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Angiogenesis modifications related with cetuximab plus irinotecan as anticancer treatment in advanced colorectal cancer patients

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Introduction: Angiogenesis has been correlated with increased invasion and metastases in a variety of human neoplasms. Inadequate inhibition of the growth of tumor microvessels by anticancer agents may result in treatment failure, rated clinically as progressive or stable disease. We designed this trial to investigate the modification of the vascular endothelial growth factor (VEGF) and interferon-γ (IFN-γ) in advanced colorectal cancer patients during treatment with a weekly combination of cetuximab plus irinotecan.

Materials and methods: Forty-five metastatic colorectal cancer patients were prospectively evaluated for circulating levels of VEGF and IFN-γ during the treatment with cetuximab (initial dose of 400 mg/m^2, followed by weekly infusions of 250 mg/m^2) plus weekly irinotecan (90 mg/m^2). The circulating levels of the cytokines were assessed at the following time points: just before and at 1, 21, 50 and 92 days after the start of cetuximab plus irinotecan treatment.

Results: Basal serum VEGF median levels were significantly decreased just at the first day (after the first treatment infusion ($P = 0.016$). The VEGF persisted at the following time points reaching the highest statistical significance 92 days after the first infusion ($P < 0.0001$). On the contrary, IFN-γ values showed a statistical significant increase one day after the first infusion ($P < 0.0001$). This effect persisted 21 days after the treatment start ($P = 0.001$), but was no more evident at the following time points. Moreover, a linear regression model with variance analysis showed a significant negative correlation between VEGF and IFN-γ values 1, 21 and 50 days after the treatment beginning ($P = 0.002$, 0.001 and 0.047, respectively).

Conclusions: This study suggests that a cetuximab may induce a modulation of VEGF circulating levels. The reduction of VEGF serum levels is a sudden and long lasting phenomenon. Moreover, in our study we identified a IFN-γ increase, even if the specific role of this behavior remains to be investigated.

Key words: angiogenesis, cetuximab, colorectal cancer, irinotecan

introduction

Colorectal cancer is the third most common cancer in the USA, with approximately 145 000 new cases expected in 2005 [1]. Estimated 5-year survival rates range from 90% for patients with stage I disease to <10% for patients with metastatic colorectal cancer [1].

Chemotherapy reliably enhances quality of life and prolongs both progression-free survival and overall survival for patients with metastatic colorectal cancer [2]. Chemotherapies, however, are limited by their lack of specificity and are often associated with frequent and potentially severe dose-limiting toxicities. The FDA recently approved two targeted agents: an anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibody (bevacizumab) and a human epidermal growth factor receptor (HER-1/EGFR)-targeted monoclonal antibody (cetuximab) as first- and second-line metastatic coloretal cancer therapy, respectively [3].

Epidermal growth factor receptor (EGFR), a member of the ErbB family of receptors, is relevant in colorectal cancer because expression or up-regulation of the *EGFR* gene occurs in 60%–80% of cases [4]. Cetuximab is a chimeric IgG1 monoclonal antibody that binds to EGFR with high specificity and with a higher affinity than either epidermal growth factor thus blocking ligand-induced phosphorylation of EGFR [4].

Cetuximab's mechanism of action in tumor cells is thought to involve the binding of cetuximab to the EGFR, preventing normal ligand binding and subsequent activation of the receptor's tyrosine kinase activity [5]. The outcome of this

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blockade is reflected in the disruption of any number of processes regulated by EGFR pathways in a given tumor cell. Several mechanisms have been identified in preclinical models whereby cetuximab inhibits the growth and survival of EGFR-positive tumors [5]. These include: inhibition of survival pathways [6], inhibition of tumor cell motility and invasion [7], inhibition of angiogenesis [8, 9] and interruption of EGFR-activated survival and proliferation signaling [10]. Moreover, the blockade of EGFR receptor seems to be able to enhance the radio-responsiveness [11] and chemo-sensitivity [12–14] *in vitro* and *in vivo* models.

New blood vessel formation (angiogenesis) is a fundamental event in the process of tumor growth and metastatic dissemination. Hence, the molecular basis of tumor angiogenesis has been of keen interest in the field of cancer research. The VEGF pathway is well established as one of the key regulators of this process. The VEGF/VEGF-receptor axis is composed of multiple ligands and receptors with overlapping and distinct ligand-receptor binding specificities, cell-type expression and function [15]. Activation of the VEGF-receptor pathway triggers a network of signaling processes that promote endothelial cell growth, migration and survival from pre-existing vasculature. In addition, VEGF mediates vessel permeability, and has been associated with malignant effusions [16]. However, the tumor is not the only protagonist of VEGF synthesis. The recruitment of monocytes, macrophages and other inflammatory cells to a tumor appears to be a common denominator for the major processes involved in tumor development and progression. Inflammatory cells contribute to tumor angiogenesis by supplying proangiogenic growth factors, such as VEGF, cytokines and proteases. They also contribute factors that promote the formation and enlargement of intratumoral or peritumoral lymphatic vessels, eventually allowing a tumor to metastasize to distant organs. Finally, they may also play a critical role in arteriogenesis by promoting the growth of the larger vessels that supply the expanding capillary bed, feeding the rapidly growing tumor mass [16]. Moreover, previous studies have reported a positive correlation between platelet number and serum VEGF level in cancer patients [17, 18], supporting the hypothesis that platelets may serve the role of storage of VEGF in the circulation.

Vascular endothelial growth factor is a potent angiogenic factor, and widely studied as a prognostic factor for cancer patients [19, 20]. The higher levels of VEGF are usually reported to be correlated with the tumor burden and also the higher levels found to be related with poor prognosis in solid tumor patients [19–21]. As a consequence, VEGF circulating levels may represent a surrogate marker of anti-tumor activity.

IFN-γ is a pleiotropic cytokine endowed with potent immunomodulatory effects and secreted by activated CD4 and CD8 T cells. The real role of serum IFN-γ increase is not known, although there is some evidence in the literature supporting an antiangiogenic action by IFN-γ through an inhibition of endothelial proliferation [22, 23].

The aims of the present study are the assessment of the VEGF circulating levels, modifications in colorectal cancer patients treated with cetuximab plus irinotecan and the evaluation of IFN-γ role as a mediator of antiangiogenic properties of this treatment.

materials and methods

study design and patients' eligibility criteria

Patients were considered eligible for the study if they had a histologically confirmed colorectal adenocarcinoma resected or not associated with distant metastases (with or without local relapse).

We considered patients eligible if they were more than 18 years of age and had stage IV, histologically confirmed colorectal adenocarcinoma. In addition, immunohistochemical evidence of EGFR expression measured semiquantitatively (> 0 on a scale of 0, 1+, 2+ or 3+) in a single reference laboratory (University Campus Bio-Medico, Rome) was required. These measurements were performed and graded using a now commercially available kit (EGFRpharmDx; Dako Corporation, Carpentino, CA) according to the manufacturer's instructions. Patients were permitted to undergo the screening process for tumor EGFR expression before meeting other entry criteria and before study entry.

Other criteria for eligibility were: a ECOG performance-status score ≤2, adequate hematologic function (hemoglobin ≥9 g/dl, neutrophil count ≥1500/mm^3, platelet count ≥100 000/ mm^3), renal function [serum creatinine <1.5× the upper limit of normal (ULN) range], and liver function (total bilirubin < 1.5× ULN range; aspartate aminotransferase and alanine aminotransferase <5× ULN values). Patients were considered ineligible for accrual when they had reported fever (body temperature >38.0°C) during the last week before study entry or had received any radiotherapy, chemotherapy, immunotherapy or growth factors during the last 4 weeks before study accrual. Moreover, if patients received radiotherapy or growth factors during our study they were excluded from the final evaluation, as well. Patients recently (less than 1 week) or simultaneously treated with steroids and with acute or chronic infections or inflammatory diseases were considered ineligible for the study. Finally, significant cardiovascular or neurological disease represented exclusion criteria.

Before being considered for the study all patients had a documented disease progression after two standard anticancer regimens: one oxaliplatin-based chemotherapy regimen (capecitabine + oxaliplatin or FOLFOX IV) as first line and one irinotecan-based chemotherapy regimen (FOLFIRI) as second line for at least 2 months.

treatment plan

Cetuximab was given at a loading dose of 400 mg/m^2, followed by weekly infusions of 250 mg/m^2. Irinotecan was administered weekly at 90 mg/m^2.

A histamine-receptor antagonist and atropine (0.25 mg) were given as premedication before every infusion. No corticosteroids were routinely administered. A standard antiemetic drug was always given in the premedication and in the following days according to the physician's opinion. All the patients were to be treated until disease progression or unacceptable toxic effects occurred.

Modifications to the cetuximab dose were made only in cases of toxic effects to the skin, and modifications to the irinotecan dose were made in cases of hematologic or non-hematologic toxic effects.

Venous blood for cytokine assessment was drawn into a EDTA anticoagulant tube just before the beginning of the first drug infusion and again at 1, 21, 50 and 92 days after the first cetuximab + irinotecan infusion (just before every subsequent infusion). After drawing, the venous blood sample was rapidly centrifuged for 10 min at 10 000 rpm and plasma stored at −80°C until tested for VEGF and IFN-γ levels. Moreover, standard hematologic parameters were tested before every single course.

cytokine analysis

VEGF and IFN-γ were assayed with R&D quantitative kits according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). The detection limit of the cytokines was as follows: 62.5 pg/ml for VEGF

and 10 pg/ml for IFN-γ. The curve for the analysis was linear until 1000 pg/ml for VEGF and 4000 pg/ml for IFN-γ; values out from this range were calculated performing a mathematical extrapolation from the standard curve.

statistical analysis

Basal cytokine levels were compared with the values observed at 1, 21, 50 and 92 days after the start of cetuximab plus irinotecan using Wilcoxon's test for non-parametric dependent continuous variables. A linear regression model with variance analysis was used to correlate different cytokines levels. A two-tailed *P* value was considered significant when less than 0.05. SPSS software (version 11.5, SPSS, Chicago) was used for statistical analysis.

results

Forty-five consecutive patients (21 males, 24 females), aged 27–79 years (median age 69), with advanced colorectal cancer were included in the study. All patients matched all the inclusion criteria. Patients' characteristics are shown in Table 1.

VEGF analysis

The median VEGF basal value (228.60 mg/dl; 95% CI 224.60–328.40 mg/dl) showed a statistically significant decrease of 16.91% just 1 day after the start of cetuximab + irinotecan anticancer treatment (median 194.90 mg/dl; 95% CI 154.57–212.60 mg/dl; $P = 0.016$). This effect persisted during the following time points. At 21 days after the first course, VEGF levels showed a decrease of 46.05% compared with the basal ones (median 123.20 pg/ml; 95% CI 104.73–196.98; $P = 0.001$). This effect on circulating VEGF levels was still evident and persisted at 50, with a reduction of 39.09% and a median value of 139.60 mg/dl (95% CI 98.17–163.50 mg/dl; $P = 0.002$). Interestingly, at the last time point (92 days after the treatment start) the decrease reached the highest statistical significance: the median value was 110.55 pg/ml with a reduction of 51.75% (95% CI 96.43–224.37 mg/dl; $P < 0.0001$). These results are summarized in Tables 2 and 3 and in Figure 1. Finally, it is of some interest to stress that at the last time point (92 days after the first infusion) 71.1% of the patients developed a reduction of at least 25% with respect to the VEGF basal levels.

IFN-γ levels

The median IFN-γ basal level was 13.03 pg/ml (95% CI 11.67–22.99 mg/dl). These levels significantly increased to 56.13% 1 day after the start of cetuximab + irinotecan (31.06 pg/ml; 95% CI 26.87–36.73 mg/dl; $P < 0.0001$). This effect persisted at day 21 after infusion, with an increase

Table 1. Baseline characteristics of the patients

Patient's characteristics	No. of patients (%)
Total number	45 (100%)
Male/female	21/24 (46.7%–53.3%)
Age (years)	
Median	69
Range	27–79
Performance Status	
Median	1
Range	1–2
Primary tumor site	
Colon	30 (66.7%)
Rectum	15 (33.3%)
No. of metastatic sites	
1	8 (17.8%)
2	18 (40.0%)
3+	19 (42.2%)
Sites of metastases	
Liver	31 (68.9%)
Lung	20 (44.4%)
Nodes	16 (35.6%)
Local	12 (26.7%)
Other	19 (42.2%)
Prior adjuvant therapy	
None	16 (35.6%)
FU/LV	29 (64.6%)
First-line regimen	
XELOX	31 (68.9%)
FOLFOX	14 (31.1%)
Second-line regimen	
FOLFIRI	55 (100%)
EGFR expression	
Score 1	14 (31.1%)
Score 2	19 (42.2%)
Score 3	12 (26.7%)

Table 2. VEGF and IFN-γ modifications during cetuximab plus irinotecan treatment

	Median (pg/ml)	95% CI (pg/ml)	*P* (Wilcoxon test)
VEGF			
Basal levels	228.60	224.60–328.40	–
1 Day	194.90	154.57–212.60	0.016
21 Days	123.20	104.73–196.98	0.001
50 Days	139.60	98.17–163.50	0.002
92 Days	110.55	96.43–224.37	<0.0001
IFN-γ			
Basal levels	13.03	11.67–22.99	–
1 Day	31.06	26.87–36.73	<0.0001
21 Days	26.22	22.33–41.86	0.002
50 Days	21.95	15.87–31. 64	0.136
92 Days	18.56	13.96–24.23	0.233

Table 3. Median changes in percentage (%) of circulating VEGF and IFN-γ levels during cetuximab plus irinotecan treatment

	Median reduction (%)	95% CI
VEGF		
1 Day	−16.91%	11.54%–22.80%
21 Days	−46.05%	46.05%–53.71%
50 Days	−39.09%	39.09%–44.78%
92 Days	−51.75%	33.71%–63.85%
IFN-γ		
1 Day	+56.13%	47.11%–64.90%
21 Days	+47.20%	34.98%–54.95%
50 Days	+26.12%	19.85%–33.71%
92 Days	+17.58%	12.94%–24.77%

of 47.20% if compared with the basal levels and a median value of 26.22 pg/dl (95% CI 22.33–41.86 pg/dl). Circulating IFN-γ levels returned to values similar to the median basal value at day 50 (21.95 pg/ml; 95% CI 15.87–31. 64 pg/ml) and day 92 (18.56 pg/ml; 95% CI 13.96–24.23); *P* values 0.136 and 0.233, respectively. These results are shown in Tables 2 and 3 and in Figure 2. The percentage of patients who developed at least a 25% increase of VEGF circulating levels at the different time-points was as follow: 62.2% at day 1, 46.6% at day 7, 17.8% at day 50 and only 6.7% at day 92.

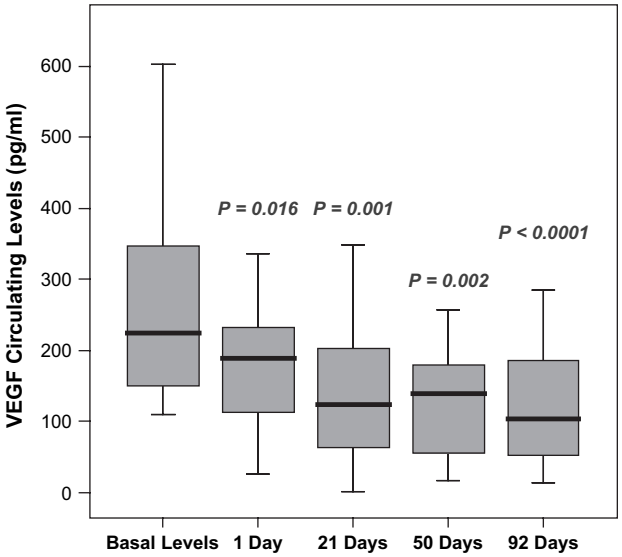

Figure 1. The behavior of VEGF levels 1, 21, 50 and 92 days after the start of treatment with cetuxuimab + irinotecan. Gray boxes represent 95 percentiles of all VEGF values. Horizontal black bar in the gray boxes represent VEGF median value. Bottom and top horizontal bars indicate minimum and maximum values. *P* values are calculated according to Wilcoxon test for non-parametric dependent continuous variables.

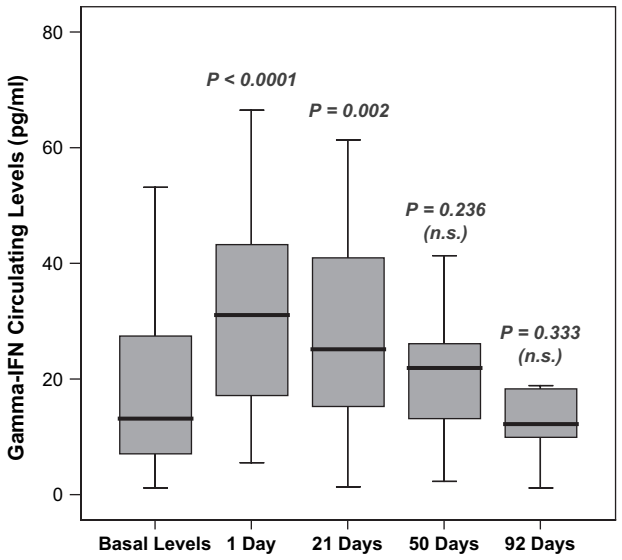

Figure 2. The behaviour of IFN-γ 1, 21, 50 and 92 days after the start of treatment with cetuxuimab + irinotecan. Gray boxes represent 95 percentiles of IFN-γ values. Horizontal black bar in the gray boxes represent IFN-γ median value. Bottom and top horizontal bars indicate minimum and maximum values. *P* values are calculated according to Wilcoxon test for non-parametric dependent continuous variables.

VEGF and IFN-γ correlations

A linear regression model with variance analysis showed a significant negative correlation between VEGF values and IFN-γ values (β regression coefficient = −0.629; $P = 0.005$). This data was obtained using all the available data regardless of the different time-points. Interestingly, performing the statistical examination at every time-point, the analysis showed that no significance is reached at the basal time point (β value = −0.322), while a statistically significant correlation is achieved 1, 21 and 50 days after the first anticancer cycle ($P = 0.002$, 0.001 and 0.047, respectively). This negative correlation is not more evident 92 days after the start of treatment. The global correlation between VEGF and IFN-γ without taking into consideration the different time-points is shown in Figure 3. In addition, the correlations performed at the basal time point and at days 1, 21 and 50 are shown in Figure 4.

When performing a linear regression analysis between the platelet count and VEGF circulating levels, we failed to identify any statistically significant correlation between the two variables. However, a borderline correlation was identified at the basal time-point (β regression coefficient = 0.291, $P = 0.09$).

discussion

The link between EGFR signaling and angiogenesis has been clearly identified [24–29]. The mechanisms by which EGFR signaling pathways regulate VEGF, interleukin 8 (IL-8) and basic fibroblast growth factor (bFGF) are unclear, but it is established that up-regulation of these factors follows activation of the EGFR signaling pathways by EGF or transforming growth factor (TGF)-α. Transcription of VEGF is potentiated by the

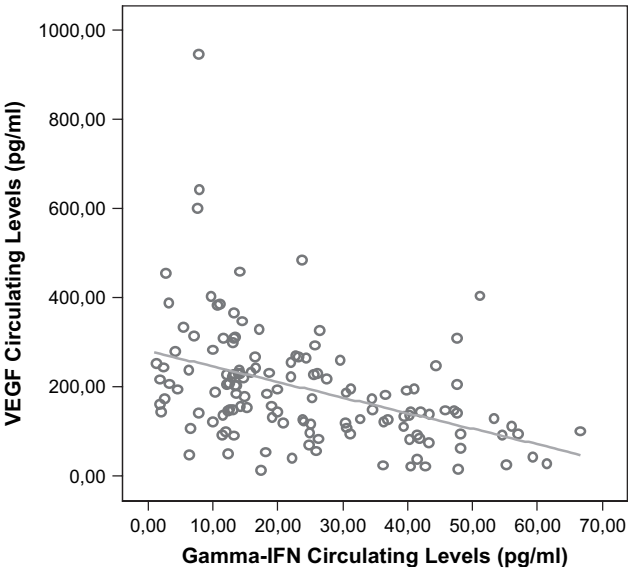

Figure 3. The correlations between VEGF and IFN-γ levels; this correlation is made without considering the different time points. *P* values were calculated using a linear regression model with variance analysis.

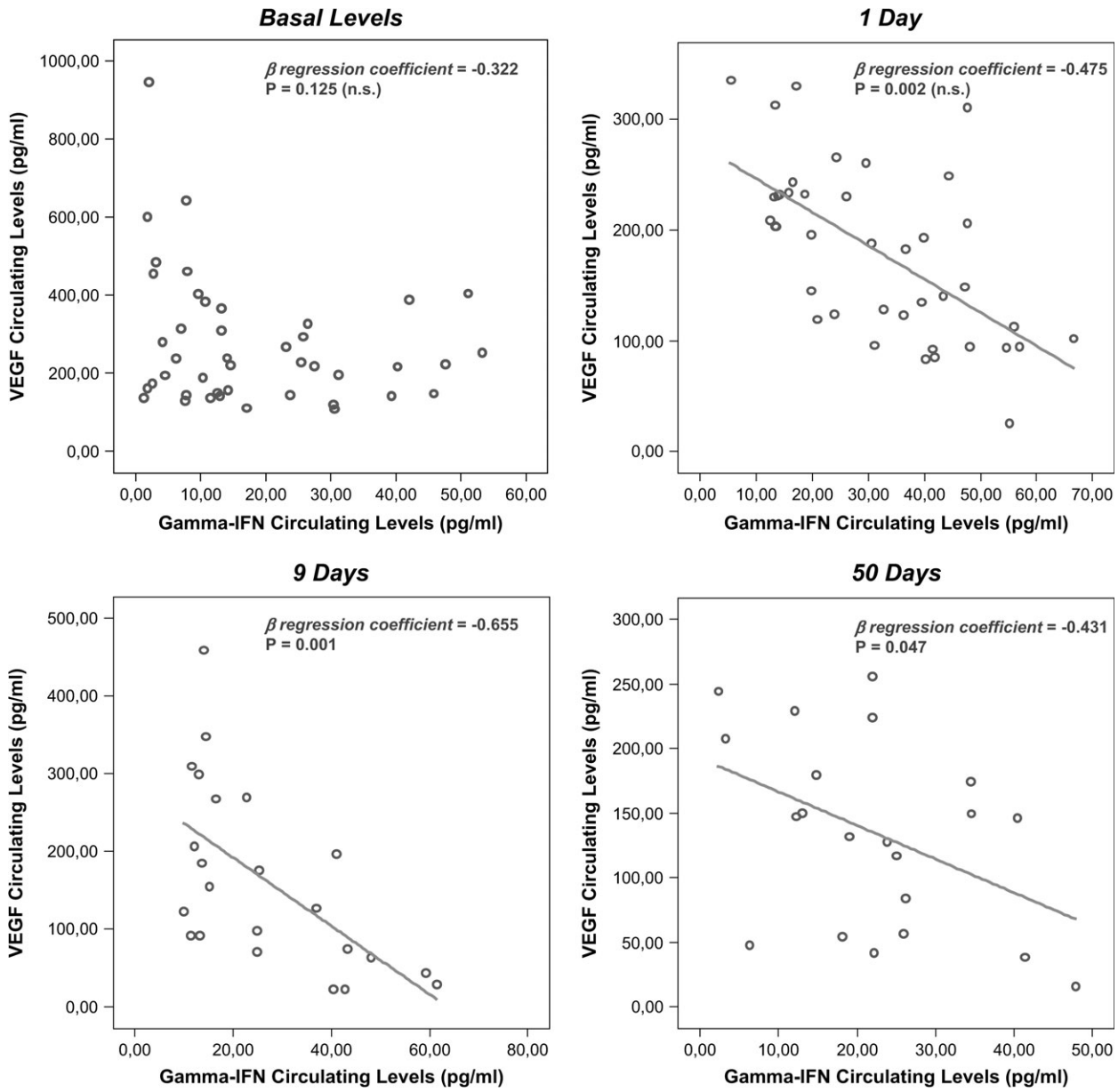

Figure 4. The correlations between VEGF and IFN-γ levels at the following time-points: basal, 1, 21 and 50 days after the start of treatment with cetuximab + irinotecan. *P* values were calculated using a linear regression model with variance analysis.

Table 4. Correlation between VEGF and IFN-γ at different time-points

	β regression coefficient	*P*
Total correlation	−0.629	0.005
Basal levels	−0.322	0.125 (NS)
1 Day	−0.475	0.002
21 Days	−0.655	0.001
50 Days	−0.431	0.047
92 Days	−0.211	0.129

activation of the four AP-1 binding sites within its promoter; the bFGF and IL-8 promoter each have one AP-1 site [30–33]. After activation of EGFR signaling pathways, *ras* and *raf* are activated, resulting in phosphorylation of c-fos and c-jun, leading to increased AP-1 activity [34–37]. This increase in AP-1 activity leads to transcription of genes with AP-1 sites in their promoter [37, 38]. Because VEGF, IL-8 and bFGF all share AP-1 binding sites, they are potential targets for therapies that down-regulate EGFR signaling pathways, which results in reduced AP-1 activity.

Perrotte and colleagues [8] clearly identified, in an *in vitro* model, a new mechanism that contributes to the antitumor effect of EGFR blockade therapy with cetuximab: the inhibition of angiogenesis. Their data suggest that the reduction in bladder cancer vascularization is secondary to down-regulation of VEGF, IL-8 and bFGF expression by EGFR blockade therapy with cetuximab. The authors hypothesize that selective down-regulation of VEGF, IL-8 and bFGF by the tumor cells after cetuximab therapy leads to involution of tumor vessels,

contributing to the growth inhibition and regression of the primary tumors and, hence, reduction in spontaneous metastases from these highly metastatic tumors.

Recently, Vallböhmer et al. [39] investigated the association between molecular markers and clinical outcome in patients with EGFR-expressing metastatic colorectal cancer treated with cetuximab. The results of this study showed that higher gene expression levels of VEGF were associated with resistance to cetuximab. This evidence confirms the tight relation between angiogenesis and EGFR pathway.

We designed this study with the aim of investigating if, in colorectal cancer patients, cetuximab in association with irinotecan can lead to a reduction of serum markers of angiogenesis. Our results demonstrated that a statistical significant reduction of VEGF circulating levels was identified just one day after the beginning of treatment and, most important, persisted along the courses. Moreover, we also investigated the concomitant modification of IFN-γ, a cytokine with anti-angiogenic properties. The results of IFN-γ analysis revealed a significant increase that persisted during the first cycles of therapy and that was lost 92 days after the beginning of cetuximab + irinotecan. In addition, using a linear regression model a statistical significant negative correlation was observed between VEGF and IFN-γ at the first time-points.

Obviously, this observation could simply be an epiphenomenon of cetuximab administration, but we cannot exclude the fact that IFN-γ could have a role in the angiogenesis modulation by cetuximab identified in this paper.

Moreover, our results do not represent clear evidence of a direct effect of cetuximab or irinotecan on the tumor cell. In fact VEGF is produced by many different cells even in physiological conditions. The VEGF modulation induced by the therapy could also be provided by an indirect action of cetuximab on tumoral stroma cells. In our study, a contribution to the VEGF circulating levels reduction could be played by irinotecan. In fact, O'Leary and colleagues demonstrated, in an *in vitro* model, that irinotecan may exert its anticancer activity by an action against endothelial cells preventing the growth of the tumor microvessels [40].

In conclusion, our study suggests that cetuximab, in combination with irinotecan, may induce a significant and long-lasting decrease of VEGF circulating levels. The modulation of this angiogenic molecule could be one expression of the anticancer properties of cetuximab and could be of interest to the investigation of the correlation between cetuximab-related serum VEGF modifications, tumor response and clinical outcome.

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