Indeed, the more stably ramucirumab binds to its ligand, the more VEGF-A increases as a result of reduced clearance by VEGFR-2: we hypothesize that the consequently more effective anti-endothelial activity of ramucirumab will lead to the subsequent release of sVEGFR-2 from apoptotic endothelial cells.

According to this hypothesis, sVEGFR-2 levels would be identified as a surrogate of drug activity. Consistently with these findings, we showed that the early increase of sVEGFR-2 levels from baseline to c2d1 takes place in patients with longer PFS and OS, suggesting its role as a predictive biomarker. To the best of our knowledge, this is the first report suggesting a predictive value of sVEGFR-2 in metastatic GC patients treated with ramucirumab.

Notably, we did not observe changes in sVEGFR-2 levels during treatment according to RECIST response (i.e. disease control or early progression). This could be due to several factors. Limited numbers of patients for each subgroup and different time points could have impacted on these findings. Moreover, the timing of sample collection could not have reliably captured the pharmacodynamic effect of ramucirumab: as stated, radiologic assessment of objective response was scheduled every 10-12 weeks while blood samples were collected at

the time of evidence of best response (which may vary in different patients). Last, objective response could not represent the best surrogate to measure benefit from the antiangiogenic treatment: some patients could have non-measurable disease by RECIST and chemotherapy might confound the effect of ramucirumab on tumour shrinkage. For all these reasons, we initially established PFS as the primary endpoint also for the identification phase of the study, as this could be more useful to capture the contribution of ramucirumab on disease control during treatment.

Some limitations of our work should be considered. Firstly, we acknowledge the limited sample size and the need for larger studies in order to confirm our data. In this regard, prospective validation of the predictive role of sVEGFR-2 changes after the first treatment cycle with paclitaxel plus ramucirumab is currently ongoing in the validation phase of PREDICTOR. Secondly, the study lacks a control arm of patients treated with chemotherapy alone, which could have helped in the interpretation of the results: however, the combination of ramucirumab and paclitaxel represents the second-line standard for fit patients with metastatic GC, and ethical concerns limit the use of chemotherapy alone in prospective studies in this population. Finally, we focused our interest on the VEGF/VEGFR pathway: as discussed, many other factors (e.g. PlGF) are involved in tumour angiogenesis [22] and may cause resistance to anti-VEGF therapy [21]. Broader analyses are therefore encouraged to better understand the complexity of the whole angiogenetic process under selective pressure of anti-VEGFR-2 therapy. Notably, we investigated the potential link between changes of biomarkers concentrations during treatment and patient outcome, while previous translational analyses of large registrative trials mainly explored the predictive or prognostic value of baseline, pre-treatment levels [11, 15, 28]. Moreover, as potentially predictive changes in sVEGFR-2 might occur early during treatment, this parameter (if validated) could be extremely useful to personalize the second-line approach in the single patient [29, 30].

In conclusion, our study confirms the negative prognostic value of VEGF-A and, despite limited by the small sample size and the lack of a control arm, provides preliminary evidence about the positive predictive role of the early increase of sVEGFR-2 after one cycle of paclitaxel plus ramucirumab in metastatic GC. If confirmed in the ongoing validation phase, our findings could lead clinicians to select patients who might experience longer benefit from antiangiogenic treatment (shifting unresponsive patients to alternative treatment options), ultimately refining the therapeutic strategy in this challenging disease.

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Disclosure of conflict of interest

None.

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