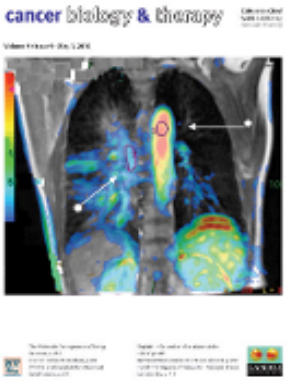

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Dose/dense metronomic chemotherapy with fractioned cisplatin and oral daily etoposide enhances the anti-angiogenic effects of bevacizumab and has strong antitumor activity in advanced non-small-cell-lung cancer patients

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Key words: cisplatin, etoposide, bevacizumab, NSCLC, metronomic therapy

Background: We designed a translational clinical trial to investigate whether a dose/dense chemotherapy regimen is able to enhance in patients with non-small-cell-lung-cancer (NSCLC) the anti-angiogenic effects of bevacizumab, a murine/human monoclonal antibody to the vasculo-endothelial-growth-factor (VEGF). We also evaluated the antitumor activity of this combination.

Results: The combined treatment induced a significant decline in the blood-perfusion of primary tumor (NMR-study); in serum levels of VEGF, angiopoietin-1, thrombospondin-1; and in the number of VEGF-transporting cells. In the group of 40 patients who received bevacizumab an objective response and a disease stabilization rate of 77.5% (95% CI, 75.63–93.17) and 15%, respectively, were recorded with a time to progression of 7.6 mo. Grade I-II hematological toxicity was the most common adverse event. Four early deaths within 3 mo, three cases of pneumonia, and six cases of mood depression at higher bevacizumab dosage were observed. The most active biological and maximum tolerated dose were 5 and 7.5 mg/kg, respectively.

Patients and Methods: Forty-eight patients (42 males and six females) with stage IIIB/IV NSCLC, a mean age of 68 y, and ECOG ≤2 were enrolled in the study. They received every 3 w fractioned cisplatinum (30 mg/sqm, days 1–3) and oral etoposide (50 mg, days 1–15) and were divided in five cohorts receiving different bevacizumab dosages (0; 2.5; 5; 7.5; and 10 mg/kg) on day 3.

Conclusion: The combination of bevacizumab with a dose/dense chemotherapy regimen resulted moderately safe but showed significant anti-angiogenic and antitumor activity.

Introduction

Non-small cell lung cancer (NSCLC) is the most common malignancy and the leading cause of cancer death worldwide. The majority of NSCLC patients cannot undergo curative surgery[1] and advanced patients have a poor prognosis with a survival time that usually does not exceed 8–10 mo.[2]

Poly-chemotherapy with platinum derivatives in combination with a second cytotoxic drug represents the standard treatment for advanced NSCLC patients.[3] More recently, the efficacy of poly-chemotherapy has been improved by the addition of bevacizumab, a murine-human chimeric monoclonal IgG1 to the vascular endothelial growth factor (VEGF) with anti-angiogenic activity.[4-6] The addition of bevacizumab to chemotherapy improved the efficacy over chemotherapy alone in NSCLC, colon, breast and over interferon alone in renal carcinomas.[7-13] VEGF is a soluble dimeric protein family produced in hypoxic conditions, which is crucial for endothelial proliferation and neo-vessel stabilization.[5,6]

However, anti-angiogenic treatments still present many limitations in NSCLC, where patients with squamous cell histology and poor performance status are considered not feasible to receive bevacizumab.[14] Therefore, the research of newest chemotherapy regimens to combine with bevacizumab in the treatment of NSCLC patients is still ongoing.

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Table 1. Patient characteristics

		All patients	Level 0	Level 1	Level 2	Level 3	Level 4
Nr. of patients		48	8	9	21	8	2
Median age		68 (42–83)	73 (62–83)	67 (54–82)	68 (47–82)	71 (42–76)	61 (54–68)
Sex	Male	37	8	9	16	7	1
	Female	6	/	/	5	1	1
PS (ECOG)	0	18	3	4	11	3	/
	1	13	3	3	6	3	/
	2	12	2	2	4	2	2
Histology	Adenocarcinoma	17	2	1	12	5	/
	Squamous cells	17	4	8	6	/	/
	Undifferentiated	9	2	/	3	3	2
Stage	IIIB	9	3	2	3	1	/
	IV	34	5	7	18	7	2
Surgery	Yes	7	3	2	/	2	/
	No	36	5	7	21	6	2

We hypothesized that the anti-angiogenic activity of bevacizumab in NSCLC patients could be improved if combined with poly-chemotherapy regimens designed on dose/dense modality including a prolonged and sequential administration of different drugs and that such combination might exert an effective antitumor activity. Therefore, we designed a bio-chemotherapy regimen by taking in consideration that full dose platinum administration at the beginning of the cycle could induce tumor debulking and endothelial cell activation and proliferation, while the consecutive bevacizumab infusion together with a prolonged oral low dose administration of etoposide could produce a more efficient anti-angiogenic, immunobiological and antitumor effect.

The rationale of this combination is derived from preclinical models where cytotoxic drugs like etoposide and cyclophosphamide, administered on metronomic modality, with short and regular inter-cycle intervals, chronically and generally at low doses,[15] showed cytotoxic activity on activated endothelial cells (anti-angiogenic activity), immunosuppressive blood cell populations like immunoregulatory T lymphocytes (with a $CD4^+CD25^+FoxP3^+$ immunophenotype) and immunosuppressive myeloid precursors (immuno-modulating activity). These drugs given with dose/dense schedules became additionally able to force cancer cells to acquire a less aggressive phenotype (epigenetic effect).[16-19] We have already shown the safety and the antitumor activity of a metronomic regimen with fractioned cisplatinum and daily oral etoposide (mPE) in advanced NSCLC patients.[20] Here we report the results of a translational Phase Ib-II study aimed to evaluate the antitumor, the anti-angiogenic and biological activity and toxicity of a novel dose/dense bio-chemotherapy regimen which combines the mPE chemotherapy with escalating doses of bevacizumab in front-line treatment of advanced NSCLC patients.

Results

Demography. Forty-eight patients were enrolled in the study receiving front-line dose/dense mPE chemotherapy and bevacizumab given at four different dose levels (**Table 1**). A control group of eight patients scheduled to receive dose/dense chemotherapy without bevacizumab (level 0) was specifically required by our institutional scientific committee in order to evaluate the biological activity of the mPE regimen.[20] A confirmative group of 12 patients was subsequently enrolled in the cohort receiving bevacizumab at 5 mg/kg which was considered the most active biological dose.

Patients' characteristics are shown in the **Table 1**.

Toxicity. Grade II hematological toxicity was the most common adverse event. Grade I-II asthenia, nausea and vomiting, and alopecia was also recorded. The most severe adverse events were observed in patients who received higher bevacizumab dose (levels 3 and 4). Along the study, there were four early deaths: two, due to a cardiovascular accident respectively after the first and the fourth cycle (level 3), and two, due to a lung hemorrhage just after the second (level 2) and third cycle (level 4). None of these deaths or severe adverse events was observed in patients with squamous histology.

Two cases of non-neutropenic fever and three cases of pneumonia were also observed. These patients recovered upon antibiotic and anti-mycotic treatment. Four patients presented an asymptomatic lung cavitation after four/five chemotherapy cycles. These lesions, whose nature is still under investigation, are likely related to a pre-existing bronchopulmunary chronic obstructive disease. Finally, we recorded six cases of severe progressive psychic depression, four of which recovered after medical treatment with serotonin re-uptake inhibitors.

No early death or grade III-IV toxicity, bronchiolytis or pneumonia was observed in the additional group of 12 patients who received bevacizumab at the dosage of 5 mg/kg.

There were four cases of thrombo-phlebitis respectively occurring at the level 0 (one case, after three cycles), at the level 2 (two cases, after two cycles), at the level 4 (one case after one cycle). The level four was closed earlier for the consecutive

Table 2. Adverse events

Adverse events (grade 3–4)	Level 0	Level 1	Level 2	Level 3	Level 4
Hematological toxicity					
Anemia	1 (G1)	1 (G2)		1 (G2)	
Thrombocytopenia	1 (G4)		1 (G1) 1 (G3)	1 (G1) 1 (G3)	
Leucopenia	1 (G1)	2 (G1) 1 (G4)	2 (G1) 2 (G3) 1 (G4)	2 (G1) 2 (G2) 1 (G3) 1 (G4)	
Gastroenteric toxicity					
Nausea/vomiting					
Mucositis/stomatitis	1 (G1) 1 (G2)				
Diarrhea					
Pulmonary adverse events					
Hemoptysis		1 (G2)	1 (G2) 1 (G4)	1 (G2)	1 (G4)
Pneumonia		1 (G3)	1 (G3)	1 (G3)	
Asymptomatic lung cavitation					
Miscellaneous					
Asthenia	2 (G1) 1 (G2)	1 (G3)		1 (G2)	
Proteinuria					
Not neutropenic fever					
Thrombosis/pulmonary embolism		2 (G2)	1 (G4)	1 (G2)	1 (G3)
Cognitive disturbance/depression			1 (G2)	1 (G2)	1 (G3)
Renal failure	1 (G4)	1 (G2)			
Total adverse events	9	10	11	14	3
Adverse events G 3–4	1	3	7	4	3

Along the study there were two cases of sudden death just after the fist and the third cycle.

occurrence of a deadly hemoptysis and a large caval thrombosis (**Table 2**). On these bases 7.5 mg/kg was identified as bevacizumab MTD.

The occurrence of mood depression and infection was mainly observed in those patients who presented a poorer performance status.

Analysis of blood perfusion in the tumor tissue. The NMR study showed a significant decline in the tumor blood flow occurring 21 d after the mPEBev treatment (0.36 ± 0.16 vs. 0.17 ± 0.04 ml/min/100 g; F = 3.815, p = 0.037) in ten patients who received mPEBev bio-chemotherapy and no changes in a control group of 3 (**Fig. 1**). This effect was not dependent upon bevacizumab dosage (data not shown) and was not observed in those patients who did not receive bevacizumab (0.328 ± 0.041 vs. 0.455 ± 0.049 ml/min/100 g) and in a further control group of patients who received bevacizumab with standard chemotherapy (data not shown).

Biological effects. We found a significant decrease in free VEGF serum levels in patients who received dose/dense chemotherapy with or without bevacizumab, even though it was more evident in the latter group. No VEGF decrease was observed in a control group of ten NSCLC patients who received conventional front-line platinum/gemcitabine chemotherapy (**Fig. 2A**).

In the patients receiving the mPEBev regimen we additionally found a bevacizumab dose-dependent decrease in the serum level of angiopoietin-1 (AGP-1) (**Fig. 2B**), another endothelial cell growth factor which could overcome VEGF inhibition.[21,22]

We also monitored serum levels of thrombospondin (TSP)-1, an endogenous anti-angiogenic factor produced by endothelial cells and whose levels have been found greatly increased in other trials testing metronomic chemotherapy.[23] It was observed a significant decrease in TSP-1 serum level in all of the patients. Its reduction was however maximal in the patients who did not receive bevacizumab (level 0 vs. I vs. 2 vs. 3; $p < 0.05$) (**Fig. 2C**). We finally measured the serum levels of collagen IV, a basic component of endothelial cells' basal membrane.[24] After three cycles of treatment, we found a significant reduction of this protein, with a maximal decrease observed again in patients who underwent dose/dense chemotherapy without bevacizumab (**Fig. 2D**).

Treatment effects on different peripheral blood cell lineages. A very large amount of VEGF is transported in the

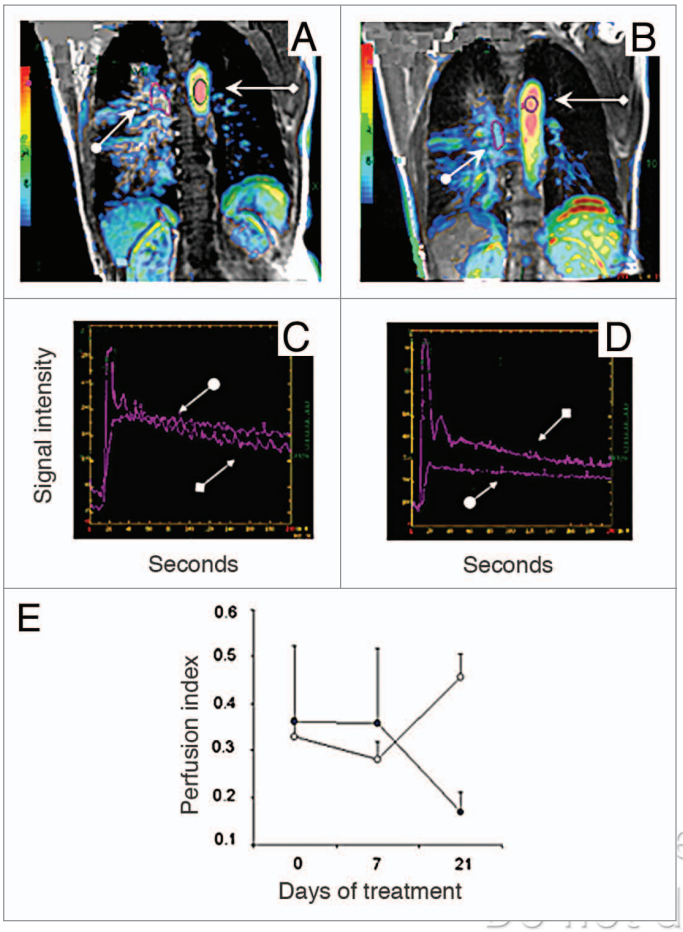

Figure 1. A Perfusional nuclear magnetic resonance analysis. (A and B) and (C and D) show NMR slides (a coronal-oblique axis) and relative curves which allowed to evaluate the blood flow at the time 0 (A and C) and after 21 d of treatment (B and D) in a representative patient who underwent mPEBev biochemotherapy. The curves show the changes in para-magnetic signal intensity in the tumor [↙] and the vascular district of thoracic aorta [↙]. The curves in the panel E show a progressive blood flux decline in the primary tumor of ten patients who received mPEBev bio-chemotherapy [–●–] and no changes in a control group of three patients who received the same dose/dense chemotherapy with no bevacizumab [–○–].

blood stream by neutrophils and platelet cells; additionally, VEGF family exerts many different receptor-mediated effects on blood cell lineages. Therefore, we evaluated potential treatment-related changes in these cell populations. Compared with the baseline and the group of patients who did not receive bevacizumab, we observed a progressive decline in neutrophils and platelet cells ($p < 0.05$) (**Fig. 3A and B**). A morpho-functional study of either platelets and neutrophils was also performed, revealing no phenotypic alterations on the first population and significant changes in the second. In fact, neutrophils derived from ten patients undergone one cycle of mPEBev resulted degranulated, pale and smaller than those isolated at the baseline (data not shown).

Furthermore, we detected a progressive reduction in lymphocyte cell counts which was correlated with bevacizumab administration and was not observed in the control group (**Fig. 3C**). A phenotypic and functional analysis of these lymphocytes is presently object of a further study.

Treatment activity. Overall, including all of the 48 patients, a 70.8% (95% CI, 69.5–84.88) (34 patients) objective response rate (ORR) and 16.6% (eight patients) stable disease (SD), respectively; with a disease control rate (OR + SD) of 87.4% (95% CI, 84.76–92.44) were recorded in our series.

Two patients (4.1%) were not evaluable for response, one, for early death (level 3) and another, due to acute kidney failure (a patient in the control group who did not receive bevacizumab) which required treatment switch to different drugs. Two patients in the level 0 without bevacizumab experienced progression of disease (PD) (5.7%) within 2 mo of treatment.

Our analysis revealed a surprising antitumor activity in the group of 40 patients who received bevacizumab, where we observed an OR in 31 (77.5%) (95% CI, 75.63–93.17), a SD in 6 (15%) and a progression of disease only in two patients (5%). One patient was not evaluable for early sudden death occurred just 1 w after the treatment. The ORs were most commonly observed in lung and lymph-nodes. It was recorded a mean TTP (time to progression) of 7.6 (95% CI 6.1–9.0) mo, a time of response duration of 6.0 (95% CI 4.6–7.5) mo and an mean overall survival of 12 mo (**Fig. 4**).

Adverse events, ORR, TTP and OS was not correlated with a particular tumor histology at the diagnosis (data not shown). Among the 14 patients with squamous histology it was recorded a PR (78.6%) in 11 and a SD in three (21.4). There was no toxic death or hemoptysis within this group of patients (**Fig. 5**).

Discussion

We report the results of a Phase Ib-II trial designed to test a novel anti-angiogenic therapeutic strategy in advanced NSCLC patients. This strategy is based on the combination of bevacizumab with dose/dense chemotherapy in a novel regimen (mPEBev) designed on translational bases to interfere at different levels with neo-angiogenesis.

Our results provide evidence that the mPEBev regimen exerts a powerful anti-angiogenic effect. In fact, we observed a significant treatment-related decrease of VEGF, AGP-1 and VEGF-transporting cells together with a significant blood flux decline in the primary tumor site as assessed by NMR in these patients. Some of these effects were not completely dependent upon bevacizumab dosage and could be observed in patients who had only received dose/dense chemotherapy. In the latter group of patients, we additionally found the lowest post-treatment level of TSP-1 and collagen IV. This finding suggests that the long-term addition of bevacizumab to metronomic chemotherapy could, on the opposite, promote vascular stabilization and normalization as reported by others.[25,26]

The mPEBev regimen was not completely safe and several grade 3–4 adverse events were observed. In line with results of much larger studies aimed to investigate bevacizumab plus chemotherapy in patients with NSCLC, colon or breast carcinoma,[9,27] we recorded a high risk of developing pneumonia and

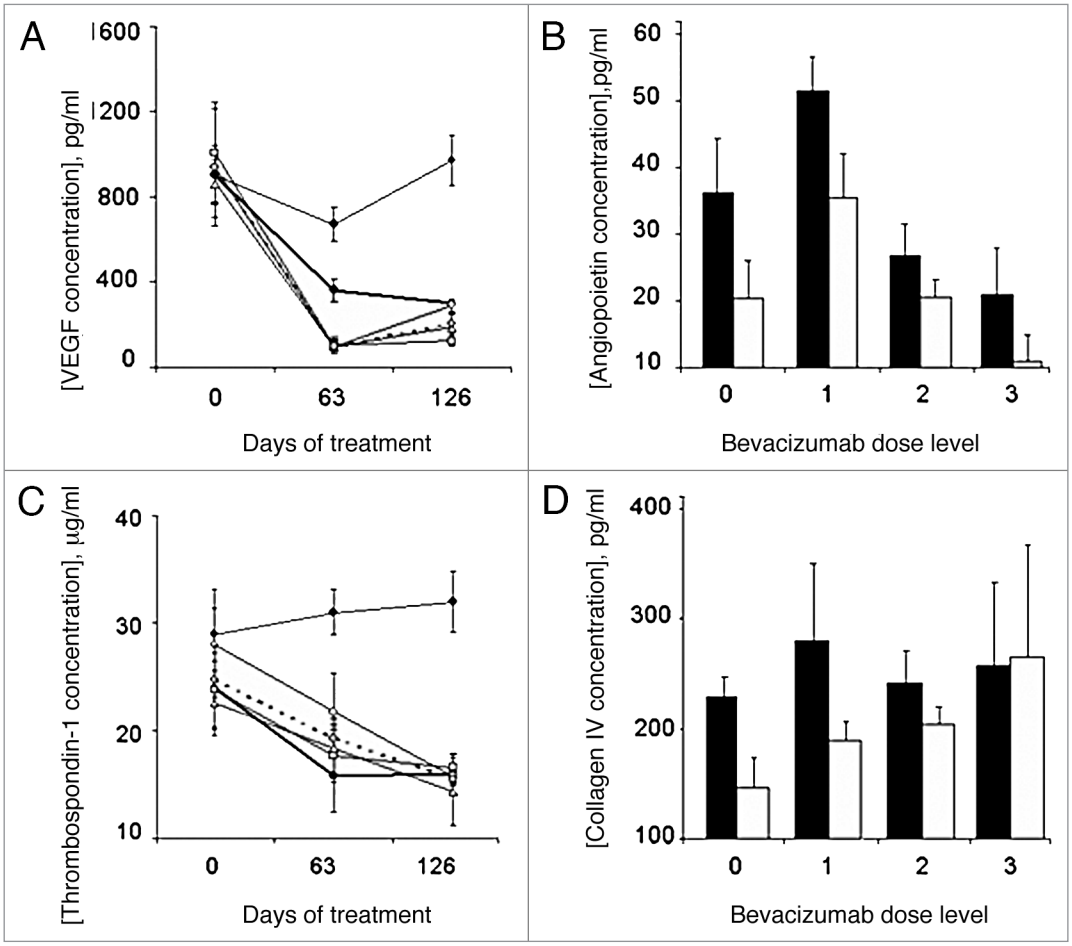

Figure 2. The curves comparing serum levels of VEGF (A), and TSP-1 (C) in advanced NSCLC patients enrolled in the mPEBev trial. Symbols represent a group of patients who received metronomic chemotherapy with no bevacizumab (level 0) [--●--], a group of patients who received metronomic chemotherapy plus bevacizumab at 2.5 mk/kg (level 1) [–○–], 5 mk/kg (level 2) [–□–], 7.5 mk/kg (level 3) [–△–], and a control group of ten patients who received standard upfront cisplatinum plus gemcitabine chemotherapy with no bevacizumab [–●–]. The latter group includes patients with advanced NSCLC (eight males and two females, average age of 69.5 y; three with spindle cell carcinoma, six with adenocarcinoma and one with poor cell carcinoma) who received treatment in our Institution. The figure shows a progressive decrease of both molecules in the serum of patients receiving metronomic chemotherapy with or without bevacizumab with values which resulted statistically significant if compared with those derived from a control group of patients who had received standard chemotherapy ($p < 0.05$). Differences among patients who had received metronomic chemotherapy with or without bevacizumab resulted statistically significant ($p < 0.05$). (B and D) The histograms represent the levels of angiopoietin 1 (B) and collagen IV (D) performed at the baseline [■] and after three treatment cycles [□] measured in the serum of the patients who had been enrolled in the mPEBev trial in the different bevacizumab dose levels. The figure shows a significant decline in the angiopoietin-1 and bevacizumab dose-dependent increase in collagen IV. The asterisks show the results which resulted statistically significant with both the baseline counts and the corresponding level 0 ($p < 0.05$).

sepsis. We believe that the enhanced risk of infection could be related to a specific bevacizumab activity on both neutrophils and lymphocyte populations. We report phenotypic alterations in neutrophils and a progressive decline in their number. This observation is not surprising considering that neutrophils transport more that 70% of VEGF in the blood and that peripheral blood cells express VEGF-2/FLt1 receptor on their membrane whose activation is important for their maturation.[28,29] An additional study is presently ongoing to explain the significant decline in lymphocytes which directly correlated with bevacizumab dosage and to evaluate which lymphocyte subset is affected by the treatment.

In the group of patients who underwent bevacizumab treatment, we also observed other uncommon adverse events like lung cavitation, and severe psychic depression and cognitive alteration, whose pathogenesis seems to be related to unknown VEGF functions. In this context, either peripheral and central nervous system adverse events have been sporadically reported in patients who have received treatment regimens containing bevacizumab or other moAbs.[30,31]

In our study the occurrence of adverse events appeared to be correlated with bevacizumab dosage and early deaths were all observed in the groups where greater moAb doses (7.5 and 10 mg/kg) were used. The mPEBev regimen showed a powerful antitumor effect fulfilling the statistical target activity of 35% which was fixed by taking in consideration a number of studies reporting the effects of frontline poly-chemotherapy.[3,32]

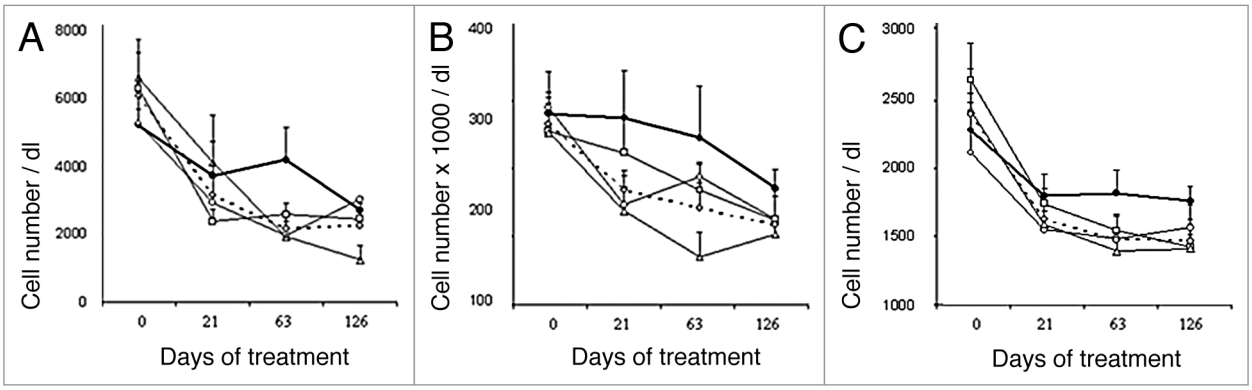

Figure 3. The curves comparing neutrophil (A), platelet (B) and lymphocyte counts (C) in advanced NSCLC patients enrolled in the mPEBev trial. Symbols represent a group of patients who received metronomic chemotherapy with no bevacizumab (level 0) [–●–], a group of patients who received metronomic chemotherapy plus bevacizumab at 2.5 mg/kg (level 1) [–○–], 5 mg/kg (level 2) [–□–], 7.5 mg/kg (level 3) [–△–]. The average of the patients who have received bevacizumab at any dose level is also represented in the figure [--●--]. The figure shows a progressive decline in the neutrophil, platelet and lymphocyte counts in those patients receiving metronomic chemotherapy with bevacizumab (any dose levels) which resulted significantly lower than those reported in the patients who had not received bevacizumab ($p < 0.05$). The asterisks show the results which were statistically significant if compared with both the baseline counts and the level 0 ($p < 0.05$).

The results of our trial become intriguing if we take in consideration the 40 patients who received bevacizumab, where a OR of 75%, a mean TTP, a mean time of tumor response and a mean OS of respectively 7.6, 6 and 12 mo were recorded. The antitumor activity of the mPEBev regimen appeared to be independent by either mAb dose level. The results appear very provocative considering that so far no bio-chemotherapy including bevacizumab has ever been reported similar results in advanced NSCLC.

In our study we also investigated the effects of the mPEBev regimen in patients with squamous histology. At present, little information is available on the effects of bevacizumab in these individuals, who account for 25–30% of all patients with NSCLC. In fact, the preliminary report of a greater risk of deadly hemoptysis in these patients, led to their exclusion by further bevacizumab experimentation. In our study, we excluded all patients with high risk of bleeding independently by tumor histology (see Patients and Methods). On these bases, we did not record any case of grade II-III hemoptysis while toxicity was similar to that observed for other histologies. On the other hand, we observed that patients with squamous cell carcinoma were very sensitive to the treatment, recording an OR in 11 out of 14 patients. Our results strongly suggest to continue the investigation in these patients.

Finally, a further consideration needs to be made regarding the cost of our regimen which resulted much less expensive if compared with the most recent treatments (including or not the newest drugs with biological targets). In fact, at our knowledge, the mPEBev regimen results twice less expensive than carboplatin + pemetrexed/docetaxel +/- bevacizumab regimens which are currently used in US.

The results of the Phase III trial (EC9446) which led to the FDA and EMEA approval of bevacizumab plus carboplatin and paclitaxel for the treatment of metastatic non-squamous NSCLC, only reported over the chemotherapy alone, an improvement of the OR from 15–35%; of the TTP from 4.5–6.2 mo and of the OS from 10.3–12.3 mo.[13] Similar results were also reported in the AVAIL Phase III trial testing bevacizumab in combination with platinum and gemcitabine.[13] Our results gain further interest if we consider that both EC9446 and AVAIL studies were restricted to patients with non squamous histology, good performance status (ECOG = 1) and no severe non neoplastic concomitant diseases. In this way patients who have a much worse prognosis were excluded.[12,13]

The antitumor activity reported in our study could be partially due to the metronomic administration modality of the mPE chemotherapy, which in a previous Phase II trials in high risk NSCLC patients resulted safe and very active. The mPE regimen compared with conventional tri-weekly intravenous PE schedules was able to reduce alone the amounts of immunosuppressive regulatory T cells, VEGF levels, and to produce a much more favorable pharmacokinetic drug profile for either etoposide and cisplatinum which allowed the achievement of a greater dose/intensity with a much lower drug blood peaks.[20,33]

Based on these results we believe that the mPEBev regimen is promising and deserves to be investigated in further a Phase II-III trial as frontline bio-chemotherapy for NSCLC patients. The results of our study suggest to use bevacizumab at the dosage of 5 mg/kg which is the dose level where we observed the most relevant biological activity, the greater response rate and less adverse events.

Patients and Methods

Study design. The study protocol code #BEVA2007 was performed in accordance to the good clinical practice guidelines and was approved by the Bioethics Committee of the University of Siena. All patients provided a written informed consent.

The study was prospectively planned according to a modified Fibonacci's schedule in order to identify the maximum tolerated dose (MTD) and most active biological dose (MABD)

of bevacizumab given in combination with mPE chemotherapy. Occurrence of both adverse and biological events per dose level were the primary endpoints for the statistical analysis in the Phase I design. In order to demonstrate that the mPEBev regimen is active in patients with advanced NSCLC, a Simon's two-stage minimax optimal design was adopted for Phase II design. This part of the study only involved those patients who received chemotherapy + bevacizumab given at any dose. The minimax two stage procedure was designed to test a null hypothesis of p = 0.150 vs. an alternative hypothesis = 0.350, with an expected sample size of 20.15 and a probability of early termination of 0.604. Therefore, if the combination is not considered to be active, there is only a 0.046 probability (4.6%) of discharging an active treatment (the target for this value was 0.05); conversely if the regimen is found to be active, there is a 0.197 probability (19.7%) that it is actually not active. The objective response rate was the primary endpoint for this statistical analysis. The treatment under investigation should be considered non-active if less than two responses out of 15 consecutive patients were recorded in the first series and fewer than 7/28 patients in the whole series. We considered the regimen as active when a response rate of at least 35% was recorded.

The inclusion criteria were: histological diagnosis of NSCLC, performance status (ECOG) from 0–2, normal renal and hepatic function, WBC count more than 2,500/mm^3, hemoglobin more than 9 g/dl, platelet cell count more than 90,000/mm^3, normal cardiac function. The exclusion criteria were: Central tumors with high risk of bleeding (excavated with large necrosis and infiltration of large arterial and venous structures), ECOG = 3, severe valvular and wall motion abnormalities or cardiac failure or a history of other severe cardiovascular disease, arrhythmia, second malignant tumors, signs of active hepatitis or liver failure, chronic or acute renal failure and active infectious disease. Considering that this is a Phase I-II trial aimed to identify the antitumor activity and toxicity of a novel bio-chemotherapy designed on translational basis also patients with squamous histology and poor performance status were included.

Treatment schedule. All of the patients received every 3 w, iv. cisplatinum (30 mg/sqm) on days 1–3 and daily oral etoposide (50 mg total dose) on days 1–15. Patients were divided into five groups of eight patients receiving every 3 w iv. bevacizumab at different doses [0 (level 0); 2.5 (level 1); 5 (level 2); 7.5 (level 3), and 10 mg/kg (level 4)] on day 3. Each dose level was aimed to be closed if two consecutive grade III-IV adverse events (with exception of hematological toxicity) were observed. A confirmative additional group of 12 patients was aimed to be enrolled in the specific cohort with the most active biological dose of bevacizumab.

Clinical assessment. A complete medical history, physical examination, complete blood count and serum chemistry were performed before starting treatment and repeated

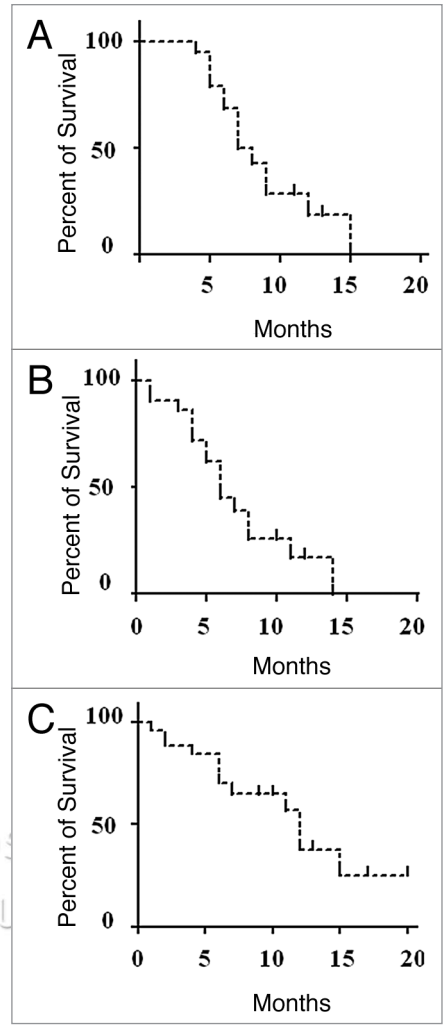

Figure 4. Actuarial Kaplan Meyer's survival curves of the 40 NSCLC cancer patients subjected to mPEBev regimen. Panels show: time to tumor progression (A); time of tumor response (B); and overall survival (C).

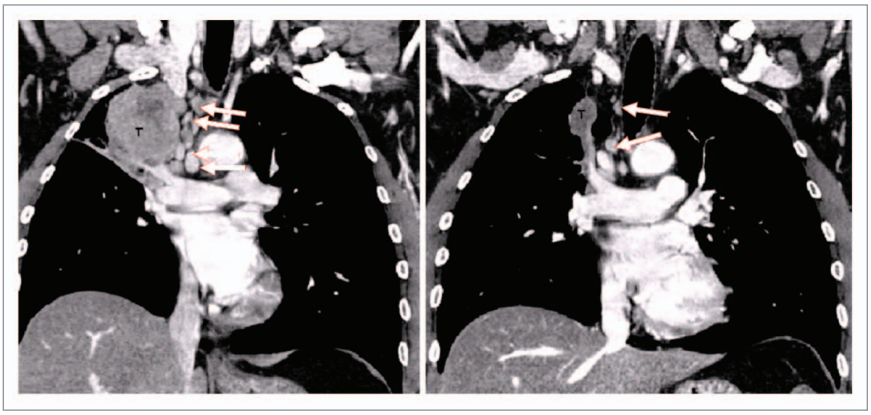

Figure 5. A representative objective response in a patient subjected to three cycles of mPEBev bio-chemotherapy. (A) The panel represents the baseline features; (B) The panel shows a significant reduction of the primary tumor burden and lymph-node metastases after three cycles of treatment. Arrows indicate the tumor sites.

every 3 w. Complete disease staging was undertaken at the baseline and after three and six cycles of treatment by computed tomography; tumor shrinkage and stabilization were confirmed after 28 d by chest X-ray and ultrasound scans. All patients were evaluable for response if they completed at least two courses of chemotherapy. Response was assessed according to RECIST criteria while toxicity was reported according to CTCAE v3.0.

Tumor blood flow evaluation. A Nuclear Magnetic Resonance (NMR) examination was performed at baseline and respectively repeated 7 and 21 d after the beginning of the treatment. Tumor perfusion was assessed by using a 1.5 T MR scanner with a receiver 8 channel phased-array body coil. Semi-quantitative data were extrapolated by a first-pass technique. In particular, a 3D gradient-echo T1 weighted sequence was acquired on coronal-oblique axis so to cover both the pulmonary lesion and the thoracic aorta; the acquisition started 5 s after the iv. administration of 0.1 mM/kg of Gd-BT-DO3A (Gadovist, gadobutrol, Bayer-Schering Pharma AG) at flow velocity of 2 ml/sec followed by 40 ml of saline solution at 2 ml/sec. On the basis of the first-pass signal-intensity (SI)/time curve a perfusion index (PI, ml/m/100 g) was calculated using a two-compartment model: $Ip = 1/\sigma^*(\Delta SI_k/t)/SI_{art}$ where $\Delta SI_k/t$ is the slope-rate of the lesion, SI_{art} is the higher SI of aorta, σ a constant of tissue density (1.05 g/ml).

Biological analysis and blood sampling. Peripheral venous blood samples (10 ml) were withdrawn at baseline and before each chemotherapy cycle (1 h before starting pre-medication and chemotherapy). Peripheral blood mononuclear cells (PBMCs) were obtained by means of Ficoll-Hypaque gradient separation, while serum samples were prepared by means of simple centrifugation; these samples were immediately frozen and stored at -80°C until their final examination.

Statistical analysis. Survival plots were constructed using the Kaplan-Meyer method, and the survival data were analyzed by using the GraphPad Instat 3.2 statistic software. Median follow-up was 12 mo. All experiments concerning molecular monitoring were performed at least three times. The statistical significance of differences in VEGF, Angiopoietin, Thrombospondin, Collagen IV etc., were evaluated with unpaired Student's t-test. Two-sided $p < 0.05$ was considered to be significant.

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