Bevacizumab targeting diffuse intrinsic pontine glioma: results of $^{89}$Zr-bevacizumab PET-imaging in brain tumor models


* These authors contributed equally

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ABSTRACT

The role of the vascular endothelial growth factor (VEGF)-inhibitor bevacizumab in the treatment of diffuse intrinsic pontine glioma (DIPG) is unclear. We aim to study the biodistribution and uptake of zirconium-89 (\(^{89}\text{Zr}\))-labeled bevacizumab in DIPG mouse models.

Human E98FM, U251-FM glioma cells and HSJD-DIPG-007-FLUC primary DIPG cells were injected into the subcutis, pons, or striatum of nude mice. Tumor growth was monitored by bioluminescence imaging (BLI) and visualized by Magnetic Resonance Imaging (MRI). Seventy-two to 96 hours after \(^{89}\text{Zr}\)-bevacizumab injections, mice were imaged by Positron Emitting Tomography (PET) and biodistribution was analyzed ex vivo.

High VEGF expression in human DIPG was confirmed in a publically available mRNA database, but no significant \(^{89}\text{Zr}\)-bevacizumab uptake could be detected in xenografts located in the pons and striatum at an early or late stage of the disease. The E98FM, and to a lesser extent the U251-FM and HSJD-DIPG-007 subcutaneous tumors, showed high accumulation of \(^{89}\text{Zr}\)-bevacizumab. VEGF expression could not be demonstrated in the intracranial tumors by in situ hybridization (ISH), but was clearly present in the perinecrotic regions of subcutaneous E98FM tumors.

The poor uptake of \(^{89}\text{Zr}\)-bevacizumab in xenografts located in the brain suggests that VEGF targeting with bevacizumab has limited efficacy for diffuse infiltrative parts of glial brain tumors in mice. Translating these results to the clinic would imply that treatment with bevacizumab in DIPG patients is only justified after targeting of VEGF has been demonstrated by \(^{89}\text{Zr}\)-bevacizumab immuno-PET. We aim to confirm this observation in a clinical PET study with DIPG patients.
INTRODUCTION

Recent advances in molecular and cellular cancer biology have resulted in the identification of critical molecular tumor targets involved in the different phases of tumor growth and spreading. This knowledge has boosted the rational design of novel drugs, especially monoclonal antibodies and tyrosine kinase inhibitors. However, broad application of these targeted therapies to treat brain tumors has lagged behind in daily clinical practice. By using molecular positron emission tomography (PET) imaging, target expression, bio-distribution can be studied concurrently in a relatively non-invasive manner. It potentially allows the identification of those patients who may, or may not, benefit from targeted therapy.

A disease that may benefit from molecular imaging is diffuse intrinsic pontine glioma (DIPG), a childhood malignancy located in the brainstem. In the past 40 years the outcome of patients with DIPG has remained unchanged, with less than 10% of the patients being alive two years from diagnosis. Given the lack of gadolinium uptake on MRI in DIPG tumors, it is plausible that the blood brain barrier (BBB) in DIPG often remains intact which might explain the resistance to systemic chemotherapy in these patients.

A well-studied drug target in gliomas is the vascular endothelial growth factor (VEGF), a signal protein stimulating angiogenesis and increasing vessel permeability. Overexpression of VEGF-A, its receptor VEGFR2, or both, have been implicated as poor prognostic markers in various clinical studies. Bevacizumab is a recombinant humanized monoclonal antibody that selectively binds with high affinity to all isoforms of human VEGF-A, and neutralizes their biologic activity. Studies in adult patients with recurrent high grade gliomas (HGG) reported high radiological response rates with bevacizumab treatment but recently two large phase III randomized studies showed no improvement in overall survival with bevacizumab treatment in an upfront setting. Bevacizumab has been studied in a number of non-randomized trials in pediatric brain tumor patients. Efficacy in these trials has been variable, with a subset of patients showing clear radiological and/or clinical improvement. The role of bevacizumab in the treatment of DIPG patients is even less clear, but is currently studied in several trials (NCT00890786 and NCT01182350; clinicaltrials.gov; NTR2391 Trialregister.nl).
No validated methods are available to identify patients who may potentially benefit from bevacizumab treatment. Lack of clinical effect may be due to either poor transport of bevacizumab into the tumor microenvironment due to an intact BBB, or a lack of VEGF expression. In this study we analyzed VEGF(R) expression in adult and childhood high grade gliomas, including DIPG tumors. Furthermore, we studied bevacizumab distribution in vivo using molecular PET imaging with $^{89}$Zirconium-labeled bevacizumab in murine DIPG models.

**MATERIALS AND METHODS**

**VEGF-A and VEGFR2 mRNA expression profiles**
VEGF-A and VEGFR2 (KDR) mRNA expression in DIPG (n=27) and pediatric high grade glioma (pHGG) (n=53) were determined *in silico*, using publicly available datasets, and compared to a dataset of non-malignant brain tissue (n=44), low grade brainstem glioma (n= 6) and adult HGG (n=284). These datasets include tumor material from biopsy, resection and autopsy (DIPG). Differences were analysed by two way ANOVA and a p < 0.01 was considered significant. As a validation of these findings, VEGF associated gene expression was studied in normal brain, low grade brainstem glioma (LG-BSG), DIPG and GBM by creating a heat map using K-means clustering. All expression analyses were performed using R2, a web-based microarray analysis and visualization platform (http://r2.amc.nl).

**Immunohistochemistry and in situ hybridization**
Formalin-fixed paraffin-embedded slides were sectioned from xenograft tumors and brain tissue and subjected to immunohistochemical (IHC) staining. Briefly, after deparaffinization and heat induced antigen retrieval, sections were incubated with primary mouse anti-Ki67 antibodies (clone MIB-1, DAKO) overnight at 4°C. Thereafter slides were washed and incubated with HRP-conjugated EnVision (DAKO) and subsequently stained by DAB with hematoxylin counterstaining.

For *in situ* hybridization (ISH), tumors were cut in 5 µm slices and incubated with VEGF probes against the human VEGF coding sequence using a previously described protocol. Samples were evaluated by microscopy with a Zeiss Axioskip microscope (HBO100W/Z), equipped with a Canon digital camera and imaging software (Canon PowerShot A640, Canon Utilities, ZoomBrowser Ex. 5.7, Canon Inc., Tokyo, Japan).
Bevacizumab uptake in preclinical brain tumor models

Labeling and quality control of 89Zr-bevacizumab

Bevacizumab was labeled with zirconium-89 using N-succinyl-desferrioxamine (N-suc-Df) as described previously. In short: the chelator, desferrioxamine, was succinylated to N-suc-Df. Next, the hydroxamate groups were blocked with iron and the succinyl group was activated as its TFP-ester (Fe-N-suc-Df-TPF ester). Bevacizumab (6 mg/mL) was reacted with two equivalents of Fe-N-suc-Df-TPF ester at pH 9 for 30 min at room temperature. Hereafter, iron was removed at pH 4.2-4.5 with an excess of ethylenediaminetetraacetic acid (EDTA) for 30 min at 35°C and N-suc-Df-bevacizumab was purified by size exclusion chromatography using a PD-10 column. Radiolabeling of N-suc-Df-bevacizumab was performed in HEPES buffer: to 200 μL 89Zr in 1M oxalic acid 90 μL 2M Na2CO3 was added. After 3 min 300 μL 0.5 M HEPES, N-suc-Df-bevacizumab and 700 μL 0.5 M HEPES were added. After 60 minutes reaction time 89Zr-N-suc-Df-bevacizumab was purified by PD10 using 5 mg/mL gentisic acid in 0.9% NaCl (pH 4.9-5.4) as the mobile phase.

Radiochemical purity and antibody integrity were determined using instant thin-layer chromatography (iTLC), high-performance liquid chromatography (HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by phosphor imager analysis. For analysis of immunoreactivity an ELISA based assay was used. iTLC analysis of 89Zr-bevacizumab was performed on TEC control chromatography strips (Biodex). As the mobile phase, citrate buffer (20 mmol/L, pH 5.0) containing 10% acetonitrile was used. HPLC analyses of bevacizumab modification and radiolabeling were performed using a Jasco HPLC system equipped with a Superdex™ 200 10/30 GL size exclusion column (GE healthcare Life sciences) using a mixture of 0.05 M sodium phosphate, 0.15 M sodium chloride (pH 6.8) and 0.01 M NaN3 as the eluent at a flow rate of 0.5 mL/min. The radioactivity of the eluate was monitored using an inline NaI(Tl) radiodetector (RaytestSockett).

Cell lines and animal models

Animal experiments were performed in accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and the Dutch Law on animal experimentation. The experimental protocol was validated and approved by the local committee on animal experimentation of the VU University Medical Center. Athymic Nude-Foxn1™ mice (six weeks old) were purchased from Harlan/Envigo (Horst, The Netherlands) and kept under filter top conditions and received food and water ad libitum.
The primary HSJD-DIPG-007 cell line was established from DIPG tumor material obtained at Hospital Sant Joan de Deu (Barcelona, Spain) after autopsy from a 6-year-old patient and was confirmed to have a H3F3A (K27M) and ACVR1 (R206H) mutation. The E98 cell line was obtained from Radboud University Medical Center Nijmegen, the U251 glioma cell line from ATCC. All cell lines were transduced in our laboratory to express firefly luciferase (FLUC) and/or mCherry. Cell lines were mycoplasma negative and were authenticated by STR-analysis modified from De Weger et al.

E98FM cells were injected subcutaneously in female athymic nude mice (7-9 weeks of age) to expand the number of cells. When the subcutaneous tumor reached a diameter of 1 cm, the tumor was removed and a single cell suspension was prepared by mechanical disruption through a 100 µm nylon cell strainer. HSJD-DIPG-007-FLUC was cultured in tumor stem medium (TSM), U251-FM in DMEM supplemented with 10% FCS and penicillin/streptomycin. Shortly before stereotactic injection, cells were washed once with phosphate buffered saline (PBS) and concentrated to 1x10^6 cells per µL. Mice were stereotactically injected with 5x10^6 cells in a final volume of 5 µL into either the pons or striatum, or injected subcutaneously with 3x10^6 cells in a final volume of 100 µL/flank. Coordinates used for intracranial injections were -1.0 mm X, -0.8 mm Y, 4,5 mm Z from the lambda for pontine tumors and 0.5 mm X, 2 mm Y, -2 mm Z from the bregma for the striatum tumors. Coordinates were previously validated and based on "The mouse brain in stereotaxic coordinates" by Franklin and Paxinos. Tumor engraftment was monitored by bioluminescence measurement of the Fluc signal. For E98FM, early stage tumors in the pons (n=8), striatum (n=7) and s.c. xenografts (n=3) were allowed to grow for 18 days and late stage tumors (n=9, striatum; n=3, subcutaneous) for 35 days after xenograft injection. E98 xenograft tumors in the pons were not available 35 days post injection, because injection of E98FM cells in the pons would result in death of the mice due to tumor growth within three weeks. For HSJD-DIPG-007-FLUC and U251-FM tumors (pons, striatum, subcutaneous) were evaluated, at days 78 and 22 respectively (Figure 2A).

Two to three days before the endpoint of the study, mice were injected intraperitoneally (i.p.) with Zr-labeled bevacizumab (40 µg, 5 MBq for PET analysis and ex vivo biodistribution or 185 kBq for ex vivo biodistribution only).
**PET imaging and ex vivo analysis**

The distribution of $^{89}$Zr-bevacizumab was determined 72 hours (ex vivo only) or 96 hours (PET followed by ex vivo analysis) after administration, as a minimum of 72 hours interval between injection and scanning has previously been shown to achieve optimal tumor-to-nontumor ratios. Scanning of E98FM tumors was performed using a LSO/LSO double layer ECAT High Resolution Research Tomograph (HRRT, CTI/Siemens, Knoxville, TN, USA): a small animal and human brain 3-dimensional (3D) scanner with high spatial resolution (2.3–3.4 mm full width at half maximum) and high sensitivity. Mice were anesthetized by isoflurane inhalation anesthesia (1.5 L $\text{O}_2$/minute and 2.5% isoflurane) before positioning in the HRRT. A static transmission scan (6 min) using a rotating 740 MBq $^{137}$Cs point source was performed. Prior to positioning the mice in the HRRT, a canula was placed i.p. to enable later injection of $^{18}$F(-). Static images of 60 minutes acquisition time were obtained. Immediately thereafter $^{18}$F(-) was injected i.p (10 MBq/mouse) for co-localization of bone structures (static images of 60 minutes acquisition). Body temperature was controlled with a heated platform (kept at 37 °C).

Mice with HSJD-DIPG-007-FLUC or U251-FM tumors and where tumor growth was confirmed by increase of BLI signal were imaged using another, preclinical, PET system, (Nanoscan PET-CT, MEDISO, Budapest, Hungary), 72 hours after $^{89}$Zr-bevacizumab injection. PET images were analyzed using AMIDE software. *Amide’s a Medical Image Data Examiner, version 1.0.1*

**Magnetic Resonance Imaging (MRI)**

Mice with representative HSJD-DIPG-007-FLUC or U251-FM tumors were selected for MRI based on the intensity of the bioluminescence signal. Gadolinium (750 µmol, Dotarem) was administered i.v. immediately before imaging. Mice were anesthetized by isoflurane inhalation anaesthesia (1.5 L $\text{O}_2$/minute and 2.5% isoflurane), placed in a preclinical PET-MRI system (Nanoscan system, MEDISO, Budapest, Hungary) and T1 and T2 weighed images with gadolinium contrast were acquired. MRI images were analyzed using MIPAV software *Medical Image Processing, Analysis, and Visualization, version 7.2.0*.

**Ex vivo analysis**

Immediately after PET imaging, animals were sacrificed for ex vivo tissue distribution analysis. Blood, urine, tumor and various tissues were excised, rinsed in PBS to remove
residual blood, and weighed. Radioactivity in blood and tissues (in percentage injected
dose per gram of tissue: % ID/g) was determined using an LKB 1282 gammacounter
(Compugamma, LKB Wallac, Turku, Finland). Differences in the amount of radioactivity in
healthy brain regions versus subcutaneous-, pontine-, and striatal tumors were analysed
by Kruskal-Wallis test with Dunn’s Multiple Comparison posthoc testing. A p<0.05 was
considered statistically significant.

RESULTS

VEGF-A, VEGFR2 and VEGF associated gene expression in DIPG

A search in a publically available mRNA expression database curated by the Amsterdam
Medical Center (R2.amc.nl) revealed that VEGF-A mRNA is overexpressed in DIPG
compared to normal brain and compared to low grade brainstem glioma (LG-BSG)
and non-pontine adult- and pediatric HGG (Figure 1a; p<0.01, ANOVA). VEGFR2 (KDR)
mRNA expression was low in both pediatric and adult glioma, compared to normal
brain (Figure 1b; p<0.01, ANOVA). A heatmap generated using K-means clustering of
expression of VEGF-A associated genes confirmed an aberrant VEGF-A pathway to
be more prominent in DIPG compared to LG-BSG. Most DIPG (red) samples clustered
together with pediatric GBM (green) and LG-BSG (blue) clustered with normal brain
tissue (purple) (supplemental Figure 1).

Figure 1 | Expression of VEGF-A and VEGFR2

1A: Box-plots representing relative median mRNA
expression in diffuse intrinsic pontine glioma (DIPG) (n=27) and pediatric high grade glioma (pHGG)
(n=53), versus datasets of normal brain tissue (n=44, blue), low grade brainstem glioma (n= 6)
and adult HGG (n=284). VEGF-A overexpression is shown in DIPG compared to normal brain and
compared to adult glioma. 1B: VEGFR2 is not overexpressed in DIPG and pediatric glioma compared
to normal brain. * P<0.01, ANOVA
Bevacizumab uptake in preclinical brain tumor models

Biodistribution of $^{89}$Zr-bevacizumab

After tumor growth was confirmed by an increase in bioluminescence (BLI) signal, $^{89}$Zr-bevacizumab was injected in these animals. 72 – 96 hours after i.p. injection of $^{89}$Zr-bevacizumab, animals were imaged by PET; a schematic overview of the experiment is given in Figure 2, with representative BLI figures of E98FM engrafted mice shown in Figure 2b.

$^{89}$Zr-bevacizumab uptake was not visible in the E98 gliomas located in the pons (only early stage, Figure 3a) or in the striatum at early or late stage. The rest of the brain showed no uptake of $^{89}$Zr-bevacizumab either, independently of the presence of a tumor. However, subcutaneous E98 tumors showed high accumulation of $^{89}$Zr-bevacizumab indicated by a red hotspot (Figure 3a).

Figure 2 | 2A Schematic overview of the experiment. Cells are injected at day 0 in the pons, striatum or subcutis (early stage tumors) or the striatum and subcutis (late stage E98FM tumors). Tumor progression was monitored by bioluminescent imaging. The time point for $^{89}$Zr-bevacizumab injection was dependent on the growth speed of used cell lines. This injection was done 72-96 hours before PET scanning. MRI, PET or PET/CT imaging was followed by ex vivo measurement of $^{89}$Zr-bevacizumab accumulation in tissues (Bio-distribution). 2B: Charged couple device (CCD) camera images of mice bearing E98FM tumors. BLI images were obtained at the study endpoint, day 18 (early stage, upper panel) and day 35 (late stage, lower panel).
**Figure 3** 3A: $^{89}$Zr-PET combined with $^{18}$F- imaging of mice with an E98 tumor located in the pons (upper panels), or subcutaneous (bottom panels). White circles indicate expected location of intracranial tumors. While no uptake of $^{89}$Zr-bevacizumab is observed in the pontine tumor, the subcutaneous tumor shows a PET hot spot reflecting $^{89}$Zr-bevacizumab accumulation in the tumor (arrow). In all animals, high intensity (visualized by red color code) is seen in the heart and liver of the mice, reflecting $^{89}$Zr-bevacizumab in the blood pool. Images acquired with Siemens HRRT. 3B: Immuno-PET scanning of mice with HSJD-DIPG-007-Fluc (upper and middle panels) or U251FM tumors (bottom panels) confirmed the lack of bevacizumab uptake in intracranial tumors. Image acquired with MEDISO Nanoscan PET-CT system. White circles indicate expected location of intracranial tumors.

Ex vivo tissue distribution measurements confirmed that no significant $^{89}$Zr-bevacizumab uptake was detected in the brain or brain tumor at any stage of disease, while there was high uptake in the subcutaneous tumor ($p < 0.01$) (Figure 4a). Besides accumulation in the subcutaneous tumor with an average level of 50% ID/g, $^{89}$Zr-bevacizumab was observed in all animals (to a lesser extent) in the blood pool and in well-perfused organs, such as the liver, spleen and lungs (Figure 5). Experiments using the two other cell lines (U251FM and HSJD-DIPG-007-FLUC) showed comparable results. Using these cell lines, no uptake of $^{89}$Zr-bevacizumab was visualized on PET in any of the xenografts (Figure 3b).

Ex vivo biodistribution analysis showed higher $^{89}$Zr-bevacizumab uptake in subcutaneous HSJD-DIPG-007-FLUC tumors ($p<0.05$) as compared to brain (areas) without tumor (Figure 4b). The subcutaneous U251FM tumors showed no significant increase in $^{89}$Zr-
Figure 4 | $^{89}$Zr-bevacizumab measured ex vivo by a gamma-counter and normalized to counts found in healthy brain tissue (brain tissue of animals without xenografted brain tumors). 4A: Uptake is significantly higher in the subcutaneous E98FM tumors (***, p<0.01), but there is no significant difference in uptake in pontine or striatal xenografts at any stage of the disease compared to normal brain. 4B: Uptake in subcutaneous DIPG7-Fluc tumors is higher than in normal brain (*p<0.05). 4C: No significant differences between U251-FM tumors (s.c. or intracranial) versus normal brain.

Figure 5 | $^{89}$Zr-bevacizumab uptake measured ex vivo by radioactivity of the specific organs and the tumor after dissection, expressed as percentage of the injected dose per gram tissue in E98FM tumor bearing animals. Note the high uptake in subcutaneous tumors, compared to the negligible uptake in the intracerebral tumors.
bevacizumab uptake (Figure 4c) as compared to non-tumor brain. Of note, both HSJD-DIPG-007-FLUC and U251FM cells formed only very small subcutaneous tumors during the time window in which mice baring intracranial tumors had reached human endpoints (78 vs 22 days after tumor injection).

**In situ-hybridization of VEGF**

To determine whether the differences in $^{89}$Zr-bevacizumab uptake in the subcutaneous and the intracranial tumors were due to an impaired distribution of $^{89}$Zr-bevacizumab into the brain or to a differential expression of its target (or both), VEGF expression was analyzed in the different xenografts. *In situ* hybridization confirmed expression of VEGF in the subcutaneous E98FM tumor (Figure 6a), while VEGF expression was absent in E98FM-brain tumors (Figure 6b) and brain tissue without a xenograft. Of note, in the subcutaneous tumors, VEGF was preferentially expressed in perinecrotic areas, while necrosis was absent in all of the striatal and pontine tumors. A Ki67 (Mib-1) staining was

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**Figure 6** | VEGF expression in DIPG tumors as assessed by VEGF in situ hybridization (ISH). 6A: Positive VEGF staining in subcutaneous E98FM tumor cells (magn. 20x, insert 2.5x). The tumor shows extensive necrosis surrounded by VEGF positive cells. 6B: No VEGF mRNA is detected in the E98FM tumor tissue in the striatum (magn. 20x, insert 1.25x). 6C: Ki67 staining shows the presence of proliferating tumor cells in the subcutaneous tumor tissue (magn. 20x, insert 2.5x). 6D: Ki67 positive tumor cells in the striatal E98FM tumor (magn. 20x, insert 10x)
performed to confirm presence of proliferating tumor tissue (Figures 6c,d). In HSJD-DIPG-007-FLUC and U251FM tumors, VEGF mRNA expression was not detectable in striatal and pontine gliomas. The subcutaneous tumors were too small to adequately perform VEGF ISH and therefore no conclusions could be drawn regarding VEGF expression in these s.c. tumors.

**Magnetic Resonance Imaging**

To visualize disruption of the blood-brain-barrier, mice with HSJD-DIPG-007-FLUC and U251-FM intracranial tumors were imaged by MRI after intravenous administration of gadolinium. In the diffusely growing HSJD-DIPG-007-FLUC tumor, gadolinium enhancement on T1 weighed images was limited (Figure 7a, arrow), whereas gadolinium enhancement was clearly visible in the U251-FM tumor (Figure 7c, arrow). On T2 weighed images, tumors were not clearly visible (Figures 7b, d; arrow).

![MRI scans of pontine tumors](image)

**Figure 7** | MRI scans of pontine tumors. The %Zr-bevacizumab uptake in the tumors of these two mice is presented as %ID/g. 7A: Limited gadolinium contrast enhancement in the tumor area (arrow) on T1 scan. 7B: HSJD-DIPG-007-FLUC tumor is poorly visible on T2 weighed MRI image (arrow). 7C: Clear gadolinium contrast enhancement in the U251-FM tumor area (T1 weighed MRI scan) indicates disruption of the blood-brain-barrier. 7D: U251-FM tumor is visible on T2 weighed MRI scan.
DISCUSSION

The potential benefit of bevacizumab in the treatment of DIPG is unclear, as efficacy depends on expression of VEGF-A as well as appropriate drug distribution. We used molecular PET imaging to study the influence of location and stage of disease on biodistribution of $^{89}$Zr-bevacizumab in three glioma mouse models (pontine, striatal, subcutaneous) using three different cell lines. The E98FM pontine and striatal and HSJD-DIPG-007 pontine xenograft models have previously been described to resemble the diffuse phenotype of human DIPG and other diffuse high grade gliomas. U251 has been described as an intracranial murine tumor model that recapitulates most of the key figures of adult GBM. We found no significant uptake of $^{89}$Zr-bevacizumab in the intracranial tumor models at any stage of the disease, nor in the normal/non-neoplastic surrounding brain. In contrast, high accumulation of $^{89}$Zr-bevacizumab was observed in the subcutaneous E98-xenograft and moderate uptake in the subcutaneous HSJD-DIPG-007-FLUC.

We initially hypothesized that lack of $^{89}$Zr-bevacizumab uptake could be explained solely by poor distribution into the brain, as large molecules like monoclonal antibodies may not be able to pass the BBB. This hypothesis is supported by the absence of enhancement of the tumor on MRI after administration of gadolinium in animals with HSJD-DIPG-007-FLUC pontine tumors. However, MRI analysis of U251-FM tumors in the brainstem of mice showed clear gadolinium enhancement, which is indicative of “leaky” blood vessels in the tumor. Furthermore, VEGF expression of the E98FM glioma cells - analyzed by ISH - also differed between tumor locations: E98FM gliomas in both striatum and pons appeared VEGF-negative, while the s.c. E98FM tumors were partly VEGF-positive. The differences in VEGF expression of the tumors in distinct locations originating from the same cell line, confirms that the orthotopic microenvironment and the resulting growth pattern significantly influence gene expression in glioma cells, a phenomenon that has been described previously. Moreover, it has been shown that in GBM, VEGF is predominantly overexpressed in hypoxic, perinecrotic cells. Indeed, in our study the VEGF expression in subcutaneous E98FM tumors was especially present around areas of necrosis, whereas in intracranial E98FM tumors necrosis and VEGF expression were lacking and this also coincided with lack of bevacizumab uptake studied ex vivo and by PET. Of note, bevacizumab does not bind to murine VEGF-A, but as typically the neoplastic cells are upregulating VEGF expression in tumor...
angiogenesis we consider it unlikely that stromal cell-derived mouse VEGF-A plays an important role in this particular xenograft model. 178

In contrast to our preclinical findings, the in silico analysis that we performed in this study, indicates that human DIPG tumors have relatively high expression levels of VEGF mRNA. However, the majority of tumors (23 out of 27) used for the microarray experiments were collected post-mortem 92,101 and therefore these samples represent the end-stage of the disease, and are post-radiation therapy. In the end-stage of the disease, DIPG is known to have necrotic areas with microvascular proliferations and blood-brain barrier disruption, compatible with the histology of a GBM, which is associated with high VEGF expression. Although the numbers are low, it is important to point out that VEGF-A expression levels in samples obtained pre-treatment were low compared to expression in post-mortem/ end-stage samples (supplemental Figure 2). In addition, biopsy samples that were included in the analysis are frequently directed at contrast-enhancing regions, and this 'biased sampling' may well lead to overestimation of the role of VEGF in the tumor as a whole. We are currently studying the differences in VEGF-A expression in autopsy-derived DIPG tissue between the perinecrotic areas and the more diffusely growing tumor parts without necrosis.

Experimental and clinical research in both adult and pediatric high grade glioma and DIPG has suggested that there is a complex relation between a histologically diffuse growth pattern of brain tumors, VEGF expression and availability and BBB integrity 163,223,236–239. Traditionally, VEGF is viewed as the main cause of increased BBB permeability in CNS tumors as represented by contrast-enhancing lesions on MRI 240. More recently, anti-VEGF therapy is thought to potentially induce a more diffuse and distant spread of tumor cells 163,237. In contrast to adult high grade gliomas, gadolinium contrast enhancement on MRI in DIPGs at diagnosis is generally limited, with 50% of the patients showing no enhancement at all 15. The lack of gadolinium enhancement suggests an intact BBB, at least for large molecules, in a substantial percentage of the patients, coinciding with low VEGF expression and inability of bevacizumab to target the tumor 164.

Results from our preclinical PET, MRI with gadolinium contrast and ISH studies suggest that this relation is not so straightforward, pressing the need for studying VEGF targeting in patients treated with anti-VEGF therapy. One could however argue, whether high local tumor accumulation of bevacizumab is at all needed to obtain potential therapeutic
effects in DIPG. Bevacizumab is capable of decreasing VEGF levels in blood to undetectable range in less than 20 days in a large cohort of adult cancer patients\textsuperscript{241}, but only a subgroup of patients responded to anti-VEGF therapy. Also in DIPG, decreased phosphoVEGR2 levels in peripheral blood mononuclear cells (PBMCs) did not correlate with treatment response\textsuperscript{219}. In the E98 xenograft model used in this study, treatment with bevacizumab did not increase survival nor did it influence the growth pattern in the diffusely growing parts of the tumor\textsuperscript{234}. This suggest simply decreasing VEGF in the blood-pool is, at least for the tumor types studied and our xenograft model, often insufficient for adequate tumor targeting and instead, local bevacizumab accumulation seems needed\textsuperscript{241–243}.

The results of immuno-PET imaging and VEGF-ISH in these DIPG models are in line with the poor clinical response rates thus far obtained with bevacizumab in children with DIPG\textsuperscript{219}. The data presented suggest that no adequate uptake of bevacizumab will occur in diffusely growing gliomas, which present with BBB disruption but without drastically increased VEGF expression. Therefore we suggest that bevacizumab treatment is only justified if targeting of VEGF by bevacizumab has been visualized by immuno-PET scan. We aim to confirm this hypothesis in a clinical PET study with DIPG patients.

**Future directions**

This study underlies the importance of using strong biological and biodistributional rationale before using any therapy in any patient. Following the results of this study, we developed a molecular drug imaging trial with 89Zr-bevacizumab in children with DIPG (study number NTR3518 www.trialregister.nl). This technique aims to further unravel the role of bevacizumab treatment in DIPG. Ideally, such molecular imaging is combined with VEGF-A and VEGFR2 expression analysis on tumor tissue originating from biopsies taken from several (contrast-enhancing and non-enhancing) parts of the tumor. Because DIPG can generally be diagnosed based on its typical radiological presentation and the delicate nature of the brain involved, taking biopsies from DIPGs is still no common practice and sampling of multiple regions is even more cumbersome. In general, integrating molecular imaging with radiolabelled drugs (classic cytostatic agents, small molecules, other monoclonal antibodies) in the treatment of childhood brain cancer provides an insight in drug targeting and might help to personalize treatment and thereby to avoid unnecessary side effects of drugs that do not reach the tumor.
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Supplemental Figure 1 | Heatmap of VEGF-A associated genes generated using k-means clustering: LG-BSG samples (blue) in the disease type histology (upper legends) cluster with normal brain (purple). DIPG (red) clusters with GBM (green). Histology confirmed in a majority of tumors: DIPG (green), pHGG/GBM (red).
Supplemental Figure 2 | VEGF mRNA expression in DIPG in comparison to normal brain (n=31) as analyzed by R2 (R2.amc.nl), clustered by stage of disease; unknown (n=10), pretreatment (n=2) and postmortem (n=23)