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**Antiangiogenic therapies for malignant gliomas:
new markers for targeted treatment**

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1. CHAPTER I - INTRODUCTION

1.1 HIGH GRADE GLIOMAS

1.1.1 Epidemiology

Gliomas are the most common primary intracranial neoplasms, accounting for 81% of all malignant brain and central nervous system (CNS) tumor (1). High grade gliomas (HGG), including grade III and grade IV gliomas according to the current classification of the World Health Organization (WHO), are the most aggressive brain tumor and grade IV astrocytoma, also called glioblastoma multiforme (GBM), is the most infiltrative subtype. Accounting for 60% to 70% of all malignant gliomas, GBM incidence is estimated to be about 2/100'000 in Europe and 3.05/100'000 in the United States of America. GBM occurs mostly in adults (2), with a median age at outcome of 64 years, and the patients median survival being at 12-18 months (with 90–95% of subjects surviving for less than 2 years), without possibility of spontaneous remission (3). For GBM standard therapy includes surgery followed by radiation and chemotherapy based on a phase III trial comparing radiation alone versus combined temozolomide (TMZ) and radiation followed by 6 cycles of TMZ, showing that addition of TMZ increases median survival

from 12.1 months to 14.6 months and improves 2-year survival rates from 10.4% to 26.5% (4)(1). For grade III gliomas, the standard therapy is basically the same since no specific golden standard has defined in this case so far (5).

1.1.2 Treatment

Although some progress has been made in the treatment of HGG, these tumor face a highly unmet medical need with limited treatment options. The current standard therapeutic approach for HGG is defined multimodal as it includes surgical resection of the tumor mass, followed by radiotherapy and chemotherapy with temozolomide (TMZ) (4).

Although surgery aimed to complete resection is considered as the first therapeutic modality, the infiltrative nature of these diseases makes a complete resection difficult; relapse remains indeed almost unavoidable. Even if an extensive resection of the tumor mass is carried out, all patients will virtually relapse within 2 to 3 cm of the original tumor (6). As curative surgery is not possible so far, its main aim is currently to perform a bulk reduction leading to a following brain decompression and lower intracranial pressure; this allows the achievement of an improvement in the quality of life and the preservation of neurological functions (7). Moreover, the acquisition of a tissue sample from the surgical procedure or biopsy (when surgery is not feasible) allows histopathological examination and confirmation of the diagnosis, hypothesised through magnetic resonance imaging (MRI) (8).

Although since the 1980's radiotherapy has followed surgery, showing an improvement of the overall survival and thus becoming a cornerstone of GBM treatment (9), the prognosis has remained extremely poor for longtime. Currently, radiotherapy, encompassing the primary tumor mass and including also 2-3 cm of margin, is commonly administered to a total dose of 60 Gy, delivered in 2 Gy fractions, five days a week, for six consecutive weeks (10). Neither stereotactic radiosurgery nor brachytherapy has significantly improved patients survival or local control (11).

Nowadays the multimodal approach for treatment of HGG includes also chemotherapy with TMZ, an imidazotetrazine derivative of the alkylating agent dacarbazine. TMZ has shown efficacy in relapsed GBM (12) and in 2002 very promising results in a phase II trial in first line treatment of GBM when combined with radiation therapy (13).

Since DNA alkylating agents, such as nitrosourea, have shown activity in brain tumor, several studies comparing treatment with nitrosourea-based chemotherapy plus radiotherapy versus radiation alone in HGG patients were carried out in the past years. Unfortunately, although a trend in favour of the addition of chemotherapy was observed in most of these clinical trials, the proposed regimens failed to attest a statistically significant benefit in terms of overall survival (OS). In any case, the addition of nitrosourea-based chemotherapy to radiotherapy was associated with a small but significant benefit in a meta-analysis based on 12 trials including HGG. In GBM, adding chemotherapy to radiation

caused a modest additional benefit of 6% and 4% in 1- and 2-year survival, respectively (14).

As a consequence, the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) sponsored a large phase III trial comparing radiotherapy alone (control arm) to the combination of concomitant radiotherapy and chemotherapy with TMZ, followed by 6 cycles of adjuvant TMZ (experimental arm), as therapy for a total of 573 newly diagnosed GBM patients. The experimental treatment resulted in a significantly longer median OS (14.6 months vs 12.1) and greater 2-year survival rate (26.5% vs 10.4%) than radiotherapy alone (Figure 1) (4). Furthermore, the median progression free survival (PFS) in the experimental arm was 6.9 months compared to 5 months of the control arm. Finally, toxicity of the combination was acceptable: only 8% of patients discontinued adjuvant TMZ because of toxicity and 67% of patients could increase TMZ dose from 150 mg/m² to 200 at the second adjuvant cycle.

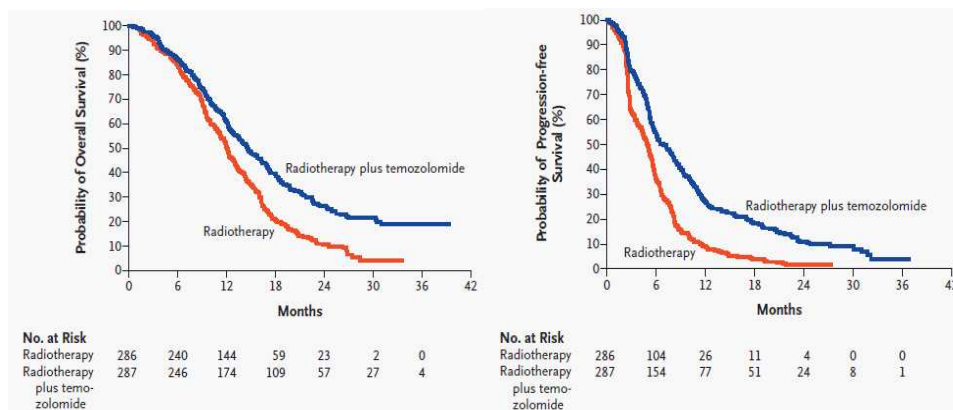


Figure 1: Kaplan–Meier curves of OS and PFS reached in the large phase III study published by Stupp et al. in 2005. The Kaplan-Meier graphs show OS (on the left) and PFS (on the right) of subjects treated with radiotherapy and TMZ (blue curves) and those treated with radiotherapy alone (red curves) (4).

Due to the clinically meaningful and statistically significant survival benefit associated with the experimental treatment with minimal additional toxicity, this study led to the adoption of concomitant TMZ and radiotherapy followed by TMZ, as a new standard of care in newly diagnosed GBM. Currently, in most centers the number of adjuvant TMZ cycles is still six, despite recently emerging evidences suggesting that to continue TMZ for longer periods when improvement on therapy is detected could be beneficial (15-17). Except for TMZ, there is currently no other standard chemotherapy for patients with HGG (18, 19).

1.1.3 Prognostic value of MGMT methylation

The methylation status of the O6-methylguanine–DNA methyltransferase (MGMT) gene, coding for an enzyme involved in DNA repair, not only plays a role as a prognostic factor in HGG, but could also be a predictive factor of TMZ treatment efficacy in this type of tumor (20).

Since MGMT removes methyl adducts at the O-6-position of guanine, one of the targets of alkylating agents such TMZ, its high activity leads to tumor protection against chemotherapeutics. Inactivation of the MGMT gene by hypermethylation of its promoter region is very common in human neoplasms (21), such as GBM, and correlates with a better prognosis in patients treated with TMZ, compared to patients with an unmethylated MGMT (Figure 2) (20-22).

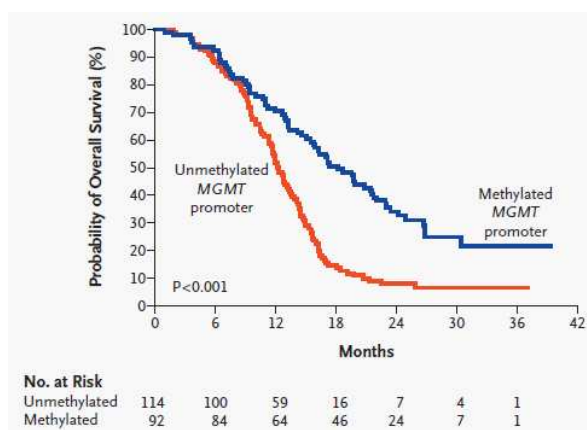


Figure 2. Kaplan–Meier curve of OS according to MGMT promoter methylation status. The Kaplan–Meier curve shows that the difference of OS of patients with a methylated (blue curve) or an unmethylated MGMT promoter

(red curve); the p value highlights the statistical significance and the prognostic value of this epigenetic modification (20).

Validation of the predictive value of MGMT gene promoter methylation is ongoing also in trials aiming at overcoming resistance to chemotherapy by a dose-dense continuous TMZ administration or in combination with MGMT inhibitors (21, 23).

MGMT methylation can be checked in blood samples of patients. Circulating DNA extracted from patient blood could represent a way to assess tumor specific DNA abnormalities: the detection of aberrant DNA methylation in serum and the comparison with tumor tissue has been recently investigated also in brain tumors (24-26). Free circulating DNA is often detected at different concentrations in serum or plasma of GBM patients (26, 27). Balana et al. demonstrated the presence of methylated MGMT in serum of GBM patients and showed that it is associated with patient response to treatment and survival (26). Moreover, a good correlation between methylation in serum and primary tumor tissue has been reported (26, 28). In 2006 Weaver et al confirmed the high level of DNA in the plasma of HGG patients and analysed primary tumor derived from those patients, reporting that 90% of the samples contained methylated gene promoters; the same promoters were methylated also in plasma DNA of more than 60% of the patients (24).

The impact of the MGMT status on the RPA Class was also analysed in a phase III trial in newly diagnosed GBM, but no conclusions were drawn due to the small number of patients (29). Thus, RPA classes,

defined by EORTC after this trial and currently used for stratification procedures in clinical research, do not take into account of the MGMT status.

1.1.4 Recurrence

Despite the aggressive multimodal approach – surgery, radiotherapy and chemotherapy with TMZ - GBM is hard to treat because of its high resistance to conventional cytotoxic chemotherapy and radiotherapy; thus, current treatments are now only palliative in nature and not curative. The refractory nature of GBM to treatment may be due to tumor cell infiltration into the surrounding brain and also to the blood–brain barrier (BBB), an obstacle for most drugs. Furthermore, cell variety and tumor mutations represent another challenge for the successful treatment of GBM that is composed of highly heterogeneous cell populations showing often high chemoresistance. Realistically, not even one of the treatments tested so far is supposed to completely destroy the tumor because a variety of genes may be mutated in different areas of it.

At relapse, chemotherapy remains the main treatment option, with the principal aim of prolonging PFS and OS, reducing morbidity, and restoring or preserving neurological function (7). Although the usually marginal effect of chemotherapy due to the difficult delivery of drugs across the BBB and the development of drug resistance by the tumor (30), a recent study demonstrated efficacy of TMZ in recurrent GBM

patients (31, 32), overcoming the results of the pooled analysis published by Wong in 1999 (33).

Recently, the use of metronomic (low-dose continuous) TMZ for HGG has been studied both *in vivo* and in the clinical setting. Kong and colleagues showed that in a rat model low-dose continuous TMZ inhibits both angiogenesis and growth of gliomas and increases apoptosis of tumor cells, whereas it reduces microvessel density in mice (34). In 2010 the same author published results of a phase II clinical trial where GBM patients, who progressed during or after the standard treatment schedule of radio- and chemo-therapy with TMZ after surgery (4), were treated with metronomic TMZ (40 mg/m² everyday in the first cohort and 50 mg/m² in the second). The experimental treatment resulted in an acceptable toxicity and a significant efficacy, since the PFS at 6 months (6m-PFS) was 32.5% and the OS at 6 months (6m-OS) was 56% (35).

Despite these results, the long-term disease outlook for HGG patients remains poor. Ultimately, the majority of patients succumb to their cancer, independently from treatments performed. Recent biological studies on the role of VEGF stressed its involvement in the tumor angiogenesis with consequences on the tumor growth and the incidence of metastasis. Thus, it seems likely that a more effective treatment for HGG could be provided with multimodal approaches using standard treatments at diagnosis (like surgery, radiotherapy and chemotherapy) with combination of novel, experimental treatments, such as antiangiogenic therapeutics, at recurrence.

1.2 ANTIANGIOGENIC THERAPY

1.2.1 Rationale for the use of anti-VEGF therapy in HGG

One of the hallmarks of GBM is its high degree of vascularisation. HGG are among the most highly vascularised tumor and express elevated levels of numerous pro-angiogenic factors, such as the vascular endothelial growth factor (VEGF), bFGF, IL-8 (36). Although several molecular mechanisms contribute to tumor angiogenesis in gliomas (37), VEGF concentrations significantly correlate with vascularity, and conditioned media of glioma cells containing high VEGF concentrations have been found to induce endothelial cell (EC) migration (38).

In physiological conditions, binding of VEGF to its receptor on endothelial cells allows the cell to have a greater permeability and capability to proliferate and migrate, as well as an increased survival. VEGF linkage is thus responsible for angiogenesis, endothelial cell integrity, vascular tone definition, prevention of blood cell adherence to endothelial cell covering vessel walls, and other important functions (Figure 3).

In the pathological setting, VEGF is secreted by tumor cells in response to a variety of stimuli, commonly characterizing many solid tumor, such as hypoxia, tissue acidosis or cellular stress. Moreover, different kinds of bone marrow (BM)-derived cells, including

hematopoietic cells (39), vascular smooth muscle cells (40), macrophages and endothelial cells (41), express VEGF receptors and are involved in tumor angiogenesis.

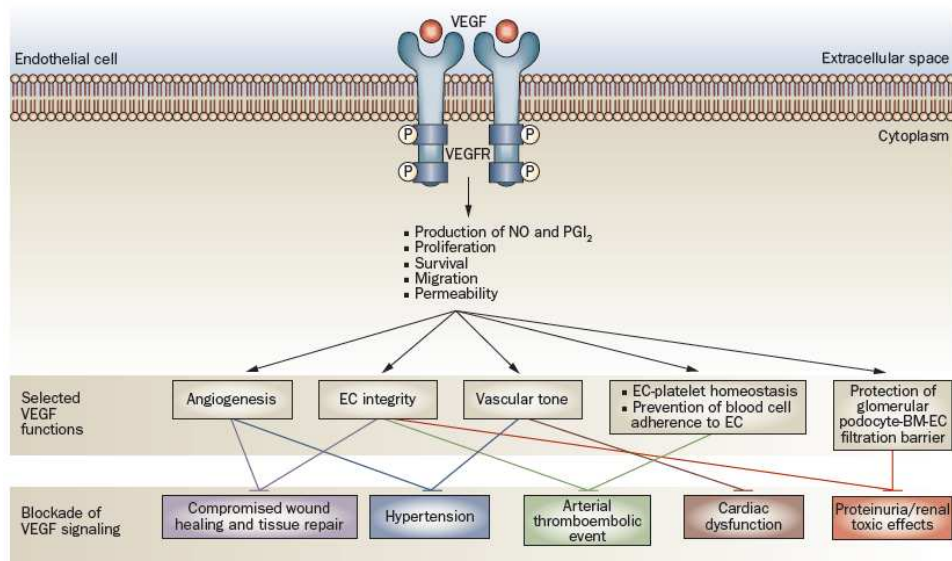


Figure 3. VEGF/VEGFR signaling: principal physiological functions and consequences of the pathway blockage. When VEGF links to its receptor, a cascade of downstream events occurs, such as increase of cell proliferation and migration and production of NO and PGI₂. These events can influence some specific aspects of the tissue, including the angiogenesis, the endothelial cell integrity, as well as the vascular tone. The inhibition of this pathway leads to a variety of consequences, depending on the blocked functions; when angiogenesis is blocked, for example, wound healing and tissue repair is compromised, whereas if modification of the vascular tone has cardiac dysfunction as a consequence. Abbreviations: BM, basement membrane; EC, endothelial cells; P, phosphorylated residues; PGI₂, prostaglandin I₂; NO, nitric oxide (42).

VEGF expression may indicate the potential of a tumor to be aggressive, infiltrating and supported by an effective supply of oxygen and nutrients. Endothelial cells normally divide about every seven years, but, in case of malignancies, this growth rate drastically accelerates reaching a division every 7–10 days. This change is also defined as “angiogenic switch”. Once new vessels are formed, VEGF functions as a survival factor inhibiting apoptosis of the poorly formed vasculature (43) and supporting tumor growth by constant blood flow and nutrition.

The identification of VEGF as one of the main stimulants of angiogenesis has recently led to the development of neutralizing antibodies (44, 45), soluble receptor constructs (46, 47), and antisense strategies (48) that either block angiogenesis or interfere with VEGF signalling. The use of agents targeting this pathway in HGG may therefore demonstrate to be effective in slowing or blocking disease progression.

1.2.2 Vascular Endothelial Growth Factor

Native VEGF is a basic, heparin binding, homodimeric glycoprotein (49, 50). Members of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factors (PlGF) 1 and 2. The human VEGF gene is localized in chromosome 6p21.3 and is formed by eight exons and seven introns; thus, the six human VEGF isoforms derive from alternative exon splicing.

VEGF interacts with three variants of receptor tyrosine kinase, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3(flt-4). While VEGFR-1 and VEGFR-2 are expressed on the cell surface of most blood EC, VEGFR-3 is exclusively present on lymphatic EC (51). Each VEGF isoform binds to a particular subset of these receptors: VEGF-A, the central regulator of physiological and pathological angiogenesis (52, 53), binds both VEGFR-1 and VEGFR-2, whereas VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3, and PLGF and VEGF-B interact only with VEGFR-1 (51) (Figure 4).

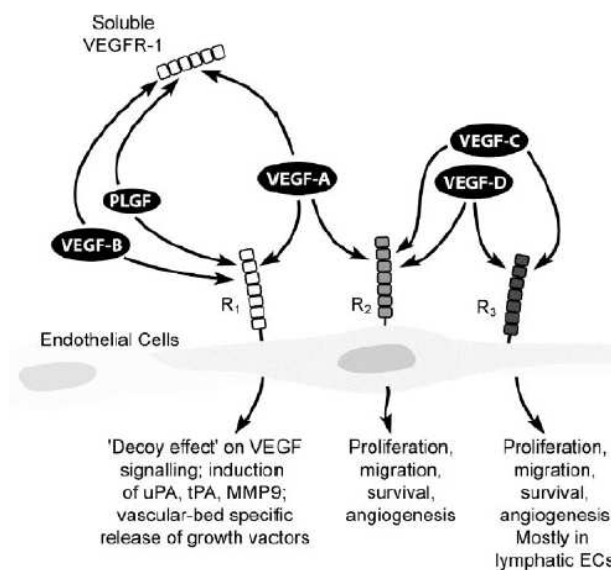


Figure 4. Role of VEGFR tyrosine kinases in EC. The VEGF family members, VEGF-A, -B, -C, -D and PLGF, bind specific receptors on EC, called VEGFR-1, -2, and -3. VEGFR-2 seems to be the main regulator of EC mitogenesis, survival and permeability, whereas VEGFR-1 does not mediate the mitogenic signal but may sequester VEGF and prevent its binding to VEGFR-2,

finally resulting in the inhibition of its signalling, in particular during early embryonic development, This inhibitory consequence is also called “decoy” effect and could be obtained also by the alternatively spliced soluble VEGFR-1 binding free VEGF. uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator (51) .

VEGFR-2 appears to be the main receptor responsible for mediating pro-angiogenic effects of VEGF (54, 55). Recruitment of co-receptors, such as neuropilins, heparin sulfate, integrins or cadherins, further modulates signalling specificity of VEGF receptors.

Recognition of VEGF as one of the primary stimulants of angiogenesis has led to the development of neutralizing antibodies (44, 45), soluble receptor constructs (46, 47), and antisense strategies (48) that either block angiogenesis or suppress tumor growth by interfering with VEGF signaling.

1.2.3 Bevacizumab, an anti-VEGF antibody

Bevacizumab is a humanized monoclonal anti-VEGF antibody, produced by Roche as antiangiogenic for treatment of cancer and also known as Avastin[®].

The mechanism of action of bevacizumab consists in sequestering any isoform of VEGF, thus making it unable to bind its receptors (VEGFR-1 and -2) on the target cells and to activate a cascade of events, influencing cell proliferation, migration, survival and permeability (Figure 5). In the

tumor setting, inhibition of these important cellular functions leads to a reduced blood supply and to the following slowing of the tumor growth.

Since gliomas are highly vascularised tumor and have been shown to over-express VEGF-A (56), blocking VEGF pathways may normalize tumor vasculature and improve chemotherapy delivery. Furthermore, according to the WHO 2007 classification, GBM can be distinguished from other astrocytic tumor also because of its peculiar presence of microvascular proliferation. This microvascular hyperplasia stresses the relevance of angiogenesis in GBM that was shown to express both VEGF mRNA and protein (57-59). Finally, pathological studies have demonstrated that, within GBM tissue, VEGF colocalizes with regions of viable tumor immediately bordering necrotic areas; coherently it is amply reported that VEGF expression is hypoxia-driven (60-62).

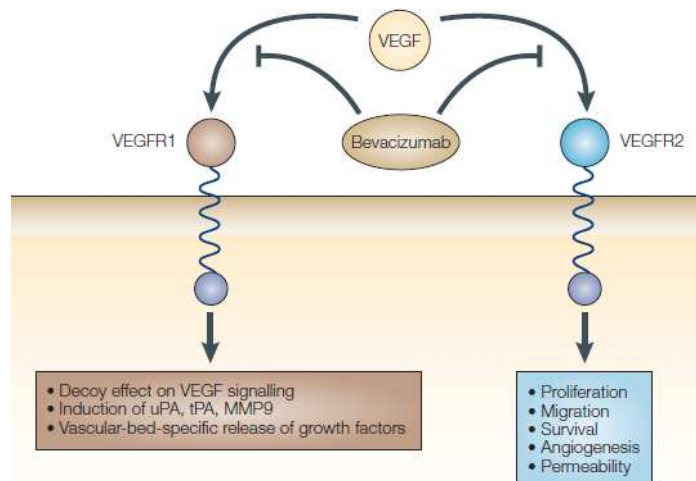


Figure 5. VEGF pathway and tumor angiogenesis. The surface of EC expresses VEGFR1 and VEGFR2. The first one is responsible for induction of

plasminogen activators and metalloproteinases (MMPs), as well as release of specific growth factor, whereas VEGFR2 plays a role in proliferation, migration and survival of cells. Tumor angiogenesis and metastasis growth are thus mainly mediated by VEGFR2. tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator (63).

If combined with chemotherapy, bevacizumab was shown to improve survival in patients with metastatic colorectal cancer, breast cancer and lung cancer (64), whereas promising results have been obtained in clinical trials with HGG patients treated with a combination of bevacizumab and irinotecan, a topoisomerase I inhibitor (65, 66). The efficacy of anti-VEGF therapy in GBM patients could be explained also by several other mechanisms of action. For example, since VEGF, as a relevant mitogenic factor, plays a significant role in the growth of astrocytes, it is expected its efficacy in astrocytic tumor, such as GBM (67). Moreover, it is hypothesized that in glial tumors the effects of anti-angiogenic therapies could be due to the selective targeting of brain tumor stem-like cells, reversing their stem cell phenotype and capacity (68)(53). Moreover, exposure to radiation has been proved to increase VEGF expression in GBM cells (69) and bevacizumab-mediated blockage of VEGF may decrease the potential angiogenic response due to radiation.

1.2.4 Preclinical activity of bevacizumab

Tumor angiogenesis is a fundamental process of the pathological blood vessel growth. Although several molecular mechanisms contribute to tumor angiogenesis in gliomas (37), VEGF concentrations in HGG correlate significantly with vascularity, and conditioned media of glioma cells containing high VEGF concentrations have been found to induce endothelial cell migration (38).

Bevacizumab, the humanized IgG1 version of the murine anti-human VEGF monoclonal antibody (muMAb VEGF) A4.6.1 (70), was extensively examined in preclinical models (71).

First of all, it was found to exert a potent inhibitory effect on the growth of three human tumor cell lines injected subcutaneously in nude mice (45). The three human tumors considered were SK-LMS-1 leiomyosarcoma, G55 GBM, and A673 rhabdomyosarcoma; their growth inhibition ranged from 70% to more than 95%, with a maximal effect observed with 5 mg/kg bevacizumab administered intraperitoneally twice weekly. Furthermore, the density of blood vessels was significantly lower in tumor from bevacizumab-treated mice compared with control. Many other tumor cell lines were then found to be inhibited by treatment with muMAb VEGF A4.6.1 (41, 72). Since neither the antibodies nor VEGF had any effect on the *in vitro* growth of the tumor cells (45, 72-74), inhibition of VEGF activity may result in suppression of tumor growth *in vivo*.

Bevacizumab resulted in tumor growth inhibition of twenty different human tumor cell lines (thirteen tumor types) implanted into nude mice

independently from route of administration or tumor location (75). Moreover, the precursor of bevacizumab, A4.6.1, was shown to decrease tumor vascularity, enhance tumor apoptosis and prolong survival of rats implanted intracranially with GBM cells (76).

To evaluate the biologic activity of bevacizumab in combination with cytotoxic chemotherapy, several studies were performed in animal tumor models. The combination of anti-VEGF treatment with cisplatin resulted in markedly enhanced biologic activity of the drug against tumor, compared with the activity of either agent alone (77). Similarly, the combination of muMAb VEGF A4.6.1 and doxorubicin resulted in significantly increased efficacy, compared to either agent alone (74).

Bevacizumab pharmacokinetic studies were conducted in mice, rats, and cynomolgus monkeys, showing a slow clearance of the drug from the serum, with a terminal elimination half-life of 1-2 weeks, as expected for monoclonal antibodies (78, 79).

1.2.5 Clinical activity of bevacizumab

A variety of clinical trials have been conducted so far to test toxicity and efficacy of bevacizumab as treatment for patients suffering from different kinds of solid tumor (77). Furthermore, several clinical studies examined the feasibility of combining anti-VEGF therapy, such as bevacizumab, with cytotoxic or biological agents. Reduction in interstitial fluid pressure as well as changes in vascular functions, including decreased vessel diameter, density, and permeability, were frequently

reported in response to treatment (77); in some cases, these modifications resulted in an increase in tumor uptake of chemotherapy, implying that the most effective use of anti-VEGF therapy is in combination with chemotherapy (36, 75).

Bevacizumab has been hitherto approved by FDA for the treatment of: metastatic colorectal cancer, with intravenous 5-fluorouracil-based chemotherapy for first- or second-line treatment; non-squamous non-small cell lung cancer, with carboplatin and paclitaxel for first line treatment of unresectable, locally advanced, recurrent or metastatic disease; metastatic breast cancer, with paclitaxel for treatment of patients who have not received chemotherapy for metastatic HER2-negative breast cancer (FDA however rescinded its approval on November 2011, because of insufficient evidence for activity); GBM, as a single agent for patients with progressive disease following prior therapy; and metastatic renal cell carcinoma with interferon alpha. However, bevacizumab had a proven efficacy also against non-small cell lung cancer (80) when administered in conjunction with traditional chemotherapeutics, as well as recurrent HGG, whose standard second line therapy has not been identified yet. The published experience with bevacizumab for recurrent HGG is encouraging; in GBM patients it was shown to reduce tumor and edema with an approximate 50% response rate (65, 81).

Although immunoneutralizing antibodies to VEGF, such as bevacizumab, suppress the growth of solid tumor, including malignant gliomas (45, 82, 83), many tumor cells survive with this treatment during which tumor growth is not blocked completely (82). That's why the

combined treatment of patients suffering from recurrent HGG with bevacizumab and chemotherapy was tested, showing radiographic response rates of at least 50% (65, 66, 81) and a median PFS of 24 weeks (65, 66).

Bevacizumab was tested in combination with irinotecan, a topoisomerase I inhibitor. The activity of irinotecan as a single agent in malignant glioma was evaluated in several prior studies. Since in these trials the reported response rates ranged from 0 to 16% and the 6m-PFS from 0 to 26% (84-90), implying that irinotecan alone has little efficacy (91), promising results in HGG patients using bevacizumab plus irinotecan suggest that the addition of bevacizumab enhances the anti-tumor activity of irinotecan. In 2007 at the Duke University 35 patients with recurrent GBM were enrolled in a phase II clinical trial and divided into two cohorts (66). Since the terminal half-life of bevacizumab is 17-21 days, the first cohort (23 subjects) was treated with bevacizumab 10 mg/kg and irinotecan at 125 mg/m² or 340 mg/m², according to the concomitant use of enzyme-inducing antiepileptic drugs (EIAED), every 2 weeks, whereas the second cohort (12 subjects) received bevacizumab 15 mg/kg every 3 weeks and irinotecan at 125 mg/m² or 340 mg/m² on days 1, 8, 15, and 22, every 6 weeks. There were at least 20 partial responses (57% objective response rate overall), 6m-PFS was 46% and 6m-OS was 77%. Compared to historical controls, the improvement was surprising.

In 2009 these promising results were strengthened by another phase II trial testing bevacizumab and irinotecan. Friedman and colleagues have

randomized 167 patients with recurrent GBM to bevacizumab alone (10 mg/kg q 2 weeks, Arm 1) or bevacizumab+irinotecan (340 mg/m² or 125 mg/m², depending on the simultaneous use of EIAED, Arm 2) (92). Clinical outcomes included 35% and 50% 6m-PFS in Arm 1 and Arm 2, respectively; mean OS was over 9 months for both arms. One intracranial hemorrhage occurred in each of the treatment arms. Finally, corticosteroid use was reduced in treated patients.

A large phase II trial was designed by the company producing bevacizumab to further evaluate effects of the drug when used alone (control arm) and when combined with irinotecan (experimental arm) for treatment of recurrent GBM. 167 patients, previously treated according to the Stupp protocol (4), were enrolled (93). Treatments in both arms were well tolerated with no new safety recordings; intracerebral hemorrhage rate was 2.4% and 3.8% in the control and experimental arms, respectively, whereas wound healing complications related to surgical resection were less frequent in the experimental arm. In terms of efficacy, the use of bevacizumab as a single agent or in combination with irinotecan in relapsed GBM resulted in a prolonged objective response and a higher 6m-PFS, when compared to salvage therapy or irinotecan alone (33). The extent of benefit over historical controls detectable for patients treated with bevacizumab was not observed with any other experimental or standard therapeutic approach so far.

Given the anti-tumor activity of bevacizumab, in terms of delayed progression and increased OS, in the GBM setting, both as single agent and in combination with irinotecan, the potential of bevacizumab as a

first line treatment in newly diagnosed GBM, in combination with radiotherapy and chemotherapy with TMZ (4) was explored. Initial reports of the combination of bevacizumab with the Stupp protocol showed a manageable toxicity and encouraging mean PFS (94, 95), providing the rationale for a large phase III study where the safety profile of bevacizumab with chemoradiation can be further characterized. Enrollment for this multicentre international clinical trial ended in the Spring 2011 and the first interim analysis is still ongoing.

1.3 THERAPEUTIC PROTOCOL

1.3.1 History of the protocol approval and amendments

In Italy the use of bevacizumab as treatment for recurrent gliomas has not been approved yet by the Regulatory Agency (*Agenzia Italiana del Farmaco*, AIFA). For this reason, the drug for this therapeutic indication is allowed only in case of use on a compassionate basis (therapeutic use according to the Italian Decree Law of May 8, 2003 “*Uso terapeutico di medicinale sottoposto a sperimentazione clinica*”) or in case of off-label use within the Hospital.

In 2009, January 14th, a therapeutic protocol on the treatment of high grade glioma patients with bevacizumab and irinotecan was approved by the Ethical Committee of the Neurological Hospital “Carlo Besta” of Milan, Italy. The title of the therapeutic protocol was “Therapeutic use of bevacizumab and irinotecan in patients with recurrent glioblastoma multiforme and without valid therapeutic alternatives”.

Later, in the same year, the protocol was amended for addition of grade III glioma patients as potential population who can benefit from the experimental treatment; the amendment was approved by the Ethical Committee in June 3rd and the title was “Therapeutic use of bevacizumab and irinotecan in patients with recurrent glioma grade III and IV in absence of valid therapeutic alternatives”.

One further amendment was approved by the Ethical Committee on November, 3rd 2010; the protocol criterion excluding patients with previous malignancy was better detailed adding “except squamous cell skin cancer and in situ basal-cell carcinoma of the cervix”. The title of the protocol did not change in this case.

1.3.2 The therapeutic protocol

1.3.2.1 Experimental treatment and inclusion criteria

The experimental treatment included bevacizumab, 10 mg/kg, in combination with irinotecan (340 mg/m² in patients treated with anti-epileptic drugs that induce enzymes involved in its metabolism, EIAED, or 125 mg/m² in patients not receiving these drugs, non-EIAED). Bevacizumab and irinotecan were administered i.v. every two weeks, as described by Vredenburgh in 2007 (65). The experimental treatment was administered only to patients with grade III and IV glioma relapsing after standard treatment and with no valid therapeutic alternative.

1.3.2.2 Radiological follow up

After MacDonald criteria were first described, imaging technology, therapeutic strategies, and requirements of clinical studies have substantially evolved, showing the limitations of these criteria, as well as the ambiguity of some of their key features.

Furthermore, the mechanism of action of antiangiogenic therapeutics determines a lack of association between the Response Evaluation Criteria in Solid Tumor (RECIST) and survival benefit of patients after treatment. In fact the antiangiogenic effects on the tumor vasculature do not necessarily correlate with tumor shrinkage.

In 2009 innovative morphological CT imaging criteria were evaluated in a clinical trial testing bevacizumab and cytotoxic chemotherapy in patients suffering from colorectal liver metastases; overall attenuation, definition of the interface between the tumor and healthy tissue, and the presence of a marginal rim of enhancement were used as criteria (95, 96). The study proved that these morphological criteria correlated better with OS than the RECIST criteria. Nevertheless, due to the missing control arm, the study did not clarify whether these new criteria were truly assessing the benefit of the investigational combination or were solely better at predicting the efficacy of cytotoxic chemotherapy (97).

The Response Assessment in Neuro-Oncology (RANO) Working Group, an international effort to develop new standardized response criteria for clinical trials in brain tumors, has proposed new criteria for the follow-up of malignant gliomas that are particularly suited for the analysis of antiangiogenic treatments (98).

1.3.2.3 Clinical follow up and Quality of Life analysis

Clinical follow up of patients treated according to the therapeutic protocol included the objective and neurological visit with Folstein Mini-

Mental State Examination (MMSE), as well as blood test and urine analysis. If appropriate, an eye examination and a CT scan were carried out in order to exclude the onset of optic neuropathy and bleeding due to the experimental treatment. Finally, since mood disturbances may influence cognitive function, the quality of life (QOL) of treated patients was monitored performing QOL-C30 and QOL-BN20 every 2 months.

1.4 PROGNOSTIC/PREDICTIVE FACTORS FOR ANTIANGIOGENIC TREATMENT

1.4.1 Biomarkers for antiangiogenic therapy

Since tumors have been traditionally treated with cytostatic chemotherapeutics, the recent introduction into clinical practice of antiangiogenic drugs led to mandatory changes in the way of evaluating novel treatments efficacy, in order to avoid the inadequate assessment of their activities based only on reduction of the tumor. To truly reflect the biological efficacy of antiangiogenic therapies, specific direct or indirect biomarkers of their efficacy must be identified and validated. Many potential biomarkers, both tumor and systemic, are under evaluation in clinical trials, but anyone has not been able to carry out a close monitoring of the vascular structure within the tumor during the different clinical stages and in relation to treatment undertaken (99, 100).

Several ways to measure changes in tumor angiogenesis are reported in literature. Originally, the measurement of microvessel density was the principal method; however a variety of limitations induced researchers to find other procedures. Microvessel density assessment is, however, invasive and difficult to standardize; moreover, the biopsy performed for angiogenesis evaluation with this technique does not always mirror the real aspect of the whole tumor status (101). Finally, angiogenesis changes

do not necessarily induce modifications of the microvessel density level thus not warranting a clear correlation with the clinical outcome after antiangiogenic therapy (101).

Angiogenesis could also be analysed by DCE-MRI, but in this case limitations are due to the necessity of a specific and expensive machine with a relevant standardization procedure (102). 3D-Power ultrasound is also potentially useful for detection of changes in the tumor angiogenesis, but is a very innovative technique which needs practiced staff for manipulation of results (103).

Finally, levels of circulating molecules involved in angiogenesis, such as VEGF, BFGF, HGF, IL-8, PLGF, VEGFR2, could be detected and used as markers to follow the process fluctuations. In our case, the obvious marker to check during treatment with bevacizumab is VEGF, the drug's specific target-molecule, as for other molecularly targeted drugs. However, the analysis of tumor fragments from patients with metastatic colorectal cancer (97, 103) and metastatic or advanced breast carcinoma (104) did not show a predictive effect of VEGF expression. VEGF concentration was shown to correlate with tumor vascularity, grade and prognosis (59, 105-107), but its changes during the antiangiogenic treatment are not necessarily predictive of benefit for multiple reasons. First of all, VEGF is the principal factor responsible for tumor early angiogenesis (108) and, as a consequence, detection of possible differences in its levels of expression could be difficult. Moreover, VEGF expression increases in the presence of hypoxia which is induced by antiangiogenic treatment; this could be overcome

performing just a baseline assessment and not dynamic evaluation of VEGF expression during treatment. Third, since VEGF has multiple isoforms and only VEGF-A activity is blocked by bevacizumab, other ligands could link to VEGFR, limiting the drug effects on tumor angiogenesis. Furthermore, VEGF is not the only molecule responsible for angiogenesis in tumor; a variety of other factors, such as circulating endothelial cells (CEPs) or progenitors (CEPs), are involved, especially in the advanced forms of cancer (109). Finally, there are several issues related to the lack of consensus in procedures and relative scoring systems, as well as the absence of a quantitative reliable method (97). Some of the mechanisms leading to drug resistance are graphically summarised in Figure 6.

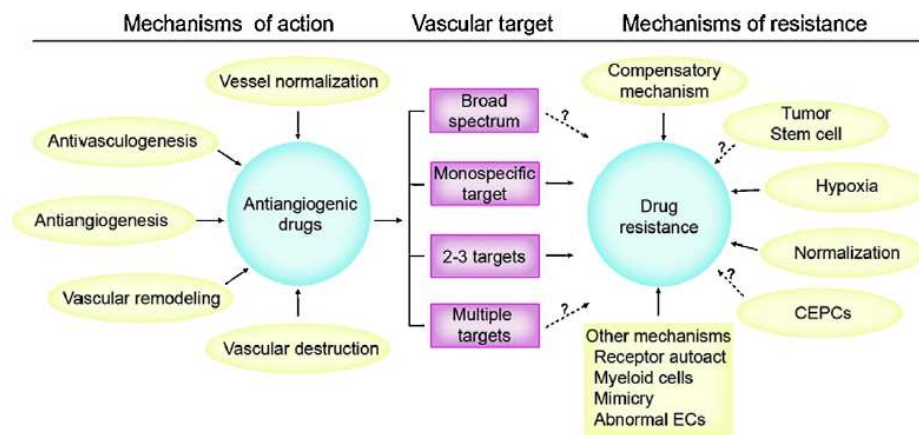


Figure 6. Possible mechanisms of resistance to the antiangiogenic therapy. Antiangiogenic drugs act inducing vessel normalization, antivasculogenic and antiangiogenic processes as well as vascular destruction and remodeling. These drugs are classified as highlighted in the violet squares.

Resistance to antiangiogenic therapy can be due to compensatory mechanisms, presence of tumor stem cells or circulating endothelial progenitor cells, hypoxia, etc. CEPC, circulating endothelial progenitor cells; EC, endothelial cells (110).

As a consequence of all these limitations in using VEGF as single marker to check efficacy of the antiangiogenic therapy, the current approach is to identify an angiogenic signature (111), i.e. a group of predictive markers, to be analysed and keep monitored. This theory is strengthened by the fact that the optimal biomarker should have a proved biological relevance with respect to VEGF inhibition, should allow its continuous evaluation so as to identify possible variations of response and resistance onset during therapy, should predict OS, and have a high predictive value (97).

Different candidate predictive markers of tumor angiogenesis have recently entered the clinical arena, such as antiangiogenic therapy-induced hypertension (97), VEGF and VEGFR polymorphisms (97), pro-angiogenic molecules, such as Bv8 (Bombina Variegata peptide 8) and PDGF (platelet-derived growth factor) (97), but their reliability is often limited by the small sample size of the studies and the impossibility to compare data regarding different tumor types treated with different therapies (111). These candidate predictive markers include CECs and CEPs.

1.4.2 Circulating endothelial cells (CECs)

Although literature on CECs extends back over several years, one of the most relevant issues in using apoptotic CEC as a surrogate marker of vessel damage and viable CECs as a marker of vascular remodelling, is that their phenotypes overlap to that of many other cell types, such as platelets and some hematopoietic cells (111) (Table 1).

| CEC PHENOTYPE | EPC PHENOTYPE | Ref |
|----------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-------------------------------------------|
| CD45 ⁻ CD146 ⁺ CD31 ⁺ CD34 ⁺ | CD45 ⁻ CD31 ⁺ CD133 ⁺ | Asahara T. Am J Physiol Cell Physiol 2004 |
| CD146 ⁺ CD31 ⁺ vWF ⁺ VEGFR2 ⁺ | NA | Beerepoot LV. Ann Oncol 2004 |
| CD34 ⁺ CD146 ⁺ CD105 ⁺ CD11b ⁻ | CFU-EC | Zhang H. Blood 2005 |
| CD45 ⁻ CD31 ⁺ CD34 ⁺ CD146 ⁺ | CD45 ⁻ CD31 ⁺ CD34 ⁺ CD133 ⁺ | Wierzbowska A. Eur J Haematol 2005 |
| NA | CD133 ⁺ CD34 ⁺ VEGFR2 ⁺ | Massa M. J Clin Oncol 2005 |
| CD45 ⁻ CD31 ⁺ CD146 ⁺ | NA | Go RS. Ann Hematol 2008 |
| CD45 ⁻ CD34 ⁺ CD146 ⁺ CD133 ⁻ | NA | Della Porta MG. Leukemia 2007 |
| CD45 ⁻ CD146 ⁺ CD31 ⁺ CD34 ⁺ | CD34 ⁺ VEGFR2 ⁺ | Furstenberger G. Br J Cancer 2006 |
| NA | CD34 ⁺ VEGFR2 ⁺ | Richter C. Breast Cancer Res Treat 2007 |
| NA | CD133 ⁺ VEGFR2 ⁺ | Naik RP. Breast Cancer Res Treat 2008 |
| NA | CFU-EC | Kim HK. Cancer Lett 2003 |
| NA | CD34 ⁺ VEGFR2 ⁺ | Dome B. Cancer Res 2006 |
| NA | CD45 ⁻ CD133 ⁺ VEGFR2 ⁺ and CD45 ⁻ CD34 ⁺ VEGFR2 ⁺ | Pircher A. Oncol Rep 2008 |
| NA | CFU-EC | Ho JW. Hepatology 2006 |
| NA | CFU-EC | Yu D. Clin Cancer Res 2007 |
| CD34 ⁺ CD146 ⁺ VEGFR2 ⁻ | CD133 ⁺ CD34 ⁺ VEGFR2 ⁺ | Zheng PP. Ann Neurol 2007 |
| CD45 ⁻ CD146 ⁺ CD105 ⁺ | NA | Rowand JL. Cytometry A 2007 |
| CD45 ⁻ CD34 ⁺ CD133 ⁺ CD105 ⁻ | CD45 ⁻ CD34 ⁺ CD133 ⁺ CD105 ⁻ | Twardowski PW. Cancer Invest 2008 |
| CD45 ⁻ CD31 ⁺ P1H12 ⁺ CD133 ⁻ | NA | Norden-Zfoni A. Clin Cancer Res 2007 |

Table 1. Lack of consensus on CEC and CEP definition. Consensus on CEC and CEP definition is still lacking nowadays; this is proved by the

numerous and discordant data on their phenotype, published so far in literature (112).

To define the lineage of this cell type is complex even because no unique way to detect them is currently available. Most of works tended to measure CECs by immunomagnetic beads (113-115) and used CD146 as a defining antigen. However, additional studies aimed to assess CEC phenotype in a clinical setting failed to provide clarity, as other different markers were declared to be expressed by CECs. As an example, Zhang in 2005 defined CECs as those cells expressing CD146, CD34 and CD105, whereas Mancuso some years before excluded CD105 expression (116-119). The currently developing view of CECs defines them as those peripheral blood cells expressing CD146 and including the intra-cellular von Willebrand factor (vWf).

The morphology usually completes the definition (113-115). CECs are well or terminally differentiated mature endothelial cells, with low proliferative potential, which are shed from the intima of the blood vessel walls and enter the circulation reflecting vascular damage or dysfunction. CECs were proved indeed to correlate positively with plasma and physiological markers of vascular damage, like soluble E selectin and flow mediated dilatation (120).

CECs are rare in healthy individuals, with a frequency of 0.5-2 cells/ml, whereas their levels are often increased up to 10-fold or more (120), in a variety of vascular disorders (121-123) and in the peripheral blood of cancer patients at diagnosis appear to correlate with tumor

progression (124) and tend to return to normal values in case of complete remission (102, 118, 125, 126).

CEC values, however, vary according to cancer typology. In breast cancer for example high CEC levels at baseline reflected a better outcome than low levels (125, 127, 128). On the contrary, in colorectal cancer a better outcome was warranted by low CEC baseline levels (129, 130). Differences in the number of CECs and in their clinical meaning could be due to diverse protocols used for detection or to a different vascular turnover depending on the tumor origin. Discrepancies on CEC vital status are also evident in literature; they are indeed reported as viable, apoptotic and necrotic with different proportions depending on the study (120).

Although a multi-centre consensus document on the definition of a common protocol to isolate and measure CECs has been proposed in 2006 (131), the overall complexity regarding management and use of these cells in the clinical setting is still an issue as no sure and clear therapeutic value have been reported for these cells so far.

CD109-positive CEC subpopulation

CD109 is a monomeric glycosylphosphatidylinositol (GPI)-anchored protein of about 170 kDa (132), working as a TGF beta co-receptor. TGF beta, a multifunctional growth factor controlling a variety of cellular processes, links to TGF beta receptor 1 and 2 on the cell surface. The following receptor internalization via the clathrin-coated pits pathway

induces the SMAD-mediated signalling; on the contrary, receptor internalization via the caveolae-pathway leads to degradation via the proteosoma (133). In this setting, CD109 is associated with caveolin-1 in the membrane lipid bilayer of caveolae, and is able to increase the percentage of TGF beta receptors that are internalized via caveolae; in case of excessive amount of TGF beta, this process can inhibit TGF beta responses. This ligand-dependent degradation of TGF beta receptors, induced by CD109 via the caveolar route, is consistent with what was discovered for several other receptors, like those for insulin and EGF, as well as beta2 adrenergic receptors.

CD109 expression has always been associated to foetal and adult bone marrow CD34+ mononuclear cells with a peak of its expression in the most primitive hematopoietic stem cells (132). CD109 seems to be highly expressed by the brightest CD34+ subpopulation from both foetal and adult bone marrows (134). Until recently, CD34 was the only hematopoietic antigen that was expressed exclusively by the stem cell population. However, this CD109+ minor subset of the CD34+ bone marrow-derived population was shown to be enriched in both primitive stem cells and non-lymphoid lineage-committed hematopoietic progenitors of the myeloid and erythroid lineages; most of CD34+CD109- cells were indeed lymphoid restricted (134).

Apart from its involvement in hematopoiesis, CD109 may play a role in cell-mediated immunity and hemostasis, since it is also expressed as an activation antigen on T cells and platelets (132). It is hypothesized that its cleavage could make it interact with adjacent molecules and mediate cell-

substrate, cell-matrix, or cell-cell contacts in hemaotopoiesis, hemostasis, as well as immune responses (132).

CD109 involvement in many relevant cell functions justifies its mutations and deregulated expression in numerous human tumor cell lines, such as those of GBM, squamous cell carcinoma, sarcoma and adenocarcinoma (135). CD109 expression was also significantly high in some of the 33 human lung cell carcinomas tested and 17 esophageal squamous cell carcinomas (135). Hence, CD109 expression on CECs shed from the tumor vessel walls could be used as marker to detect these cells and to study their behaviour during antiangiogenic therapy. The physical vicinity of tumor vessel walls and relative endothelial cells to the tumor mass could be the reason why genetically-altered CECs have been identified (102). Possible explanations could be the common origin of endothelial and tumor cells; the possible fusion of the two cell types; or the chromosomal transfer or dedifferentiation of tumor cells induced by microenvironmental factors which in turn cause differentiation towards the endothelial phenotype (102).

In conclusion, due to its potential usefulness as molecular target for the development of innovative drugs against tumor or monitoring of tumor response to therapy, CD109 is currently deeply studied both at preclinical and clinical levels.

1.4.3 Circulating endothelial progenitors (CEPs)

Literature on CEPs extends back almost as that on CECs, but, as for CECs, no common definition of these cells combines the published papers. Furthermore, whilst works on CECs quite unanimously proved their increased levels in several diseases and their correlation with the mentioned disease severity, papers on CEPs hitherto failed to show the same (120).

The interest on CEPs is due to their potential as stem cells and thus possible providers of therapeutic neovascularisation in case of vascular diseases (120). In tumor, however, the focus is more on the role of CEPs in tumor vascularization and, as a consequence, on their fluctuations during and after antiangiogenic therapy.

CEPs are mobilized from the bone marrow following tissue ischemia and may be recruited to complement local angiogenesis supplied by existing endothelium (136, 137). Their levels correlate with the potential for repair of vascular damage (138) and in cancer patients, high CEP counts reflect an ongoing tumor vasculogenesis (112, 118, 139, 140). Tumor vascularisation depends indeed on the sprouting of the surrounding blood vessels due to migration and differentiation of the existing mature endothelial cells and on the recruitment of bone-marrow derived endothelial progenitor cells; the first process is called angiogenesis, the latter vasculogenesis (141).

Although very different for many aspects, there is evidence of some commonality between CEPs and CECs; some of the shared characteristics are the expression of some CD molecules, morphology and growth

features *in vitro* (120). The panel of markers used to select CEPs, indeed, is not completely defined, because of its partial overlap with that currently used for selection of hematopoietic cells and CECs. CEPs were shown to express mainly the glycoprotein CD34, a marker of hematopoietic progenitor cells, and VEGFR2 (130), but also CD31, CD146 (111), CD45, CXCR4, VE-cadherin and the typical marker of hematopoietic stem cells CD133 (112) have been reported as possible markers of CEPs. CD133, in particular, has been reported to allow distinction between early endothelial progenitors, expressing CD133, and CEPs which gradually lose it (112); however, a consensus of the scientific community on it has not been reached yet.

The most evident difference between CECs and CEPs is that CEPs have a high proliferative potential. A smart experiment, conducted by Lin in 2000, showed that only 5% of peripheral blood CECs from patients who had bone marrow transplant were of donor origin and had a greater proliferative capacity than recipient cells *in vitro* (132). These results stressed once more the proximity of the two cell types, which were also thought to be “two sides of the same coin”, as said by Blann in 2006 (120).

A variety of factors which stimulate CEP mobilization is reported in literature, such as administration of recombinant human erythropoietin (rHuEPO), presence of granulocytemacrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF), placental growth factor (PlGF) (142), angiopoietin-1 (Ang-1) (143), platelet-derived growth factor-CC (PDGF-CC) (144), stromal cell-derived factor-

1 (SDF-1) (145), nitric oxide (NO) (146), 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) (147). Moreover, when estrogens (148) and physical training (149) increase CEP mobilization, tumor necrosis factor-alpha (TNF-a) and C-reactive protein (CRP) decrease their levels (150, 151), whereas NO synthesis is improved (120). Among them, however, VEGF, interacting with their receptors, plays a key role in CEP mobilization.

VEGFR2+ CEP subpopulation

As for other endothelial subpopulations, VEGFR2+ CEP have not been hitherto identified with a specific and unambiguous phenotype. It seems quite established in literature that CEPs are CD34+VEGFR2+7AAD- (120). More uncertainty is reported for the expression of CD45, the hematopoietic stem cell marker, which could be dim or absent (141).

Bone-marrow derived progenitor cells involved in tumor vasculogenesis include hematopoietic (VEGFR1+) and endothelial (VEGFR2+) cells, which initiate the pre-metastatic niche and promote the metastasis vascularisation, respectively (141) (Figure 7).

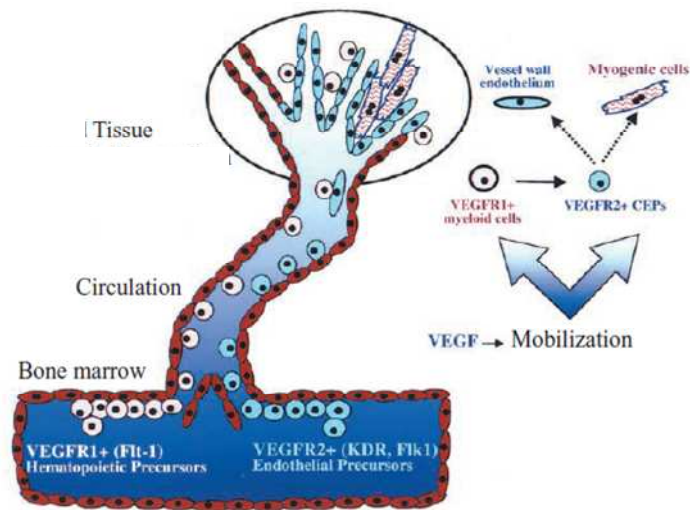


Figure 7. Hematopoietic and endothelial as bone-marrow derived progenitor cells. The first express VEGFR1, whereas the latter VEGFR2. Both types of cells are involved in angiogenesis and stroma formation. In particular, endothelial progenitors play a relevant role in tumor angiogenesis and growth. Angiogenic factors released by the neoplastic cells induce recruitment and co-mobilization from the bone marrow of both haematopoietic progenitors and VEGFR2+ CEPs, whose functional incorporation into the tumor vasculature is essential for neoplastic angiogenesis (152).

VEGFR2 (KDR) expression is not present in the CD45- CEP subset of paediatric patients with solid malignancies, whereas it is significantly expressed in the rare CD45dim subpopulation, thus confirming the common origin of endothelial and hematopoietic progenitors (141). Moreover, Farace and colleagues recently published a correlation between a baseline level of CD45dimCD34+VEGFR2+7AAD- cells

higher than 2% and a higher risk of progression in patients with metastatic renal cell carcinoma (153). Patients with a stable or increased level ($\geq 2\%$) of these cells at baseline and after 14 days during treatment with sunitinib or sorafenib, two tyrosine kinase inhibitors of VEGFR as well as PDGFR and other receptors, had a lower risk of progression (153). Blann et al. defined CEPs as CD45⁻ cells and reported that although typical prostate cancer molecular markers increased, no change was detected in CEP count (154). Du Bois defined CEPs as CD146⁺CD31⁺CD45⁻CD133⁺ and showed that their count did not differ in osteosarcoma paediatric patients compared to controls (155). The author supposed that selection of CEPs using CD133 may be responsible for the difference of his results from those of other papers previously published. However, it is hypothesized that also cancer histology might play a role in fluctuations of CEP and CEC counts.

As anticipated above for CECs, also CEPs have several aspects in common with the hematopoietic cell lineage. Figure 8 clarifies the role of hemangioblasts and their relationship with CECs and CEPs. Endothelial cells and hematopoietic cells have the hemangioblast as common precursor (112, 156). Hemangioblasts are endowed with long-term proliferative capacity and ability to reconstitute both endothelial and hematopoietic lineages, expressing CD34 and VEGFR2 on both early progenitors; these markers are then gradually lost during hematopoietic differentiation and conserved in completely differentiated endothelial cells (Figure 7) (156, 157). Nevertheless, a clear identification of hemangioblasts currently still lacks, since another cell population with

the hemangioblast bilineage potentiality but without expression of CD34 and CD45 (as well as CD133) was recently isolated from peripheral blood (158).

Proximity of endothelial and hematopoietic precursors is once more stressed by the fact that tumor vascularization is supported also by hematopoietic cells, despite their principal localization in the periendothelial tumor site; examples of these supporters are mast cells, tumor-associated macrophages, natural killer cells, VEGFR1+ hematopoietic progenitors, CD8+ T cells, Tie2-expressing monocytes, etc (112).

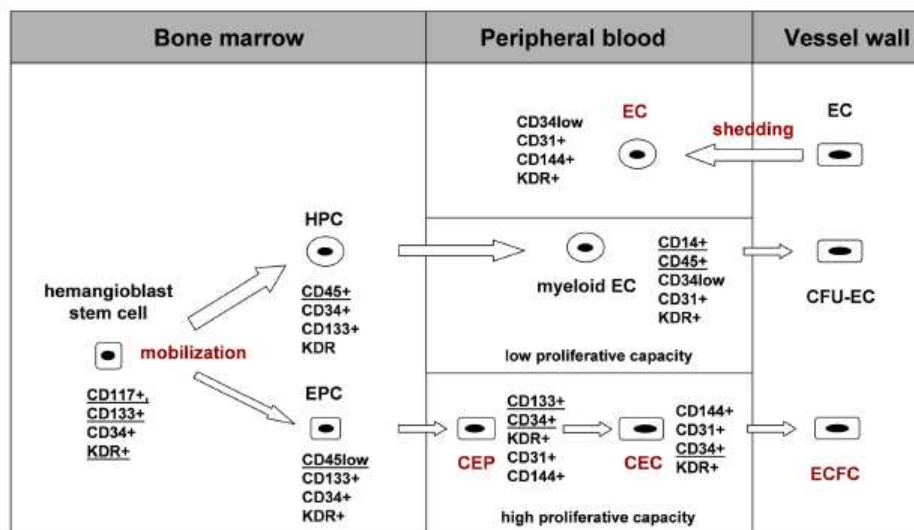


Figure 8. Origin and phenotype of CEC and CEP. Hemangioblasts originate from the pluripotent stem cells of the bone marrow. Hemangioblasts have a bilineage potentiality, thus the ability to differentiate as hematopoietic or endothelial cells. VEGFR2 and CD45 are the major markers distinguishing the

two populations. Both CEC and CEP originate from endothelial cells, whereas hematopoietic progenitors give rise to myeloid cells, like monocytes, and myeloid endothelial cells. HPC, Hematopoietic Progenitor Cells; EPC, Endothelial Progenitor Cells; EC, Endothelial Cells; CFU-EC, Colony Forming Unit Endothelial Cells; ECFC, Endothelial Colony Forming Cell Clones (159).

1.4.4 Methods for CEC and CEP detection

In 2006 Blann reported that CECs are mainly detected by immunobeads, whilst CEPs through flow cytometry (120). This is basically still valid, despite the presence of evidence regarding other laboratory techniques.

Since CECs are rare in peripheral blood, the main aim of protocols used for CEC measurement is cell enrichment (120). Immunomagnetic separation and flow cytometry are currently the most common techniques (112) (Figure 9). Immunomagnetic separation allows exclusive selection of CECs thanks to immunomagnetic beads coated with antibodies that bind only cells expressing specific molecules on their surface. Selection ends then with magnet retrieval (160). The most common antibodies in this case are CD146 (however expressed by pericytes, bone marrow fibroblasts as well as activated lymphocytes, and frequently increased in cancer patients) and CD31 (112); moreover, leukocyte markers, such as CD14 and CD45, are used as negative markers. Finally, cell size must be higher than 10 μm (112).

CECs are currently also detected by flow cytometry and protocols for their phenotypic enumeration are based on use of: i) CD146, as a specific but not exclusive endothelial marker; ii) CD45 to exclude haematopoietic cells; iii) CD105 expression, which is expressed in activated endothelial cells; and CD45 as negative marker to exclude hematopoietic cells (161). Finally a visible nucleus (DAPI positive) and round to oval morphology are needed to define CECs. Mancuso et al. included in the panel also CD31, as a differentiation marker of endothelial cells, and CD140b (162). 7AAD is usually used to determine the viability status of cells, whereas the nuclear staining Syto16 allows discrimination between DNA containing cells, platelets and cell debris (162). Mancuso et al. defined necrotic cells as Syto16low/7AAD+, apoptotic cells as Syto16low/7AAD- and viable cells as Syto16bright/7AAD- (162).

Ronzoni et al. published a way to distinguish resting CECs from active CECs using flow cytometry: the first are defined as CD45-CD146+, CD34+, and CD106-, whereas active cells express CD45-, CD146+, CD34+, and CD106+. Total CEC are CD45-, CD146+, CD34+ and CD133- (130).

CEPs are principally defined by flow cytometry and/or tissue culture (120); however, as it happens for CECs, there is still no consent on their antigen-expression profile (102) (Table 1 and Figure 9).

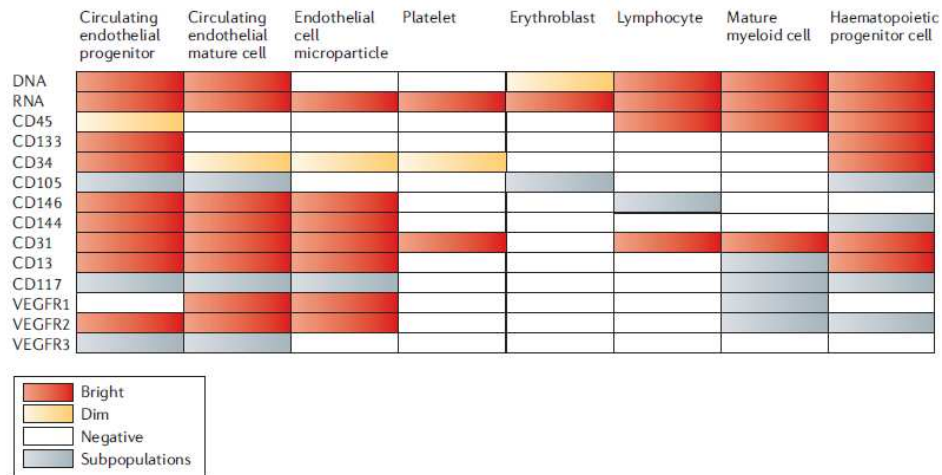


Figure 9. Phenotype of CEC, CEP and other cell populations. The majority of the antigens were detected by flow cytometry; however, some of them were also confirmed using other techniques, such as immunohistochemistry and molecular biology. With “subpopulations” the authors intend that the antigen is only expressed in a fraction of the cell population (102).

Furthermore, real time-PCR has been considered for measurement of circulating RNA levels of CEPs, and was found to be an easy and reliable methodology. Through this technique for assessment of VE-cadherin RNA, a decrease of its levels was detected in apoptotic compared to viable cells; CEC viability seems thus to correlate with VE-cadherin RNA levels (101). This technique is still not as useful as FACS since it can not distinguish patients with a prevalent angiogenesis, mainly driven by mature endothelial cells, from those with a prevalent vasculogenesis, basically due to involvement of endothelial progenitor cells (101).

In conclusion, all available tools for detection and quantification of CECs and CEPs provide complementary information which may give the overall view of involvement and fluctuations of these cells during tumor angiogenesis and vasculogenesis. Nowadays, the diversity due to the use of different techniques for measurement of CECs and CEPs has decreased, showing overlapping results with different methods (163); in breast cancer (127, 164), for example, CEP and CEC counts obtained by CellSearch system are in line with those got by flow cytometry. Moreover, rather than phenotype only, it is recommended to use the function as well, to distinguish CECs, CEPs and other more specific subpopulations involved in the complex tumor angiogenic process (102). The overlapping information obtained using diverse techniques and the multifaceted analysis of these molecular and cellular markers can help in planning new therapeutic approach and test innovative targeted therapies.

1.4.5 Circulating endothelial cells and progenitors in tumors

In cancer patients, elevated CEC levels, probably due to endothelial perturbation were detected (124), but the implication of this increase is still unknown. Although standardized enumeration of CEC counts is still required to minimize variability and allow cross-studies comparisons, the most common variability is not relative to the methodology used to detect CEPs and CECs, but rather to values obtained when analysing different types of cancer (161).

In haematological patients, high levels of CECs have been recorded in acute and chronic leukemias, myelodysplastic syndromes, lymphoma and myeloma (116, 165-168); these levels decrease markedly after treatment with chemotherapy, but they do not normalize, remaining higher than in healthy controls. A possible explanation for these findings is that chemotherapy-induced endothelial damage makes CEC levels remain higher than in controls despite the relative decrease (169). The study carried out by Kideryova highlighted that levels of endothelial progenitor cells are lower in haematological patients than healthy controls, maybe due to the influence that comorbidities and medications could have on this population (169).

Although the possible association between CEC or CEP levels and outcome in patients suffering from tumors is very attractive, researchers are nowadays aware that the predictive value of the baseline counts of these populations could vary depending on cancer type and patient subset, thus reflecting tumor-specific endothelium activation. In contrast with results in breast cancer (125), indeed, patients with colorectal cancer and lower baseline CEC levels have longer PFS (130). The same inverse correlation between baseline CEC count and OS was reported for metastatic pancreatic cancer patients treated with erlotinib and bevacizumab by Ko et al (170). The possible reasons for these differences might be related to a different vascular turnover among cancer types.

Matusaka and colleagues published data on metastatic colorectal cancer patients treated with bevacizumab-based chemotherapy; the purpose of their study was to identify the threshold of CEC count,

measured by CellSearch system, necessary to claim a response to treatment (161). They found that CEC baseline levels were associated with outcome: higher levels correlated with a shorter median PFS and OS. Moreover, the threshold between high and low baseline CEC amounts in peripheral blood was 65 cells/4 ml. Ronzoni et al. confirmed that lower CEC levels are positive predictive factors for clinical outcomes in advanced colorectal cancer (130).

In renal cell carcinoma patients, treatment with a tyrosine kinase inhibitor, called sunitinib, targeting VEGFR1-3, PDGFR and leading to inhibition of tumor vessel formation, correlated with a further CEC increase in subjects with a PFS above the median as an early biological response with a positive outcome. In patients with a PFS below the median, CEC increase after treatment was not recorded. In this setting CEC increase could thus be considered as a marker for clinical activity (163).

Data regarding breast tumor are not so clear; CEC baseline levels were not predictive for response to metronomic chemotherapy (126), but a higher baseline count was associated with a higher PFS after combination of metronomic chemotherapy and bevacizumab (130). Furthermore, apoptotic CECs are candidate predictive marker of clinical outcome after metronomic therapy in breast cancers (171).

In conclusion, although the use of CECs as a diagnostic and predictive marker in cardiovascular disease has led to the analysis of these cells in different cancer types, CECs are probably more useful as a biologic marker of tumor vascular status and possible response to

antiangiogenic therapy, than as a long-term clinical prognostic marker (172).

In any case, a positive or a negative correlation between CEC and CEP counts could be hypothesised according to two different points of view. In the presence of endothelium damage, CEC and CEP counts should both increase, respectively due to their shedding from the vessel walls and to their recruitment from the bone marrow intended for repairing the damage. On the other hand, persisting vascular damages could also be the result of a CEP failure in repairing them; in this case a decrease in CEP count and a concurrent increase of CEC number should be expected.

1.4.6 CD140b-pericyte progenitor cells

Endothelial cells express several different surface molecules involved in a variety of vascular functions. Apart from endothelial cells, also pericyte cells play a relevant role in the generation and homeostasis of blood vessels. While endothelial cells form the inner lining of the vessel wall, pericytes wrap around the whole blood vessel and are responsible for its stabilization and hemodynamic processes (173-175).

Evidence of a tight crosstalk between VEGF and members of other signaling pathways, like PDGF (162), are reported in literature. As an example, CD140 absence *in vivo* is associated with vascular leakage and hemorrhages (110); moreover, CD140b+ viable cells decrease after high dose chemotherapy. Furthermore, *in vivo* failure of the interactions

between endothelial cells and pericytes results in severe and even lethal cardiovascular defects, whereas abnormalities in their interactions are implicated in many human pathologies, such as tumors, diabetes and stroke (176).

In normal conditions, to stabilize and mature new vessels, endothelial cells recruit pericyte cells via the expression of the platelet-derived growth factor receptor beta (PDGFR beta or CD140b) (173-175, 177). When neoplastic angiogenesis occurs, pericytes are reduced and show abnormalities in their contact with the surrounding endothelial cells (178, 179); hence, tumor vessels are heterogeneous in their pericyte coverage (176).

Since pericytes seem to be spared by antiangiogenic therapies (180), the idea of a combination of antiangiogenic and antipericyte drugs, which can act synergistically, has been proposed. Bergers et al., for example, tested this concept in an in vivo model of pancreatic islet tumor, recording complementary and synergistic antiangiogenic and antitumor effects (181). Additional studies reported that PDGF inhibition decreases the interstitial tumor pressure and, thereby, enhances effects of chemotherapy (182, 183).

Mancuso et al. in 2010 published that number and viability of CD140b+ progenitor perivascular cells (PPCs) are always higher in cancer patients than in healthy controls (162). In addition, a decrease of these parameters is always detectable in patients after treatment with chemotherapy; two are the possible reasons for this. PDGFRb+ PPCs could be reduced due to the block of recruitment from the tissue reservoir

or because of the inclusion of PPCs within the vessel walls for repairing the vascular damage induced by chemotherapy (162). In any case, PPCs seem worth of deeper investigations, especially for their role in tumor angiogenesis.

In conclusion, data available in the literature indicate potential benefits of targeting pericytes in the treatment of tumors and, as a consequence, in the analysis of their possible role as a marker of antiangiogenic and antipericyte treatment efficacy.

1.5 SCOPE OF THE STUDY

Despite the multimodal treatment strategy for newly diagnosed HGG, including surgical resection of the tumor mass, followed by radiochemotherapy, recurrence is almost universal and overall prognosis is still poor.

Due to the high vascularization of these tumors, antiangiogenic drugs, such as bevacizumab, are being tested in clinics. Chapter II reports our findings on efficacy and safety of this drug as treatment for patients with recurrent HGG and no other therapeutic option.

Effectiveness of the experimental antiangiogenic therapies is however jeopardized by the lack of markers for the selection of patients who are likely to benefit from treatment. The scope of this study was to analyse circulating endothelial cells and progenitors, as well as well-known clinical and radiological parameters, to identify markers facilitating the stratification of patients and allowing treatment in a more selected subpopulation.

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2. CHAPTER II - PAPER

High levels of CD109+ circulating endothelial cells and progenitors at baseline are associated to longer survival in recurrent high grade gliomas treated with bevacizumab and irinotecan.

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2.1 ABSTRACT

Purpose: Bevacizumab, an anti-VEGF antibody, has shown significant activity in high grade gliomas (HGG). Previous studies emphasized the need for predictive markers of response.

Experimental Design: We treated 63 recurrent HGG patients with poor prognostic factors with bevacizumab (10 mg/kg) and irinotecan (125 or 340 mg/m²) every 2 weeks, and investigated the predictive potential of circulating endothelial cells (CECs) and their progenitors (CEPs).

Results: After a median follow-up of 27 weeks, median OS and PFS were 33 and 18 weeks, respectively. PFS at 6 and 12 months were 32% and 12%. OS at 6 months was 60%. No complete response but fourteen partial responses according to RANO criteria were observed. Toxicity and side effects were mild.

Patients with distant intracerebral disease or leptomeningeal dissemination at baseline MRI had shorter PFS (p=0.002; p=0.01) and OS (p=0.005; p=0.03).

Baseline CEP over 32.8 cells/ml (1st quartile) or CD45dimCD34+CD133+ hematopoietic committed progenitors over 27 cells/ml (1st quartile) were associated with an increased PFS (p=0.01; p=0.001, respectively). Baseline CD109+ CECs over 47.5 cells/ml (2nd quartile) were associated with longer PFS and OS (p= 0.001; p=0.02). Patients who progressed after 18 weeks of therapy or more (n=22) had

baseline levels of CD109+ CECs and CD45dimCD34+VEGFR2+ cells significantly higher than others ($p=0.008$; $p=0.04$, respectively). At progression no significant change in CEC and CEP was detected.

Conclusions: The data point to baseline CD109+ CECs, CEP and CD45dimCD34+CD133+ hematopoietic committed progenitors as promising markers for the selection of patients who could benefit from bevacizumab in the treatment of HGG.

2.2 INTRODUCTION

Accounting for 35-40% of all primary brain tumor, high grade gliomas (HGG), including grade III gliomas and glioblastoma multiforme (GBM), are the most frequent malignant brain tumor (1). Although local invasion is the hallmark of malignant gliomas at recurrence, dissemination or second distant lesions can also occur intracerebrally; the prognostic significance of these different radiological patterns is not well established yet (2).

Since HGG are highly vascularized tumor, several antiangiogenic compounds have been investigated so far in the clinical setting. Bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor (VEGF), represents one of the front-runners among currently available antiangiogenic drugs. However, despite the significant number of trials based on treatment with bevacizumab in HGG (3), predictive markers to distinguish patients who are likely to benefit from treatment are still lacking.

Circulating endothelial cells (CECs) and progenitors (CEPs) seem promising predictive and escape biomarkers (4, 5). CECs, rare and mostly apoptotic/necrotic in healthy individuals (<1/100 circulating blood cells (6), increase in a variety of vascular disorders and tumor, and are considered to be shed from vessel walls and enter the blood stream as a consequence of vascular turnover or damage. They appear to correlate with tumor progression (7, 8) and tend to normalize at complete remission

in other solid tumor (5, 9-11). CEPs are mobilized from the bone marrow following tissue ischemia to complement local angiogenesis supplied by the existing endothelium; a similar process takes place during tumor vasculogenesis (12, 13). In particular, levels of CEPs, defined as CD34+CD133+ VEGFR2+ cells, are higher in HGG patients than in healthy controls and metastatic patients, and correlated with higher tumor blood vessel densities in the GBM subgroup (14).

One relevant limit in using CECs and CEPs as biomarkers to predict benefit from bevacizumab is the lack of consensus about their phenotype. CECs are usually defined as DNA+CD45-CD31+CD146+ cells (15), whereas CEPs as DNA+CD45-CD34+ (16). Several subpopulations of these vessel-lining endothelial cells with different antigenic profiles may have a predictive clinical potential. CD109 has been proposed as a tumor-specific endothelial cell antigen (17). CEP subpopulations might express VEGFR-2 or CD133, two antigens also expressed by endothelial and progenitor cells, respectively. Finally, progenitor perivascular cells (PPCs) expressing CD140b+ (the platelet-derived growth factor receptor beta, PDGRFbeta) may play a role in tumor angiogenesis as they regulate vessel stability (18). Indeed, there is evidence of a tight crosstalk between VEGF and members of other signaling pathways, like PDGF (19): *in vivo* the absence of CD140b is associated with vascular leakage and hemorrhages and CD140b+ viable cells decrease after high dose chemotherapy (18).

Here, we report on clinical and radiological outcome of recurrent HGG patients treated with bevacizumab and irinotecan, and on the potential predictive value of CEC, CEP and PPC counts in these patients.

2.3 PATIENTS AND METHODS

2.3.1 Patient selection

Subjects involved in the therapeutic protocol were adult patients (≥ 18 years) with radiologically proven progression of grade III (n=10) or grade IV (n=53) gliomas (1) and Karnofsky Performance Score (KPS) ≥ 40 (Table 1). All patients signed an informed consent. The therapeutic protocol was carried out according to the Italian Decree Law May 8th, 2003 which allows the off-label use of drugs not approved by the Italian Regulatory Agency when the patient has no other therapeutic options. Before treatment with bevacizumab and irinotecan, GBM patients underwent prior surgical resection and radiotherapy with concurrent temozolomide (TMZ) according to the Stupp's protocol (20), followed by second line chemotherapy in 22 patients and third line chemotherapy in 2. Grade III patients were submitted to prior surgery followed by radiotherapy; subsequently eight were treated with PCV (procarbazine, lomustine and vincristine) or TMZ, and five received a second line chemotherapy at progression.

Treatment was not possible in the presence of: unstable angina; myocardial infarction within 6 months; clinically significant peripheral

arteriopathy; hemorrhagic foci; presence of deep venous thrombosis and/or pulmonary embolism within 3 months; history of abdominal fistula, gastrointestinal perforation, or intra-abdominal abscess within 6 months; chronic intestinal active inflammatory disease; serious non-healing wound, active ulcer or untreated bone fracture; pharmacologically uncontrolled hypertension; any previous malignancy, other than basal spino-cellular carcinoma of the skin and carcinoma in situ of the cervix; previous treatment with bevacizumab or irinotecan; serious unstable systemic disease, including active infections or serious cardiac arrhythmia; pregnancy or lactating.

The therapeutic protocol was approved by the Ethical Committee of the Neurological Institute “Carlo Besta” of Milan.

2.3.2 Drug administration

Bevacizumab and irinotecan were supplied by Roche S.p.A. (Monza, Italy) and Hospira (Napoli, Italy), respectively.

Irinotecan was administered at a dose of 125 or 340 mg/m², depending on the concomitant use of enzyme-inducing anti-epileptic drugs (EIAED), according to Vredenburgh et al. (21). Bevacizumab was dosed at 10 mg/kg. Each agent was administered i.v., every 2 weeks until intolerable toxicity, tumor progression, or patient consent withdrawal. If patients developed low tolerance to irinotecan, they continued the treatment with bevacizumab alone.

2.3.3 Patient evaluation

Patients were clinically and neurologically evaluated before initiating therapy and in correspondence of bevacizumab and irinotecan administrations; the visits included blood count and urine analysis. MGMT methylation was assessed as previously described (22). If necessary, an ophthalmological visit and a computerized axial tomography scan were performed to exclude the onset of optical neuropathies and bleedings.

Toxicities were evaluated at each visit and graded according to the NCI CTC-AE, version 3.0. In case of intolerable toxicity or disease progression, patients were treated according to the Hospital normal clinical practice.

Patient underwent conventional contrast enhanced MRI soon before starting treatment, every 8 weeks and in case of neurological worsening, until tumor progression. Patients were scanned on a 1.5T MR system (Siemens, Avanto) with an 8 channels head coil. MRI sequences included axial T1 weighted spin-echo (TE/TR=9.1 ms/500 ms, FA=70°, slice thickness=5 mm, no gap, matrix =187x256, FOV=230x187 mm, number of NEX=2), axial turbo spin-echo T2 and proton density weighted (TE/TR=39-79 ms/3500 ms, FA=180°, slice thickness=5 mm, no gap, matrix=256x256, FOV=240x240 mm, NEX=1), coronal FLAIR (TI=2500 ms, TE/TR=121 ms/8000 ms, FA=150°, slice thickness=5 mm, no gap, matrix=149x320, FOV=250x194 mm, NEX=1). After the administration of contrast medium (Gadovist, 0.1 mmol/kg) axial and 3D T1 weighted images (TE/TR=4.24 ms/1160 ms; FA=15°, voxel size 0.90x0.90x0.90,

gap 0,45 mm, matrix=192x256 and FOV=230x172.5 mm, NEX=1) were acquired.

2.3.4 MRI and response evaluation

Response to the experimental treatment was determined by clinical and radiological examinations. The following radiological features were considered: width of the enhancing tumor (on the volumetric enhanced T1 weighted. sequence), pattern of contrast enhancement (ring-like, nodular, patchy and faint), extension and pattern of long-TR signal alteration (infiltration, lack of homogeneity), presence of edema, mass effect, leptomenigeal and/or leptomenigeal seeding, multifocality in the controlateral hemisphere or in the same hemisphere but far from the primary lesion.

Patterns of radiologically defined disease were characterized as local, distant, diffuse and multifocal, using the radiographic classification published by Chamberlain (23). However, because in our cohort patients with 3 or more non contiguous lesions were not observed, differently from Chamberlain we adopted the definition of leptomenigeal dissemination instead of multifocal disease.

Therapy response assessment was performed independently from the neurologists' clinical examinations, according to the RANO criteria (24)(10). Tumor volume measurements were determined on the 3D post gadolinium T1 weighted images by manually outlining the enhancing portion of the lesion in MRIcro (<http://www.mricro.com>). The number of

enhancing voxels was multiplied by the voxel volume in order to obtain the total enhancing volume of the tumor.

2.3.5 Circulating endothelial cells and progenitors analysis

Number and viability of CECs and CEPs were measured on day 0 and every 8 weeks by six-color flow cytometry. In brief, viable and apoptotic CECs were defined as Syto16(DNA)+CD45-CD31+CD146+ (15), and the combination of Syto16 and 7-AAD was used to discriminate between nucleated viable (Syto16bright/7-AAD-) and apoptotic/necrotic (Syto16dim/7-AAD+) endothelial cells, and to exclude from analysis platelets and endothelial macroparticles (Figure 1). The expression of CD109 (25) in CECs was also investigated in combination with Syto16 and 7-AAD.

According to Mead L.E. et al., CEPs were evaluated as Syto16(DNA)+CD45-CD34+ (16). However, as a consensus on the phenotype of these cells has not been achieved, we also investigated the kinetic of Syto16(DNA)+CD45dimCD34+VEGFR2+ described as VEGFR2+ hematopoietic progenitor cells according to Case et al. (26), as well as Syto16(DNA)+CD45dimCD34+ and Syto16(DNA)+CD45dimCD34+CD133+ as hematopoietic committed progenitors (5, 16, 26, 27) (Figure 2).

As PDGFRbeta(CD140b)+ progenitor perivascular cells (PPCs) can differentiate into pericytes and regulate vessel stability and vascular survival in tumor, Syto16(DNA)+CD45-CD31-CD140b+ PPCs were also

enumerated (18). To define reference values, CECs and viable CECs were investigated also in 72 age- and sex-matched healthy controls (age range 30-50 years), whereas the remaining cell populations were evaluated in 36 healthy subjects.

2.3.6 Statistical analysis

Progression Free Survival (PFS) was calculated from the start of the experimental treatment until disease progression and death/last follow-up, if censored. Overall Survival (OS) was calculated from the start of the experimental treatment until death/last follow-up, if censored. Patients who interrupted the therapeutic protocol because of intolerable toxicity, consent withdrawal or other reasons were followed for progression and death and were included in PFS and OS analyses. The Kaplan Meier method was used to estimate survival functions. The log rank test was used to test for differences in progression or survival between patients with different clinical, radiological or biological parameters. Clinical (age ≤ 40 years; age ≤ 60 years; KPS ≤ 70 ; KPS ≤ 80 ; EIAED use; glioma grade; dexamethasone use at enrolment, among 0, < 4 mg/die, < 8 mg/die) and radiological parameters (tumor volume at enrolment, cut off values) as well as biological parameters (CECs and CEPs) were set at 25^o, 50^o, 75^o, 90^o percentile and separately evaluated in all patients and in the GBM subgroup.

Cox proportional –hazards regression was used to determine univariate and multivariate hazard ratios for potential predictors of PFS

and OS. Correlation between biological markers and clinical parameters or treatment response was assessed using Wilcoxon-Mann-Whitney test. A Wilcoxon rank sum test was used to evaluate differences between biological markers levels at baseline and at week 8 or progression. A multivariate analysis and a Cox proportional hazard regression model analysis were performed on variables showing statistically significant differences at univariate analysis to investigate their independent prognostic role.

All statistical analyses were performed using Statview software. All tests were two-sided.

2.4 RESULTS

2.4.1 Patients

Clinical characteristics of HGG patients treated between January 2009 and December 2010 and included in this study are described in Table 1. Of note, since treatment was administered according to a therapeutic protocol (see Patients and Methods for definition), many patients had poor prognostic factors (age >60 years in 19% of patients, KPS <70 in 25%, leptomeningeal disease dissemination in 19%). None of the patients was previously treated with bevacizumab or other anti-angiogenic drugs. Six patients were treated with bevacizumab alone, whereas 9 patients interrupted irinotecan due to low tolerance.

Thirty patients of 63 (48%) had more than one progression before treatment with bevacizumab/irinotecan. Sixteen of 53 GBM patients (30%) experienced progression during the first six cycles of conventional adjuvant TMZ therapy (28) . Two patients progressed <12 weeks after radiation therapy and performed a second MRI confirming progression after 6 weeks, according to RANO criteria (24); no patient had pseudoprogression.

2.4.2 Toxicity

Adverse events are summarized in Table 2. Four patients interrupted the treatment before radiological assessment of disease progression due to consent withdrawal (n=2) or intratumoral bleeding (n=2). Dates of their disease progression and death after therapy interruption were included in statistical analysis.

Four patients died before neurological and radiologically-assessed disease progression due to: pancreatic neoplasia (n=1); sudden death for unknown reason (n=1); epigastric discomfort, nausea, vomit, anorexia (n=1); acute heart failure (n=1). Their date of death was included in both OS and PFS statistical evaluations.

2.4.3 Response Rate and MRI patterns of relapse

No complete response was observed. Fourteen patients had a partial response according to RANO criteria (24); in most of them (13 patients) the partial response was recorded by week 8.

Within the first 8 weeks of treatment, 33 patients had a stable disease and 17 a disease progression.

Before starting treatment, 63.5% (40/63) of patients had local disease, as defined by Chamberlain (23). No patient with multifocal disease was observed; leptomeningeal dissemination was present in 19% of cases and distant disease in 16%; in one patient (grade III) a diffuse pattern of disease was detected at baseline (Table 1).

At progression, 32% of the radiographically assessable patients converted to a diffuse pattern (8 patients starting from local pattern, 4 from leptomeningeal dissemination and one from distant pattern), 7% to leptomeningeal dissemination (2 patients with local disease and one with distant pattern at baseline) and 62% did not show changes of their disease pattern with respect to baseline. An example of change of the radiological pattern from distant disease to diffuse disease is reported in Figure 3. Finally, survival did not show differences associated to the radiographic patterns of disease recurrence.

2.4.4 Survival

Overall, median follow up was 27 weeks (5-107). Eleven of 63 patients are still alive; four patients died for non-neurological causes before neurological and radiologically-assessed disease progression; the remaining 48 for tumor progression. Median OS was 33 weeks (5-107) overall; it was higher in the GBM subgroup (36 weeks, 8-96) than in grade III gliomas (25 weeks, 7-65). OS at 6 and 12 months (OS-6; OS-12) were 60% (95% CI 48-72%) and 25.5% (95% CI 14-37%), respectively; in GBM they were 64% (95% CI 51-71%) and 25% (95% CI 12-38%) respectively, whereas in grade III gliomas 40% (95% CI 9.6-70%) and 26% (95% CI -2.7-56%).

Median PFS was 18 weeks (5-78) overall; 18 weeks (5-78) in GBM and 14 weeks (5-53) in grade III glioma patients. Overall PFS at 6 and 12 months (PFS-6; PFS-12) were 32% (95% CI 20-43%) and 12% (95% CI

1.9-18%), respectively. PFS-6 was 34% (95% CI 21-47%) in GBM and 20% (95% CI 4.8-44%) in grade III gliomas.

PFS and OS were not affected by the following clinical parameters: age>40 years; age>60 years; KPS>70; KPS>80; early progression during adjuvant TMZ according to the Stupp's protocol (20); *de novo* versus secondary GBM (Table 3).

Tumor volume ≤ 13.1 cc (25^o percentile) at the time of first administration of bevacizumab was associated with PFS and OS longer than in the remaining patients, but this was not statistically significant (Table 3). Although tumor volumes in patients with leptomeningeal disease dissemination and multifocal tumor (median volume 34.4 and 36 cc, respectively) were not significantly higher than in the remaining subjects, both subgroups had shorter PFS (p=0.002 and 0.01, respectively) and OS (p=0.005 and 0.03, respectively; supplementary data). No difference in PFS or OS was detected in the EIAED group versus the non-EIAED group, neither in patients who discontinued irinotecan versus those who received both bevacizumab and irinotecan, and in patients who developed hypertension versus those who did not. MGMT promoter methylation status did not influence PFS and OS.

2.4.5 Biomarker results

2.4.5.1 *Baseline values*

Figure 4 reports the baseline levels of CECs, CEPs and other cell populations in patients and healthy controls. Significantly higher levels of CD109+ CECs ($p=0.0001$), CEPs ($p=0.0001$), CD45dimCD34+CD133+ and CD45dimCD34+ hematopoietic committed progenitors ($p=0.0001$ and 0.008 , respectively) were found in patients compared to healthy controls, also when p values were adjusted for multiple comparisons. A slight increase of viable CECs and decrease of CD140b+ PPCs was also observed in patients compared to controls. GBM and grade III glioma patients did not show significant differences in their CEC and CEP values at baseline (data not shown). Furthermore, no correlation was observed between CEC and CEP values and clinical parameters, such as age, presence of multifocal disease or leptomeningeal dissemination, steroid dosage and time of progression before treatment with bevacizumab and irinotecan, using stratification criteria proposed by Perry et al (28).

2.4.5.2 *Dynamic analysis*

CEC and CEP subpopulations were assessed at baseline and after 2 months from the treatment onset by serial analysis (Figure 5). In all patients available for this analysis ($n=53$), levels of CD109+ CECs, CD140b+ PPCs and CD45dimCD34+VEGFR2+ hematopoietic progenitor cells significantly decreased after 2 months ($p=0.02$, $p=0.001$, $p=0.004$, respectively), whereas those of CD45dimCD34+ hematopoietic

committed progenitors increased (Figure 5, panels A and B). Patients who progressed at 2 months according to the RANO criteria (non-responders, n=15) were compared to those who did not (responders, n=38) (Figure 5, panels C and D). Interestingly, CD109+ CEC counts decreased significantly after treatment in responders only (p=0.008). Also variations of CD45dimCD34+CD133+ and CD45dimCD34+ hematopoietic committed progenitors counts were statistically significant for responders only (p=0.02 in both cases). CD140b+ PPCs and CD45dimCD34+VEGFR2+ hematopoietic progenitors significantly decreased in both responders and non responders.

2.4.5.3 CECs, CEPs and disease progression

Baseline CD109+ CEC count higher than 47.5/ml (2nd quartile) was significantly associated with increased PFS (19 versus 10 weeks, p=0.001; Figure 6A) and OS (36 versus 24 weeks, p=0.02; Figure 6B). CD45dimCD34+CD133+ hematopoietic committed progenitors higher than 27/ml (1st quartile) and CEP baseline values higher than 32.8/ml (1st quartile) were significantly correlated with an increased PFS (18 versus 9 weeks, p=0.001 and p=0.01 respectively; Figures 6C and D). In a cohort of 22 long-term responders, who progressed after at least 18 weeks of treatment, levels of CD109+ CECs were significantly increased at baseline (Table 4) and reduced after 2 months of therapy (Table 5). In the same cohort CD140b+ PPCs were significantly higher at baseline than at follow-up (Table 5). CEC and CEP values detected at the time of

progression in the 38 patients investigated so far were not significantly different from those collected at the previous assessment.

We performed a multivariate analysis with the use of the Cox proportional-hazards model of all biological (CEPs, CD109+ CECs and CD45dimCD34+CD133+ hematopoietic progenitor cells) and radiological parameters which were found to significantly affect PFS or OS at the univariate analysis. The multivariate analysis showed that PFS was negatively affected by distant disease pattern at baseline MRI ($p=0.03$, RR 2.5, 95% CI 1.05 – 6.03) and positively affected by CEP numbers $>32.8/\text{ml}$ ($p=0.004$, RR 3.76, 95% CI 1.24– 11.4). Distant disease at baseline also affected OS ($p=0.01$, RR 2.9, 95% CI 1.2 – 7.1).

2.5 DISCUSSION

Our data on treatment of patients with recurrent grade III and IV gliomas with bevacizumab and irinotecan confirmed that the combination is quite safe and effective in this setting. Median and 6-month PFS and OS were slightly lower than those reported in recent meta-analysis of 548 patients (3); this is likely due to the different inclusion criteria adopted for enrolment. The patients we considered had lower median KPS and were characterized by poor prognostic factors. Our clinical results in terms of OS are similar to those reached by Desjardins and colleagues who treated seriously impaired recurrent GBM patients with bevacizumab and metronomic TMZ (29). Our GBM patients however reached higher PFS-6 and median PFS, maybe because of the exclusion of cases previously treated with bevacizumab. Recurrent GBM patients treated with continuous dose-dense TMZ, acting as an anti-angiogenic drug, showed PFS similar to ours if treated at late relapse, and shorter in case of early relapse (28). Compared to the historical data by Wong et al, who combined 375 HGG patients from eight phase II trials (30), we observed a doubling of PFS-6 in GBM and similar and higher median PFS and OS, respectively. Interestingly, the experimental treatment seems more effective in GBM patients than in grade III glioma patients, as median OS and PFS were greater in the first subgroup. Similar findings were already observed in patients treated with bevacizumab (21, 31, 32), suggesting

that neoangiogenesis and susceptibility to the drug could differ according to the glioma subtype.

Safety data obtained in our population are comparable to those reported in literature so far. Hypertension and proteinuria in our patients were common, but not associated with longer PFS and OS. Gastrointestinal events, quite frequent in our patient population, were mostly attributable to irinotecan. We did not observe a difference in terms of PFS and OS between patients treated with bevacizumab and irinotecan and those for whom irinotecan was interrupted, in good agreement with previous observations by Friedman et al. (33).

Although not yet validated, the RANO radiological criteria were used for assessment of disease response to treatment (24), as they are better suited for the study of effects of antiangiogenic factors. A decrease of the gadolinium enhanced lesion at MRI scans is indeed frequent after bevacizumab administration, but could be due to vessel normalization, rather than reduction of the tumor mass (34, 35).

The pattern of recurrence observed in our patients is somehow different from those previously reported; at baseline local disease was radiologically detected in 63.5% of our patients, instead of 80% and 72% reported by Chamberlain and Pope, respectively (23, 36), whereas the leptomeningeal dissemination was present in 19% (6.25% in Chamberlain et al). Interestingly, 66% of our patients with baseline local disease showed local progression, similarly to what reported by Chamberlain and Dejardins (23, 29) and 50% of patients with baseline leptomeningeal disease developed a diffuse pattern at recurrence after bevacizumab.

Although bevacizumab has been used in combination with irinotecan as a valid treatment with manageable toxicities for recurrent HGG (33), optimization of the treatment strategy could lead to reduce costs to the health system, avoiding serious adverse events in patients that would not benefit from treatment and addressing them to alternative treatments. Hence, it is of foremost relevance to identify markers of drug activity or failure in order to select and treat only patients who are likely to benefit from the antiangiogenic therapy.

Well-known clinical parameters, such as age, KPS, etc, were not predictive for response to treatment in our cohort. However, our findings suggest that patients with a diffuse disease or leptomeningeal dissemination are less responsive to antiangiogenic therapy. The leptomeningeal dissemination has not been previously mentioned as a prognostic factor for patients treated with bevacizumab (29, 37, 38) and previous studies on these patients did not show a correlation between baseline radiological patterns of disease and PFS or OS.

Among biological parameters recently entering the clinical arena (39), circulating biomarkers are particularly appealing because they are minimally invasive, highly repeatable and dynamic. As reported in other solid tumor (8, 15) and confirmed in malignant gliomas by Rafat et al. (14), viable CECs, CEPs and hematopoietic progenitors populations at baseline were higher in HGG patients in comparison to healthy controls, probably as a consequence of tumor hypoxia inducing neovascularization.

The baseline count of CD109+ CECs $\geq 47.2/\text{ml}$ identifies a subgroup of patients with longer PFS and OS; moreover, baseline amounts of

CD45dimCD34+CD133+ hematopoietic committed progenitors and CEPs higher than 27/ml and 32.8/ml, respectively, characterize patients with longer PFS. In agreement with this, in 22 long-term responder patients, baseline CD109+ CECs and CD45dimCD34+VEGFR2+ hematopoietic progenitors were significantly higher than in the remaining patients ($p=0.008$ and 0.04 , respectively; supplementary data).

CD109 is a glycosylphosphatidylinositol-anchored cell surface glycoprotein, which was found highly expressed in several solid tumor (40), but not yet related with response to therapy. Further studies may clarify whether the limited presence of circulating endothelial cells mirrors a tumor status less dependent on neoangiogenesis and, as a consequence, less responsive to antiangiogenic therapy.

In our patients the number of viable PPCs was lower than in healthy controls ($p=0.02$). GBM are indeed characterized by disorganized, large-diameter vessels with diminished pericyte coverage (41). Treatment with bevacizumab and irinotecan further decreased viable PPC counts ($p=0.0001$), maybe due to the block of PPCs recruitment from tissue *reservoirs* or PPCs inclusion in blood vessels to repair the damage induced by chemotherapy (18).

In conclusion, our data indicate that the investigation of baseline counts of CD109+ CECs, CEPs and CD45dimCD34+CD133+ hematopoietic committed progenitors by flow cytometry has potential for the stratification of HGG patients who are most likely to benefit from antiangiogenic treatment. These observations encourage the study of the predictive value of CECs and CEPs on larger number of patients.

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2.7 TABLES

2.7.1 Table 1

Table 1. Patients characteristics at baseline.

| Characteristic | No. of pts | % |
|---------------------------------------------------------------------|------------|--------------------|
| Gender | | |
| Male | 40 | 64 |
| Female | 23 | 36 |
| Age, y | | |
| Median [all pts] (range) | | 51 (15-76) |
| < 40 | 14 | |
| 40-60 | 37 | |
| > 60 | 12 | |
| KPS | | |
| Median [all pts] (range) | | 70 (50-100) |
| < 70 | 16 | |
| 70-80 | 43 | |
| 90-100 | 4 | |
| Histological diagnosis | | |
| De novo GBM | 46 | 73 |
| Secondary GBM | 7 | 11 |
| Grade III astrocytoma | 7 | 11 |
| Grade III oligodendroglioma | 3 | 5 |
| Time from first diagnosis, mos (range) | | 15.5 (4.5-158) |
| GBM (range) | | 14 (4.5-144) |
| Grade III gliomas (range) | | 26.5 (6-158) |
| Disease recurrence | | |
| 1 st /2 nd /3 rd | 33/27/3 | 52/43/5 |
| Prior therapy | | |
| 1 st /2 nd /3 rd surgery | 63/22/1 | 100/35/2 |
| Radiotherapy | 63 | |
| Radiosurgery | 2 | 3 |
| 1 st /2 nd /3 rd line chemotherapy | 63/27/2 | 100/43/3 |
| Systemic therapy | | |
| No Dex/Dex<8mg/Dex≥8mg | 7/27/29 | 11/43/46 |
| EIAED therapy | 10 | 16 |
| Tumor volume, cc (range) | | 26.43 (0.97-173.2) |
| GBM (range) | | 26.57 (0.97-173.2) |

| | | |
|------------------------------|------------------|-------------------|
| Grade III gliomas (range) | | 19.28 (5.96-60.2) |
| MRI patterns at baseline | | |
| Local | 40 (4 grade III) | 63.5 |
| Leptomeningeal dissemination | 12 (2 grade III) | 19 |
| Distant | 10 (3 grade III) | 16 |
| Diffuse | 1 (1 grade III) | 1.5 |

Abbreviations: cc, cubic centimetres; EIAED, enzyme-inducing anti-epileptic drugs; GBM, glioblastoma multiforme; mos, months; MR, magnetic resonance; pts, patients; y, years.

2.7.2 Table 2

| Adverse events. | | |
|-----------------------------------|-------------------|------------------|
| Adverse event | All grades | ≥ grade 3 |
| Asthenia | 27 | |
| Blood arterial hypertension | 21 | 2 |
| Abdominal pain | 19 | |
| Diarrhoea | 18 | |
| Proteinuria | 17 | |
| Vomiting | 15 | 1 |
| Nausea | 10 | 1 |
| Headache | 8 | |
| Seizures | 5 | 1 |
| Leukopenia | 3 | |
| Intralesional bleeding | 3 | |
| Alopecia | 2 | |
| Anemia | 2 | |
| Vertebral fracture | 1 | 1 |
| Anorexia | 1 | 1 |
| Conjunctiva bleeding | 1 | |
| Epigastric discomfort | 1 | 1 |
| Epistaxis | 1 | 1 |
| Herpes infection | 1 | 1 |
| Ischemic heart failure | 1 | 1 |
| Pancreatic neoplasia | 1 | 1 |
| Sudden death | 1 | 1 |
| Suspected cerebral ischemic event | 1 | 1 |

2.7.3 Table 3

| Univariate analysis of the most relevant clinical parameters. | | | | | | |
|----------------------------------------------------------------------|-------------------|----|----------------|------------------|----|----------------|
| | Median PFS | | p value | Median OS | | p value |
| | wks | | | wks | | |
| KPS ≤70 vs >70 | 10 | 19 | n. s. | 33 | 37 | n. s. |
| Age ≤40 yrs vs >40 | 10 | 19 | n. s. | 23 | 36 | n. s. |
| Age ≤60 yrs vs >60 | 17 | 20 | n. s. | 30 | 38 | n. s. |
| De novo vs secondary tumor | 20 | 15 | n. s. | 37 | 27 | n. s. |
| Tumor volume ≤13.1 mm ³ vs >13.1 mm ³ | 39 | 17 | n. s. | 42 | 29 | n. s. |
| Dex treatment vs dex free | 17 | 39 | n. s. | 30 | 67 | n. s. |
| EIAED use vs EIAED free | 39 | 17 | n. s. | 59 | 29 | n. s. |
| Distant disease vs no distant disease | 9 | 19 | 0.01 | 24 | 37 | 0.03 |
| Leptomeningeal diss. vs no leptomeningeal diss. | 10 | 20 | 0.002 | 19 | 37 | 0.005 |
| Bevacizumab+irinotecan vs bevacizumab alone | 17 | 18 | n. s. | 32 | 33 | n. s. |

Abbreviations: dex, dexamethasone; diss., dissemination; EIAED, enzyme-inducing anti-epileptic drugs; wks, weeks; yrs, years.

2.7.4 Table 4

| Baseline levels of CEC, CEP and other cell populations in 53 patients (38 GBM and 9 grade III gliomas) with (n=22) or without (n=31) clinical benefit. | | | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|---------------|----------------|
| | No CB | CB | p value |
| CEC | 83.2 ± 41.8 | 111.2 ± 53.2 | 0.046 |
| Viable CEC | 28.5 ± 24.6 | 29 ± 16.6 | n. s. |
| CD109+ cells | 77.3 ± 6 | 124 ± 86 | 0.008 |
| CD140b+ PPC | 22.1 ± 18.2 | 27.8 ± 27.4 | n. s. |
| CEP | 91.1 ± 86.5 | 102.1 ± 67.1 | n. s. |
| CD45 dim hcp | 177.1 ± 208 | 207.2 ± 188.3 | n. s. |
| CD45dimCD34+VEGFR2+ hp | 38.5 ± 44.3 | 52.6 ± 41 | 0.04 |
| CD45dimCD34+CD133+ hcp | 947.2 ± 1152.6 | 840.5 ± 696.5 | n. s. |

Abbreviations: CB, clinical benefit; CEC, circulating endothelial cells; PPC, progenitor perivascular cells; CEP, circulating endothelial progenitors; VEGFR, vascular endothelial growth factor receptor; hcp, hematopoietic committed progenitors; hp, hematopoietic progenitor cells; n. s., not significant.
Notes: All p values were calculated by Mann-Whitney test.

2.7.5 Table 5

| Levels of CEC, CEP and other cell populations at baseline and 2 months in patients, included in the serial study, who showed clinical benefit (n=22). | | | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------|----------------|
| | Baseline | 2 months | p value |
| CEC | 111.2 ± 53.2 | 96.1 ± 85.8 | n. s. |
| Viable CEC | 29 ± 16.6 | 25.3 ± 21.8 | n. s. |
| CD109+ cells | 124 ± 86 | 66.2 ± 37.6 | 0.01 |
| CD140b+ PPC | 27.8 ± 27.4 | 8.6 ± 12.6 | 0.01 |
| CEP | 102.1 ± 67.1 | 88.8 ± 85.6 | n. s. |
| CD45dim hcp | 207.2 ± 188.3 | 188.8 ± 196.1 | n. s. |
| CD45dimCD34+VEGFR2+ hp | 44.6 ± 40.6 | 28.5 ± 24.4 | n. s. |
| CD45dimCD34+CD133+ hcp | 840.5 ± 696.5 | 1114.6 ± 1007.2 | n. s. |

Abbreviations: CEC, circulating endothelial cells; PPC, progenitor perivascular cells; CEP, circulating endothelial progenitors; VEGFR, vascular endothelial growth factor receptor; hcp, hematopoietic committed progenitors; hp, hematopoietic progenitor cells; n. s., not significant.

Notes: All p values were calculated by Wilcoxon.

2.8 FIGURES

2.8.1 Figure 1

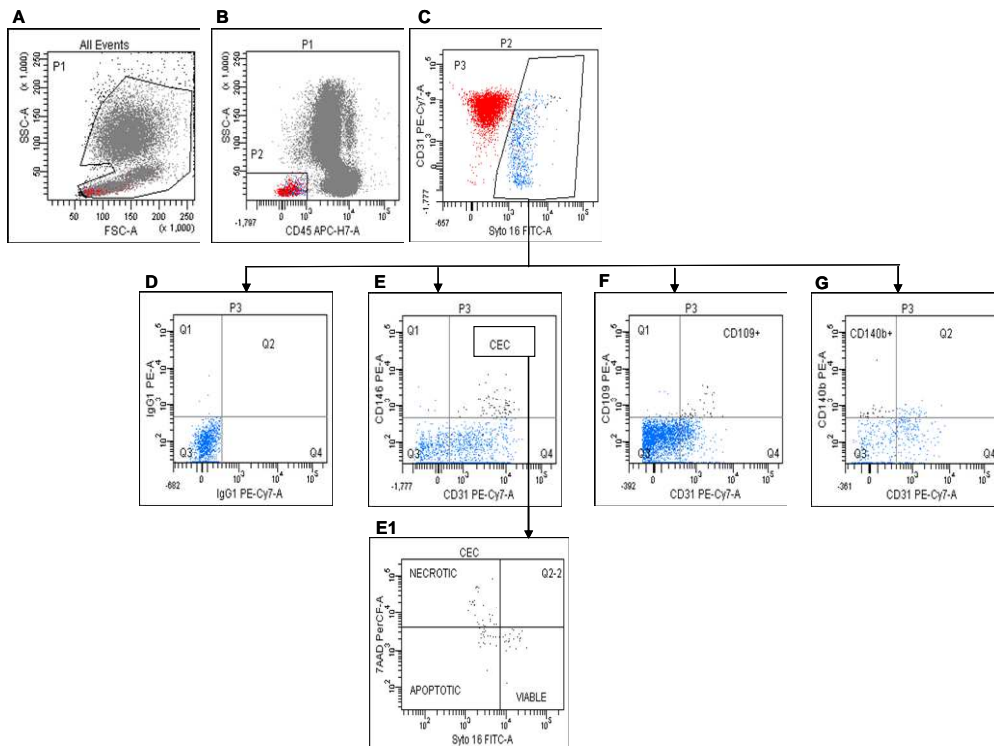


Figure 1. CEC evaluation by flow cytometry. *A* Gate used to exclude cell fragments and debris. *B* Gate made to identify CD45- cells. *C* CD31 expression and Syto16 staining in CD45- cells. *D* Negative control for *E* (CD31+CD146+,CECs), *F* (CD31+CD109+ CECs) and *G* (CD31-CD140b+, PPCs). *E1* Distribution of viable, apoptotic, and necrotic CECs.

2.8.2 Figure 2

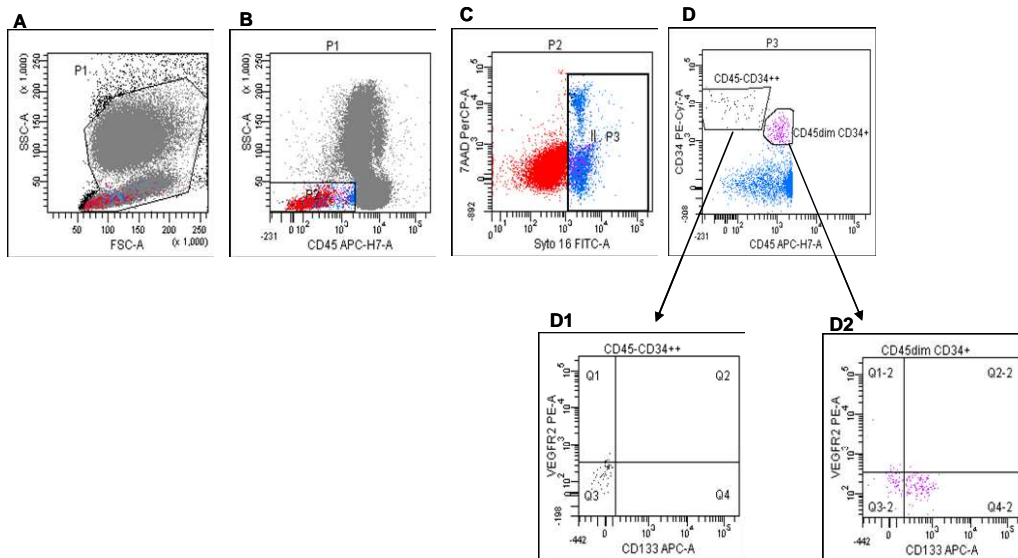


Figure 2. Progenitor cell evaluation by flow cytometry. **A** Gate used to exclude cell fragments and debris. **B** Gate made to include CD45- and CD45dim cells. **C** Gate on Syto16+7AAD+ cells. **D** Identification of 2 different populations: CD45-CD34++ and CD133-VEGFR2- (**D1**), and CD45dimCD34+ and CD133+ cells (**D2**).

2.8.3 Figure 3

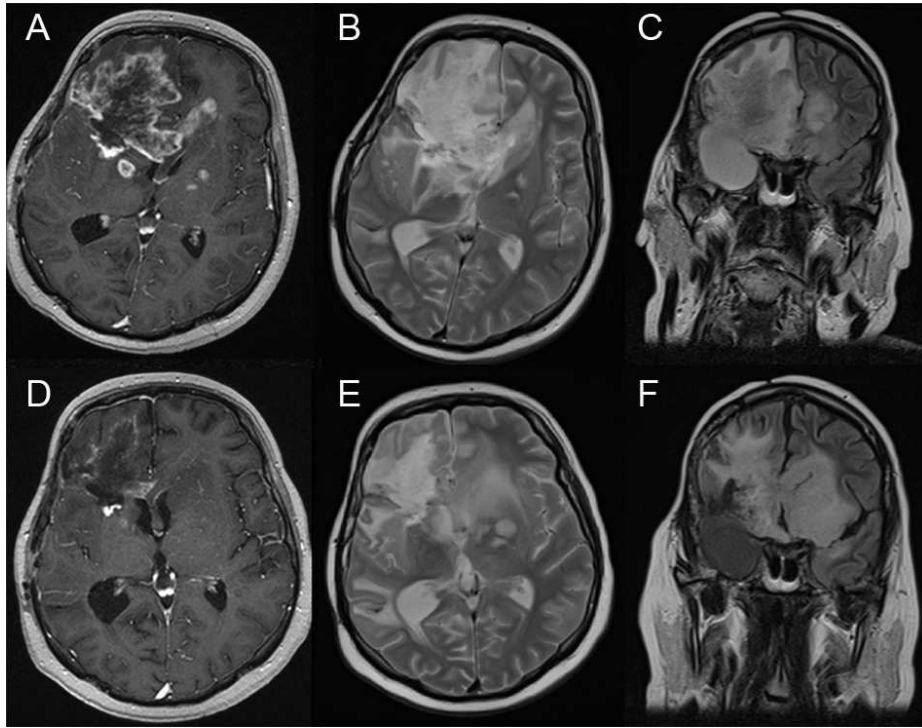


Figure 3. MRI of one patient (A, B, C before treatment. D, E, F two months later). From the left to the right: axial T1-weighted image (T1WI) with contrast injection, axial T2WI and coronal Flair image. A Recurrent GBM with irregular and marked enhancement and cystic-necrotic appearance. Invasion of genu of the corpus callosum is well demonstrated. Small areas of enhancement are visible in the basal ganglia region bilaterally. B and C The corresponding T2 and Flair of inhomogeneous hypersignal. In C the surgical cavity is also visible. D We can see a marked reduction of the enhancement in the left frontal region and corpus callosum, disappearance of enhancement in basal ganglia

*region and lowering of the mass effect. **E and F** Conversely T2 hypersignal is increased and infiltration of controlateral frontal and basal regions is evident.*

2.8.4 Figure 4

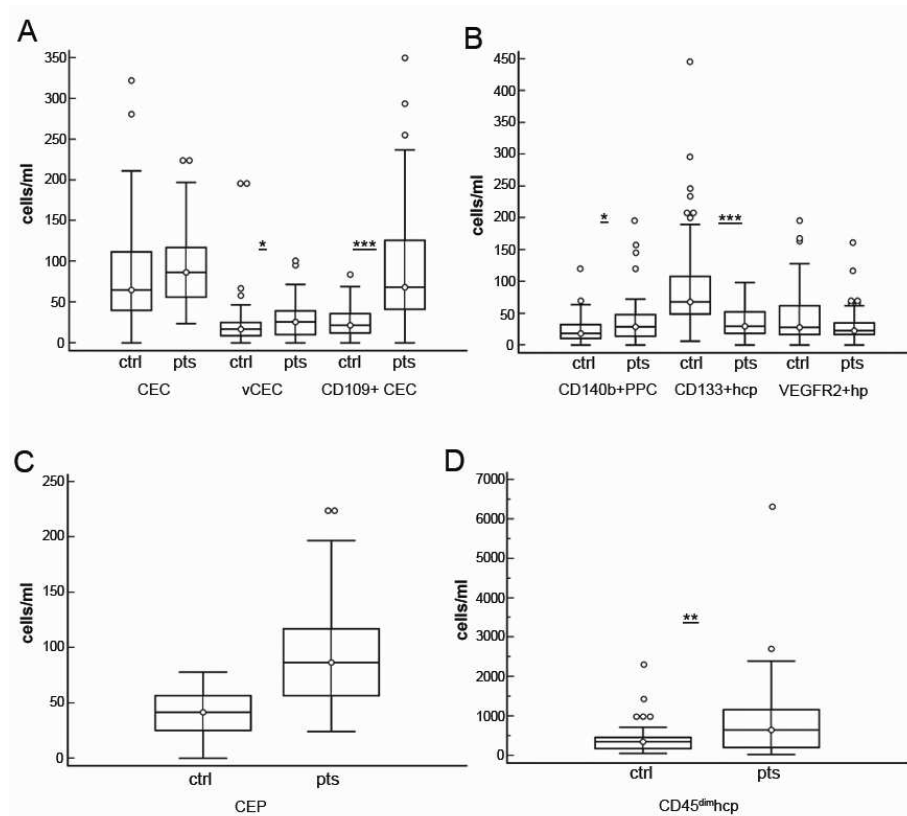


Figure 4. A Baseline levels of CEC, viable CEC and CD109+ CEC in patients and healthy controls. Patients tested were 58. Boxes, the interquartile range; lines, location of first quartile, median, and third quartile. \circ , outliers beyond the standard span. All p values were calculated by the Mann-Witney test. P values: vCEC, $p=0.01$; CD109+ CEC, $p=0.0001$. **B** Baseline levels of CD140b+ PPC, CD133+ hcp and VEGFR2+ hp in patients and healthy controls. P values: CD140b+ PPC, 0.02; CD133+ hcp, $p=0.0001$. **C** Baseline levels of CEP in patients and healthy controls. $p=0.0001$. **D** Baseline levels of

CD45dim hcp in patients and healthy controls. $p=0.008$. Abbreviations: CEC, circulating endothelial cells; vCEC, viable CEC; CEP, circulating endothelial progenitors; ctrl, healthy controls; hcp, hematopoietic committed progenitors; hp, hematopoietic progenitor cells; PPC, progenitor perivascular cells; pts, patients; VEGFR, vascular endothelial growth factor receptor.

2.8.5 Figure 5

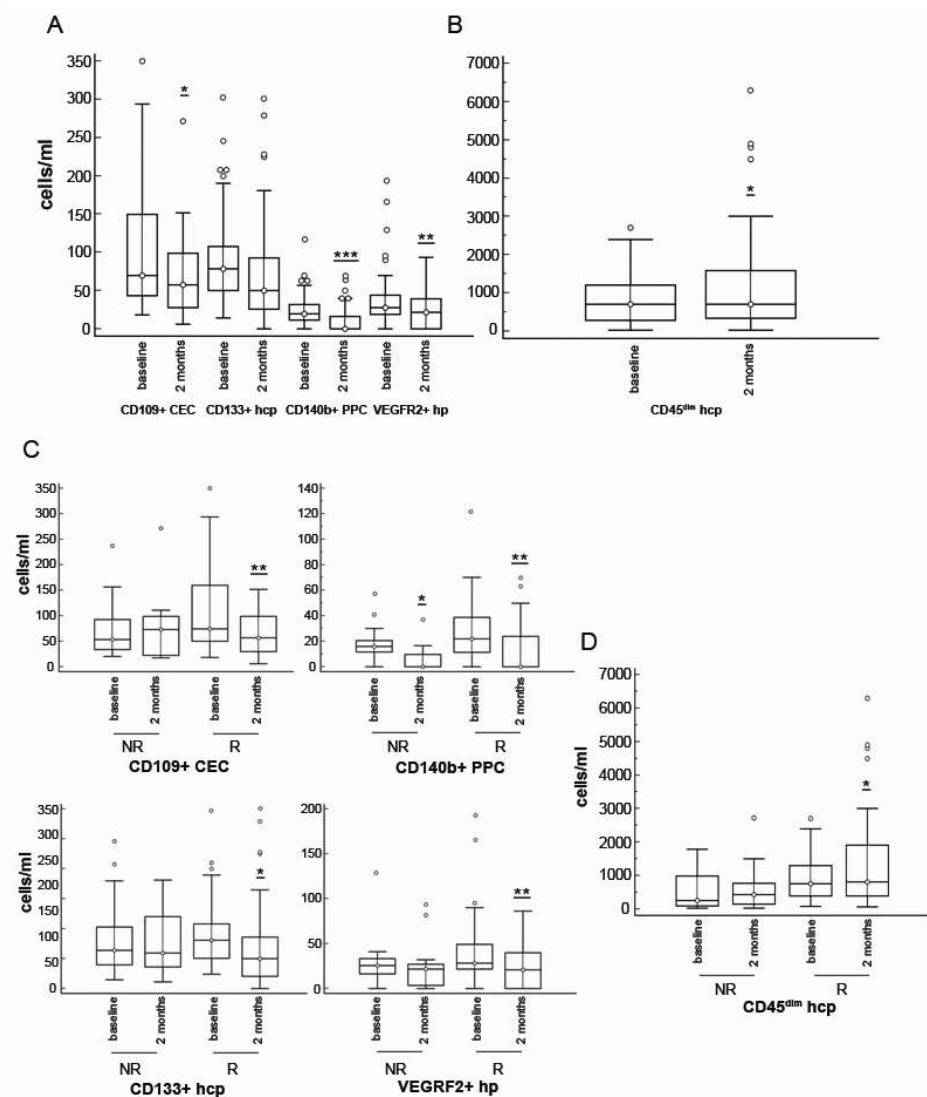


Figure 5. A and B Levels of five selected cell populations at baseline and at 2 months after beginning of therapy in 53 patients. Boxes, the interquartile

range; lines, location of first quartile, median, and third quartile. ○, outliers beyond the standard span. All *p* values were calculated by Wilcoxon test. *P* values: CD109+ CEC, *p*=0.02; CD140b+ PPC, *p*=0.0001; VEGFR2+ hp, *p*=0.004; CD45dim hcp, *p*=0.01. ***C and D Levels of five selected cell populations at baseline and at 2 months in non-responders (NP) and responders (R) included in the serial study.*** Non-responders (*n*=15) were defined as those patients who progressed after 2 months of therapy. *P* values: CD109+ CEC in R, *p*=0.008; CD133+ hcp in R, *p* value=0.02; CD140b+ PPC, *p*=0.03 in NR, *p*=0.001 in R; VEGFR2+ hp in R, *p*=0.005; CD45dim hcp in R, *p*=0.02. Abbreviations: CEC, circulating endothelial cells; CEP, circulating endothelial progenitors; hcp, hematopoietic committed progenitors; hp, hematopoietic progenitor cells; NR, non-responders; PPC, progenitor perivascular cells; R, responders; VEGFR, vascular endothelial growth factor receptor.

2.8.6 Figure 6

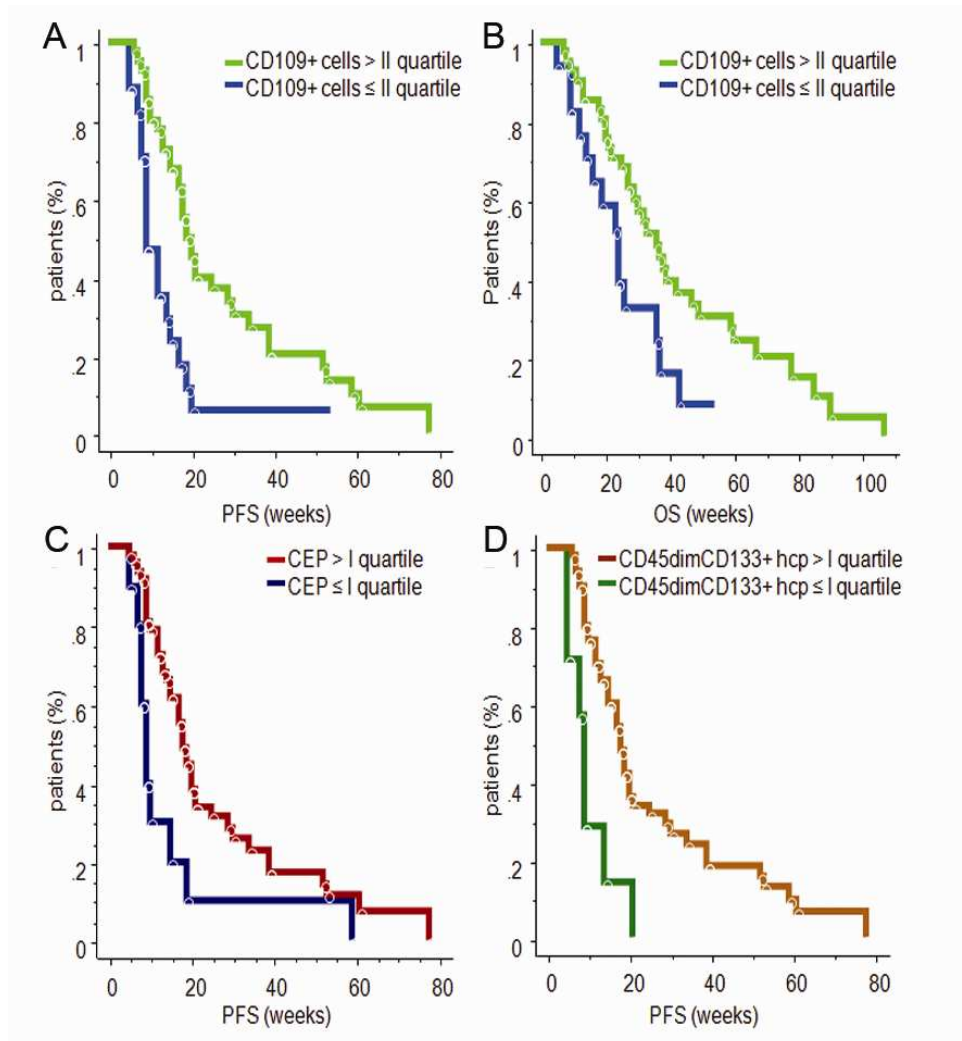


Figure 6. A and B Correlation between baseline CD109+ CECs and PFS or OS respectively. Baseline CD109+ CEC count > 47.5/ml (II quartile) was associated with an increased PFS (19 vs 10 weeks, $p=0.001$) and OS (36 vs 24 weeks, $p=0.02$). C Correlation between CEP and PFS. CEP baseline values

higher than the I distribution quartile were associated with an increased PFS (18 vs 9 weeks, $p=0.01$). **D Correlation between CD45dimCD34+CD133+ hcp and PFS.** Baseline values of CD45dimCD34+CD133+ hcp higher than the I distribution quartile were associated with an increased PFS (18 vs 9 weeks, $p=0.001$).

3. CHAPTER III – CONCLUSIONS

3.1 CONCLUSIONS

Despite the multimodal sophisticated treatment strategy, the overall prognosis of HGG is still poor in adults (1) and children (2), especially after relapse (2-4). For this reason, there is an impelling need for well-tolerated, long-term therapeutic strategies that may improve lifespan and quality of the life of HGG patients, either through indirect effects - facilitating delivery of chemotherapeutics or targeting the tumor vessels - or directly - by killing residual tumor cells infiltrating in the adjacent areas of the brain.

The theory that tumor neovascularization is characterized by a biphasic pattern (5, 6), where CEP recruitment occurs as a second event, could explain the greater efficacy of bevacizumab in GBM, a particularly infiltrating and aggressive tumor (7). In addition, we observed that raised levels of CECs, particularly CD109+ CECs, may support the idea of a positive correlation between CECs and CEPs in patients; as in cardiovascular disease, if increased CECs reflect damage to the endothelium, raised CEPs should be expected for repairing it (8).

The baseline higher value of CD45dimCD34+CD133+ hematopoietic committed progenitors that we recorded in patients and not in healthy

controls, and its further increase after two months of antiangiogenic therapy may be due to the drug-induced “normalization” of the tumor vasculature (9), which needs recruitment of hemangioblasts from the bone marrow and their differentiation to hematopoietic or endothelial progenitor cells (10).

The discordance in findings published so far by different research groups and difficulty in comparing them may be due to the lack of a consensus on CEC and CEP phenotypes and of a common methodology to detect them. As an example, CECs in colorectal cancer and in breast cancer are defined differently although the same behaviour in case of progression is claimed (11, 12). Furthermore, low baseline levels of CECs in peripheral blood have been shown to correlate with a better clinical outcome in terms of PFS and OS in colorectal cancer treated with bevacizumab and chemotherapy (11), although the same is not valid for advanced colorectal cancers and is even opposite in other tumors, such as breast cancer (13, 14). This difference, however, could be due to either the tumor type or the detection method used.

To date, anti-VEGF therapy seems to be one of the most innovative and effective ways to treat recurrent HGG. However, the possible benefit of antiangiogenic therapy, as in general for molecularly targeted anticancer drugs, is limited by relevant factors.

First, antiangiogenic drugs, such as bevacizumab, are expensive and partly responsible for increase of cancer care costs; therefore the biological activity of the drug should be monitored in treated patients in

order to facilitate their stratification and allow treatment in a highly selected subpopulation (5, 15). The selection of a patient subpopulation may be performed using biomarkers exclusively detectable in subjects who respond or resist to therapy and allowing the reliable monitoring of patients. The possible use of circulating endothelial cells (CECs) and progenitors (CEPs) as markers of effectiveness has been already reported in literature and considered for several different kinds of tumors (8, 13, 16-18). However, due to the lack of a consensus on CEC and CEP phenotype and behaviour during therapy, an *ad hoc* study on the use of bevacizumab in HGG patients and its possible biological and cellular markers of efficacy, is recommended. Results of our study on HGG patients treated with bevacizumab, if confirmed, may help decreasing the potential impact on the national health system thanks to a more selected use of bevacizumab. Moreover, since bevacizumab may present relevant side effects, the early stratification of patients would make the efficacy/safety ratio more favourable. Until no marker for prediction of the drug's benefit is available, the clinical efficacy of bevacizumab is virtually reduced.

Second, we need a deeper understanding of the alternative proangiogenic pathways which can be activated following treatment with bevacizumab to overcome VEGF-inhibition. These pathways were first hypothesised when progression after an initial response to the antiangiogenic treatment, such as VEGF decrease (19), was reported. This possible complementary pathway may be activated in response to VEGF inhibition and provide means of escape from treatment with bevacizumab.

Rubenstein et al. in 2000 observed a co-option of pre-existing blood vessels by the tumor and a consecutive increased invasion of surrounding tissues (20). Similar findings were reported by Shaked who suggested that the predictive and prognostic value of CEPs is limited as their mobilization is influenced by antiangiogenic treatment and also other factors, like the granulocyte colony-stimulating factor (21).

Apart from the molecular heterogeneity and the secondary activation of parallel proangiogenic pathways after antiangiogenic therapies, there is also evidence of tumors which are intrinsically resistant to anti-VEGF treatment because of the pre-existence of the same alternative pathways, not targeted by the therapy. Innovative therapeutic strategies presently include the simultaneous targeting of multiple molecules involved in different angiogenic pathways (22), called “horizontal targeting” (23, 24); however, studies suggesting a specific combination are still lacking. In our case, patients were treated with an anti-VEGF drug and chemotherapy because of the ability of bevacizumab to transiently “normalize” the tumor vasculature thus facilitating chemotherapy delivery (23, 25). The combined treatment with bevacizumab and irinotecan for patients with recurrent HGG was proven to be an active regimen with tolerable toxicity (26, 27).

In conclusion, although much is still unknown about the mechanisms of resistance to these therapies, FDA approval of bevacizumab for treatment of several tumors, including recurrent GBM, highlights that this drug currently represents an important option for patients with different

cancers. In any case, a deeper investigation on the molecular, cellular and radiological profiles of patients who respond or not to treatment, should be carried out so as to identify predicting signs and allow earlier treatment decisions and more tailored therapies. This would save patients from adverse effects of ineffective therapies, trying alternative treatments sooner.

3.2 FUTURE PERSPECTIVES

The use of antiangiogenic drugs, such as bevacizumab, for treatment of cancer has strongly emerged from extensive preclinical and clinical research and markedly increased in the recent years, although their clinical benefits are relatively modest (28). A deeper understanding of the cellular and molecular mechanisms underlying tumor angiogenesis would likely lead to relevant improvement of the clinical benefit of these drugs because of their consequent more tailored use in selected patient subgroups.

In our case, the study of CECs, CEPs and other cell subpopulations could be completed by the investigation of important molecules, such as VEGF, PDGF β , TNF α and IL-6, even if a correlation was not observed in other settings (29, 30). Experiments on this regards are ongoing and will show the possible involvement of some selected molecules in the mechanisms of resistance or response to bevacizumab in recurrent HGG patients.

A randomized study showed that addition of irinotecan to bevacizumab does not give a statistically significant advantage in terms of survival (31). However, the best drug to be combined with bevacizumab is still unknown, although its association with a chemotherapeutics is highly recommended due to the capacity of the antiangiogenic drug to normalize tumor vessels and improve chemotherapy delivery (25). New treatments combining bevacizumab with innovative chemotherapeutics or other

targeted-inhibitors, according to the molecular profile of the tumor, should be tested in the clinics. Due to the complexity of such approach, “proof-of-concept” studies should be carried out on a limited number of molecularly selected cases, so as to rapidly identify the optimal patient population and drug dose (23).

Finally, the presence of a pro-invasion effect of bevacizumab is under scrutiny (32, 33); patients with radiographic evidence at relapse should be deeply investigated in order to identify a molecular profile which is preferentially correlated with the higher risk of invasion or local recurrence.

3.3 SUMMARY

Bevacizumab has shown activity in different tumor types, including high grade gliomas (HGG). However, the use of bevacizumab and other antiangiogenic drugs in the clinical setting limited by the lack of markers to predict responses.

We report that the combined treatment with bevacizumab and irinotecan is effective in recurrent HGG patients, particularly in those with local disease, with mild toxicity. Median OS and PFS were 33 and 18 weeks, respectively. PFS at 6 and 12 months were 32% and 12%. OS at 6 months was 60%. Patients with distant intracerebral disease or leptomeningeal dissemination at baseline magnetic resonance had shorter PFS and OS.

We analyzed circulating endothelial cells (CECs) and their progenitors (CEPs), as previous studies supported their involvement in responses to bevacizumab. Higher levels of CD109+ CECs, CEPs and CD45dimCD34+CD133+ hematopoietic committed progenitors before treatment were associated with longer PFS. Moreover, long-term responders showed higher baseline CD109+ CECs and CD45dimCD34+VEGFR2+ hematopoietic progenitors.

These findings pave the way for larger studies further addressing the potential of CECs and CEPs as biomarkers to target patient populations that may benefit from bevacizumab and possibly other antiangiogenic drugs.

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