Bevacizumab impairs oxidative energy metabolism and shows antitumoral effects in recurrent glioblastomas: a $^{31}$P/$^1$H MRSI and quantitative magnetic resonance imaging study

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Bevacizumab shows unprecedented rates of response in recurrent glioblastomas (GBM), but the detailed mechanisms are still unclear. We employed in vivo magnetic resonance spectroscopic imaging (MRSI) and quantitative magnetic resonance imaging to investigate whether bevacizumab alters oxygen and energy metabolism and whether this effect has antitumoral activity in recurrent GBM. $^{31}$P and $^1$H MRSI, apparent diffusion coefficient (ADC), and high-resolution T2 and T2′ mapping (indirect marker of oxygen extraction) were investigated in 16 patients with recurrent GBM at 3 Tesla before and 1.5–2 months after initiation of therapy with bevacizumab. Changes of metabolite concentrations and of the quantitative values in the tumor and normal appearing brain tissue were calculated. The Wilcoxon signed-ranks test was used to evaluate differences for tumor/edema versus control as well as changes before versus after commencement of therapy. Survival analyses were performed for significant parameters. Tumor T2′, pH, ADC, and T2 decreased significantly in patients responding to bevacizumab therapy ($n = 10$). Patients with at least 25% T2′ decrease during treatment showed longer progression-free and overall survival durations. Levels of high-energy metabolites were lower at baseline; these persisted under therapy. Glycerophosphoethanolamine as catabolic phospholipid metabolite increased in responders. The MRSI data support the hypothesis that bevacizumab induces relative tumor hypoxia (T2′ decrease) and affects energy homeostasis in recurrent GBM, suggesting that bevacizumab impairs vascular function. The antiangiogenic effect of bevacizumab is predictive of better outcome and seems to induce antitumoral activity in the responding GBMs.

Keywords: ADC, bevacizumab, glioblastoma, phosphorus spectroscopy, T2′.

Patients with recurrent glioblastomas (GBMs) have a poor prognosis, with a median duration of survival of 3–6 months.1,2 GBMs are highly vascularized tumors characterized by strong neoangiogenesis, at least partly mediated by the vascular endothelial growth factor (VEGF) secreted by the tumor cells. Bevacizumab is a humanized monoclonal immunoglobulin (Ig) G antibody against VEGF-A. Therefore, an antiangiogenic treatment targeting VEGF has a strong biological rationale, aiming at reduced perfusion and tumor starvation due to oxygen and nutrient depletion. Bevacizumab has been shown to induce high response rates, but the effects on overall survival have been disappointing.3,4 However, only 2 recently published clinical studies have thus far provided circumstantial evidence for the hypothesis that bevacizumab does increase tumor hypoxia in patients with glioma.5,6 Although oxygen deprivation may have negative short-term effects on cell growth, evasive/adaptive responses to hypoxia can enhance migration and induce resistance toward radiotherapy, chemotherapy, and targeted therapy.7–11 Hypoxia has even emerged as a major factor that influences malignant progression and is mainly regulated by hypoxia-inducible transcription
factor 1 (HIF-1) or carbonic anhydrase IX and XII. Inadequate oxygen supply, even in highly perfused gliomas, may result from abnormal tumor vascularity, including increased interstitial fluid pressure, reduced blood flow, and even thrombosis of tumor vessels. There is evidence that anti-VEGF/VEGFR receptor therapy initially normalizes the tumor vasculature in preclinical models as well as in patients with gliomas, resulting in decreased interstitial pressure and improved tumor oxygenation. This increased oxygen delivery is possibly transient, and preclinical studies found persisting hypoxia in experimental gliomas exposed to inhibitors of angiogenesis.

Anatomical magnetic resonance (MR) imaging (MRI) is the method of choice to follow the treatment of recurrent glioma. With the development of more sophisticated MR techniques, specific tools are now available to monitor physiological and biochemical changes that can be directly linked to the cessation of oxygen and nutrient supply:

1. Blood oxygenation can be monitored by measuring susceptibility changes induced by the local content of deoxyhemoglobin (DeoxyHb). This requires the correction of quantitative T2* mapping for T2-spin-spin effects (T2′).

2. Hypoxia and other biochemical changes in the tumor environment may enhance glycolysis, augmenting lactic acid production. Therefore, increased extracellular acidosis causes an increase of intracellular pH by upregulation of H+ extrusion and buffering pathways. 31P MR spectroscopic imaging (MRSI) offers a tool to monitor intra- and extracellular pH in the brain.

3. Hypoxia in combination with an increased cellular plasma membrane pH gradient promotes cell proliferation. Biochemical markers of tumor cell proliferation can be obtained from 31P spectra by measuring compounds involved in the metabolism of the membrane phospholipids phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEth). Studies of cell lines and experimental tumors in animals indicated that the increase of the membrane phospholipid precursors phosphocholine (PCho) and phosphoethanolamine (PEth) may indicate mitogenic stimulation and tumor malignancy.

4. Depleted oxidative energy metabolism as a result of hypoxia and repressed mitochondrial function may be detectable by a decrease in high energy phosphates, such as ATP and phosphocreatine, which can be monitored by 31P MRSI.

5. Restriction of random molecular motion by cellular membranes can be measured by diffusion-weighted imaging (DWI) and is observed in brain ischemia. Bevacizumab may induce energy depletion by impairing vascular function and therefore restricting supply of oxygen and nutrients. A previous study described the occurrence of ADC reduction in the majority of responding patients with glioma, and 1 biopsy from a lesion with reduced ADC demonstrated necrosis with enhanced HIF-1-α expression, suggesting tissue hypoxia. However, a sound statement on bevacizumab-induced alterations of energy homeostasis requires additional investigations.

We have taken advantage of advanced MR methods to measure altered oxygen extraction, pH, depletion in energy metabolism, and modified membrane lipid turnover in tumor tissue, compared with normal-appearing brain tissue before and during therapy with bevacizumab. To our knowledge, this is the first study to have investigated in vivo combined 1H MRSI and 1H-decoupled 31P MRSI with additional quantitative ADC, T2′, and T2 mapping to monitor therapy-induced microenvironmental changes in recurrent GBMs and to relate these parameters with the clinical outcomes of the patients.

### Materials and Methods

#### Study Subjects

This report is based on a prospective, observational, noninterventional study including the extended MR protocol that was approved by the ethics committee of the university hospital Frankfurt, Germany (protocol number SIN 01/2009–4/09). Treatment with bevacizumab itself was not part of the study protocol, and the recommendation for the bevacizumab treatment and for any concomitant therapy (chemotherapy, radiotherapy, etc.) was solely in the responsibility of the treating physician. Patients agreeing to participate in the project, including the extended MR protocol, by written patient consent form were considered to be consecutive patients.

We investigated 16 consecutive patients (5 female and 11 male patients) with recurrent GBMs before and 1.4–2.1 months after the first cycle of bevacizumab (mean interval, 1.8 months) with multimodal MR techniques.

In between the 2 MRI measurements, all patients received bevacizumab at a dose of 10 mg/kg of body weight intravenously every other week. In 7 patients, irinotecan was coadministered at a dose of 125 mg/m², whereas the rest of the patients received bevacizumab only. The median interval between the first neurosurgical tumor resection and the MRSI examination was 11.4 months (range, 5.3–34.7 months).

Enrollment was restricted to patients with a histological diagnosis of GBM with radiologically confirmed recurrence according to the updated response assessment criteria (RANO) for high-grade gliomas. The group included 14 patients with primary and 2 with secondary GBMs. All patients were pretreated with radiochemotherapy plus temozolomide, adjuvant temozolomide, and additional chemotherapy before they were referred for bevacizumab therapy (Table 1). Treatment response after starting bevacizumab therapy was diagnosed according to the RANO criteria, except for the early control MRI after 4 weeks to confirm the response. Follow-up MRI after ~2.5 months revealed progression
in 2 of the responding patients for whom secondary therapy failure was suggested.

**MR Study Protocol**

MRSI and MRI of the brain were performed on a 3 T whole body system (Magnetom Trio; Siemens Medical AG). A double-tuned $^1$H/$^31$P volume head coil (Rapid Biomedical) was used for MRSI directly followed by the MRI, which was performed using an 8-channel phased array head coil.

**MRSI**—Spectroscopic imaging (Table 2) was planned on T2-weighted (T2-w) images in 3 orientations. The contrast-enhancing area of the recurrent tumor was known from the routine MRI.

For $^1$H MRSI, a transversal slice was positioned to cover a maximum of tumor tissue. The volume of interest (VOI), selected by a combination of the point

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Data are no. of patients, unless otherwise indicated. Fourteen patients had a primary glioblastoma (GBM), and 2 patients had a WHO grade II astrocytoma that transformed into a secondary GBM prior to bevacizumab.

$^*$One patient with secondary GBM already had radiation with 60 Gy for astrocytoma before GBM occurred.

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| Table 2. Sequence protocol of the study, performed on a 3 T whole-body system |
|-------------------------------|--------------------------------|
| Sequence                      | Number of acquisitions (NA); measurement time |
| 2D $^3$P MRSI; WALTZ4 proton decoupling | 1; 10:44 min |
| 2D $^1$H MRSI                 | 1; 4:45 min |
| 2D $^1$H-w; FLASH             | 1; 14:33 min |
| 2D $^1$H T2-w TSE             | 1; 2:08 min |
| Gradient-echo EPI PWI         | 2; 1:15 min |
| Spin-echo EPI DWI$^*$          | 2; 1:08 min |

| Abbreviations: DWI, diffusion-weighted imaging; EPI, echo planar imaging; GRAPPA indicates generalized autocalibrating partially parallel acquisition; PWI, perfusion-weighted imaging; w, weighted. |

$^*$PRESS volume (volume of interest) was normally 80 × 80 mm$^2$ (see Methods).
resolved selective spectroscopy (PRESS) and outer volume suppression, covered the center of the recurrent tumor area and the contralateral normal brain tissue. For $^{31}$P spectroscopy, a 3D MRSI slab aligned to the $^1$H MRS slice was used.

**MRI**—The MRI protocol (Table 2) included protocols for mapping quantitative $T_2$ and $T_2^*$ relaxation times, 3D $T_1$-w flash sequences before and after intravenous application of the contrast agent, a DWI sequence, and a dynamic susceptibility contrast (DSC) perfusion sequence with standardized intravenous contrast agent injection (0.05 mmol/kg gadobutrol [Gd-DOTA-butrol]) followed by a 20-mL bolus of 0.9% saline.

For quantitative $T_2^*$ mapping, a series of high-resolution $T_2^*$-w images with increasing TE was performed using a FLASH sequence (bandwidth, 300 Hz/pixel). The acquisition of 8 gradient echoes per excitation was done, increasing TE from 10 ms to 52 ms with a constant increment of 6 ms. All echoes were performed by successively inverting the readout gradient under the same readout gradient polarity to avoid misregistration due to different distortions in the presence of field inhomogeneities.

**Data Analysis**

**MRSI**—Data were sampled from voxels within the tumor and, as a control, from the respective area in the contralateral hemisphere. For each selected voxel, $^1$H spectra were analyzed with the software LCModel (Provencher; downloadable test version at http://s-provencher.com), whereas $^{31}$P data were analyzed with the program jMRUI. Signal intensities were corrected for T1 and T2 relaxations using values reported previously and averaged over the target region.

The LCModel analyzes the $^1$H MRSI spectra through a frequency domain–fitting routine using a linear combination of model spectra and was shown to be suitable for analyzing short-TE $^1$H MRSI spectra of brain tumors. Baseline correction was performed including macromolecules. The $^{31}$P data were analyzed with the program jMRUI, which we found more appropriate for these kinds of spectra because it can handle and report frequency shifts of inorganic phosphate (Pi) and β ATP. The time domain model function was composed of 14 exponentially decaying sinusoids in the frequency domain. Six of those, which had identical damping, corresponded to peaks assigned to phosphocreatine (PCr), phosphoethanolamine (PETH), phosphocholine (PCho), glycerophosphocholin (GPC), glycerophosphoethanolamine (GPE), and Pi. PCr was adjusted to 0 ppm, and constraints for the chemical shifts of the other signals except for Pi were applied as a fixed difference with regard to the position of PCr. Adenosine triphosphate (ATP) was represented by 7 exponentially damped sinusoids, defining each multiplet by the respective number of peaks with identical damping and adequate amplitude ratios. The pH values of the tumor and control tissue were determined from the chemical shift difference between Pi and PCr using the formula of Petroff et al., which is implemented in jMRUI.

All $^1$H MRSI spectra from the selected voxels were visually assessed for artifacts according to the criteria described by Kreis et al. In the fit of the $^{31}$P spectra, all signals were generally assigned correctly, leaving a flat line for the residual with equal distribution of noise.

Concentrations for $^1$H detectable metabolites were calculated by referring to an independent measurement in a spherical phantom containing an aqueous solution of 100 mmol/L acetate as a calibration standard. A repetition time (TR) of 10 s was applied to avoid T1 saturation. For $^{31}$P data, a spherical phantom with 20 mmol/L phosphate was used (TR 60 s).

**MRI**—The data analysis was performed in several steps, as follows:

1. The size of contrast-enhancing tumor tissue was delineated on the contrast-enhanced (CE) $T_1$-w image. Because we often observed T1 hyperintensities in the non–CE $T_1$-w images, final CE volumes were computed from the percent enhancement image ($T_1$-w CE/non–CE $T_1$-w).

2. The size of tumor-associated edema was delineated on high-resolution $T_2$-w images (TE = 103). Considering nonenhancing infiltrative tumor progression under bevacizumab, we also delineated tumor-like hyperintense $T_2$ areas.

3. The resulting VOIs were defined as contrast-enhancing tumor (VOI$_{CE-T}$), $T_2$-w visible tumor (VOI$_{t}$), tumor-associated edema (VOI$_{edema}$), and contralateral healthy hemisphere (VOI$_{Cn}$) (Fig. 2).

4. Quantitative maps for $T_2$ and $T_2'$ relaxation times were calculated offline with custom-built software tools (Fig. 2), whereas ADC and rCBV maps were generated using the vendor-provided software on the scanner console.

5. All VOIs were coregistered with the quantitative ADC, $T_2$, and $T_2'$ parameter maps, and mean parameter values for each VOI were recorded.

Details for quantitative MRI analysis

$T_2'$ mapping. The $T_2$ and $T_2^*$ relaxation times were computed in the native space with custom-built programs written in MATLAB (The Mathworks). $T_2$ and $T_2^*$ relaxation times were mapped pixelwise by exponential fitting of the respective image series. Maps of $T_2$ were calculated from $1/T_2 = 1/T_2^* - 1/T_2$, with $T_2 = 1/(1/T_2^* - 1/T_2)$. In contrast to previous studies, the $T_2'$ measurement in our study was based on high-resolution $T_2^*$ maps, which are prone to motion artifacts but allow optimal anatomical coregistration. Even though we cannot totally exclude such artifacts, a thorough visual inspection of the $T_2^*$-w raw images made sure that only patients without significant motion artifacts were taken into consideration. Furthermore, parameter values were only considered...
for the voxels with a high correlation coefficient between measured and fitted data. Last but not least, the fact that we observed stable T2 values in the normal-appearing tissue before and after treatment also supports minimal influence of any motion artifacts on our data. Although the T2 values are already corrected for edema-associated spin-spin effects, they are affected by susceptibility changes caused by paramagnetic substances, such as microbleeds and calcifications. Although these sources may be present in tumor region, it can be excluded to find them in edema.

Co-registration. After non–brain tissue removal using BET (part of FMRIB’s Software Library-FSL), each subject’s MRIs were co-registered with the subject’s pre-treatment T2-w image by means of linear registration using FSL’s FLIRT. T2 and T2′ parameter maps were also co-registered to the pre-treatment T2-w image.

VOIs. Parameter values before and during treatment were computed for the VOICET, VOITu, VOIedema, and VOICtr with the help of FSL. All defined VOIs excluded resection cavities.

To prevent any bias on data scattering caused by volume reduction and to maintain proportionality to edema volume, control VOIs were purposefully drawn smaller after therapy (thus, VOICtr significantly decreased in size with −40% in responders and −41% in nonresponders). VOIs were manually drawn in each image slice using the MRICroN software and were verified by an experienced neuroradiologist (E.H.). During VOI drawing, we paid special attention to consistency between the 2 measuring time points; areas that were determined to be tumor or edema on the basis of anatomical landmarks on pretreatment image were similarly designated to the same tissue group on the treatment image.

To determine the VOICET, we defined contrast enhancement as a 20% increase in T1-w image intensity after contrast agent injection. This enabled a semi-
automatic VOI\textsubscript{\text{CET}} definition by thresholding the ratio between the 2 co-registered T1-w images (T1-w CE and T1-w nonCE) and masking the results with crude VOIs drawn on the contrast enhanced T1-w image. Thresholding the ratio image not only offered an easy and more objective way of defining enhancement, but it also avoided pseudo-enhancement (eg, calcification) hyperintensity present on both the non-CE and the CE T1-w images.

The VOI\textsubscript{tu} was defined as regions of moderate T2-weighted hyperintensity showing the following characteristic pattern: (1) frequently inhomogeneous in a salt and pepper pattern and less bright than edema and CSF; (2) mass effect evident by sulcal effacement, midline shift, ventricular compression, etc.; (3) blurred gray-matter junction lacking “fingers of edema”;\(^5\) (4) thickening of the corpus callosum;\(^5\) and (5) infiltration of the cortical ribbon.

\section*{Statistical Methods}

The statistical analyses were performed using commercially available software (STATISTICA, version 7.1; StatSoft) for MRSI and freely available software (R-Statistics; R Development Core Team) for MRI parameter values. The nonparametric test for paired samples (Wilcoxon signed-ranks test) was used to test for differences in values for all metabolites, pH, ADC, T2, and T2’, comparing tumor tissue to control as well as tumor tissue before and during therapy. Also, significant differences of anatomical changes such as the contrast enhancing tumor area (VOI\textsubscript{\text{CET}}), the edema size (VOI\textsubscript{edema}), and tumor area delineated from T2-w MRI (VOI\textsubscript{tu}) were determined with the same nonparametric test. Correlations between ADC and quantitative T2 relaxation times were computed using nonparametric Spearman rank regression.

The relation of MRI parameters with progression-free survival (PFS) and overall survival (OS) after start of bevacizumab treatment was evaluated through the Kaplan-Meier analysis with the log-rank test (SPSS Statistics, version 14.0). Parameters were evaluated as quotients (percentage change during/before bevacizumab) and as normalized values (relative to the contralateral, healthy tissue) before and during bevacizumab therapy. The corresponding median value was used as a cut-off value to discriminate the 2 patient groups. \(P\) values <.05 were considered to be statistically significant for all analyses.

\section*{Results}

On the basis of RANO criteria, 10 responders and 6 nonresponders were identified. Responders were defined as patients fulfilling the RANO criteria for complete response or partial response. Nonresponders were defined as patients fulfilling the RANO criteria for stable disease or progressive disease.

In the group of responders, significant decreases for VOI\textsubscript{\text{CET}} (\(-68\%)\), VOI\textsubscript{edema} (\(-60\%)\), and VOI\textsubscript{tu} (\(-30\%)\) were found during therapy. For the nonresponder group, the decrease in edema size was significant (\(-69\%)\).

\section*{MRSI}

The results of MRSI are summarized in Table 3.

Considering all patients prior to therapy with bevacizumab, recurrent GBMs were associated with lower tNAA, tCr, and PCr concentrations as well as higher Pi/PCr and Pi/ATP ratios and higher pH\textsubscript{i}, compared with contralateral (control) tissue (Fig. \text{3}). Furthermore, PEth/GPE was increased; GPE was decreased in recurrent GBM versus control tissue.

In a subgroup analysis of responders and nonresponders, only recurrent GBMs of the responders showed significantly higher pH\textsubscript{i} at baseline. Nonresponders had significantly lower GPE and GPC. Upon treatment, the increased pH\textsubscript{i} reverted to normal values in the subgroup of responders (Figs 4 and 5). Furthermore, responders showed a significant increase in GPE. None of the other metabolites changed significantly during therapy with bevacizumab. Data from control ROI did not change significantly during therapy.

\section*{Quantitative MRI}

Before therapy, all patients had significantly higher T2 and ADC values in all VOIs, compared with the respective parameters in the control region (Fig. 6). The T2’ values in the edema, but not in the tumor region, were increased. During treatment, the mean ADC, T2’ and T2 values in VOI\textsubscript{\text{CET}}, VOI\textsubscript{edema} and VOI\textsubscript{tu} decreased significantly in the responder group (Fig. 7) while the nonresponder group did not demonstrate such a clear parameter decrease as the responder group in all of these areas. The nonresponder group demonstrated significantly (\(P = .03\)) less T2’ reduction in the contrast-enhancing VOI than the responder group did.

In all subjects and for each VOI on the affected side (but not for the control VOI), ADC significantly correlated with the T2 values (VOI\textsubscript{CE} rho = 0.6 [\(P < .001\)]; VOI\textsubscript{edema} rho = 0.8 [\(P < .001\)]; VOI\textsubscript{tu} rho = 0.4 [\(P = .03\)].

\section*{Outcome Depending on Different Parameters}

Nineteen months after onset of the study, all but 2 patients had a progression of their contrast-enhancing tumors and died of progressive disease. One patient was lost during follow-up. The median PFS duration for the remaining 13 patients was 3.7 months, and the median OS duration was 6.7 months. The 2 patients without progression had follow-up durations of 14.1 and 12.9 months.

Interestingly, the reduction of VOI\textsubscript{\text{CE}} during treatment correlated with PFS but not with OS. The volume reduction of VOI\textsubscript{\text{tu}} and VOI\textsubscript{edema} correlated with neither PFS nor OS.
Table 3. Results of $^{31}$P and $^1$H MRSI

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<th>$\text{P}i/\text{ATP}$</th>
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Data are mean values and standard deviation (SD) for the ratios and concentrations of tumor ROI and control before and under therapy with bevacizumab (BZ). Significant differences between metabolites of tumor tissue vs. controls are indicated in boldface font, significant changes of metabolites prior vs. post starting BZ are marked in boldface italic font. Control indicates contralateral hemisphere of the patients; non-resp indicates nonresponders; pts indicates patients.

Fig. 3. Metabolite ratios obtained from $^{31}$P magnetic resonance spectroscopic images for tumor and contralateral control tissue from all patients before treatment with bevacizumab. The first row shows the membrane phospholipid compounds containing choline (PCho/GPC) and ethanolamine (PEth/GPE), and the second row depicts changes of high-energy metabolites ATP and PCr related to inorganic phosphate (Pi). Stars denote significant differences between metabolite ratios of the tumor and control.
In contrast, patients with a more pronounced reduction of T2′ values (−25%) in the VOI CE upon treatment not only had longer PFS durations but also had significantly longer OS durations (𝑃 = .02 and 𝑃 = .039, respectively) (Fig. 8). In addition, patients with a more pronounced T2′ reduction in the VOI tu had longer OS (𝑃 = .021) and a tendency towards longer PFS (𝑃 = .089). Furthermore, patients with higher values for pH decrease upon treatment in the VOI CE had longer PFS durations (𝑃 = .025). Normalized values of various parameters (ADC, T2′, T2, pH, and GPE) before and during therapy demonstrated no significant relation to PFS and OS. Only higher T2′ values showed a trend toward longer PFS duration (𝑃 = .074).

Discussion

This study was aimed to monitor physiological and biochemical changes in tumor microenvironment of recurrent GBMs after treatment with the antiangiogenic agent bevacizumab. The treatment effects should be most pronounced in the GBMs responding to bevacizumab therapy and also might have implications on the clinical prognosis of the patients.

Before treatment, tumors exhibited higher ADC, T2, and pH and lower PCr and GPE concentrations than did the control region. The 31P data indicate limited energy supply under conditions of cell proliferation, which confirms the findings of previous reports.55–57 Increased ADC and T2 values, as well as a strong correlation between the 2 parameters, were also reported in gliomas.58 We found similar T2 relaxation times in the tumor and in edema, which is supported by a human study59 but not by animal models.60,61

Six to 8 weeks after onset of treatment with bevacizumab, ADC, T2, and T2′ of CE and non-CE tumor areas decreased only in responders, with ADC and T2 approximating normal values and T2′ lowering under normal values. Lowered high energy phosphates did not recover, whereas the pH decreased to almost normal values in responders.

Confining therapy response to decrease in CE tumor volume may disregard growing non-CE tumor tissue.24,62 However, an exact delineation of non-CE tumor volume is critical, because infiltrating gliomas lack defined borders and signal changes on T2/FLAIR images may not be differentiated from edema and other therapy-induced changes. Nonetheless we could delineate a tumor volume on T2-w images based on several imaging criteria (see Materials and Methods),24,63 which indeed differed from edema regarding the T2′ values.

We could show that the long-term effect of antiangiogenic therapy reduces oxygen and energy supply of the tumor and increases oxygen extraction per tissue volume.25 Persistent decrease of high-energy metabolites and their ratios to Pi as well as the decrease in T2′ values (indicating increased Deoxy-Hb) during therapy support the hypothesis that bevacizumab-induced vessel rarification depletes nutrient and oxygen supply. Reduced oxidative phosphorylation and/or shift to glycolytic pathways may also be considered. This is important because metabolic alterations, such as hypoxia and reduced mitochondrial respiration, are modulators of the tumor phenotype.8,14–16,26,29,32 Decreasing levels of high-energy metabolites in tumors have been reported previously during several therapies, such as treatment with tumor necrosis factor.64 whereas radiotherapy and chemotherapy with 1.3-bis(2-chloroethyl)-1-nitrosourea
caused increased ATP/Pi and PCr/Pi ratios in animal tumor models.\textsuperscript{55,57} This increase was explained by treatment-related tumor re-oxygenation shifting tumor metabolism from anaerobic glycolysis to glucose oxidation.\textsuperscript{57}

Our findings of bevacizumab-induced increased tumor hypoxia and impaired energy homeostasis raise the question whether these effects on the tumor environment promote or delay tumor growth. Tumor hypoxia increases the aggressiveness and tumor resistance to chemotherapy and radiation mediated by the activation of HIF-1\textalpha{} and carbonic anhydrase IX and XII.\textsuperscript{7–16} Furthermore, HIF-1\textalpha{} increases glycolysis and reduces oxidative phosphorylation, thus enhancing tumor cell resistance toward hypoxia.\textsuperscript{26} There is evidence that a hypoxic tumor environment may be a selection factor toward therapy resistance.\textsuperscript{11} It is worth mentioning that therapy-resistant tumor stem cells were increased in hypoxic tumor areas and that GBM stem cells were enriched in perinecrotic niches.\textsuperscript{16,65} Therefore, tumor hypoxia may facilitate a selection toward increasing malignancy and increasingly aggressive phenotypes. However, the effect on recurrent or primary GBMs in vivo is still discussed. Although clinical series reported a more infiltrative pattern of recurrence in GBM during bevacizumab therapy, recent clinical analyses did not support a specific predisposition of bevacizumab to induce diffuse gliomatosis cerebri–like growth pattern or distant spread at recurrence.\textsuperscript{66,67}

The results of this study also do not support the hypothesis that bevacizumab-induced hypoxia might promote a more aggressive tumor growth because therapy-induced hypoxia is not associated with shorter OS duration. Patients with pronounced T2' reduction (ie, an increase in DeoxyHb) upon treatment in the CE tumor (VOI\textsubscript{CE}) and in the non-CE tumor (VOI\textsubscript{n}) even survived for a longer period. Therefore, tumors that exhibited a more extended decrease in oxygen concentration versus their status before treatment had a more favorable outcome. Given that the pretreatment T2'
values tended to be higher in patients with longer PFS durations, it remains disputable whether the T2' decrease during treatment instead represents a normalization of luxury tumor oxygenation or indicates real hypoxia. However, the decrease in T2' values to less than normal values during treatment in the responding tumors support the thesis that bevacizumab treatment induces long-term tumor hypoxia. It is noteworthy that the reduction of the tumor volume (enhancing or nonenhancing) did not yield longer overall survival time.

Apart from T2', the ADC value is known as indirect marker of hypoxia, decreasing in acute ischemia due to critical oxygen deficiency. Chemotherapeutic agents usually induce an increase of ADC and T2 values due to tumor necrosis. In contrast, bevacizumab is causing rather a decrease in ADC and T2 values. Our findings are consistent with a recent larger series demonstrating that bevacizumab-induced ADC restriction is predictive of better outcome. Less interstitial water as result of the blood-brain barrier normalization during bevacizumab therapy may in part cause a correlated decrease of ADC and T2 in responding tumors. A decrease in ADC has been discussed as an indicator of swelling of hypoxic tumor cells, supported by the fact that expression of HIF-1-α was markedly enhanced in a lesion with reduced ADC. However, the bevacizumab-induced T2 shortening is not explained by cell swelling but may result from a decrease in the blood water fraction, which has longer T2 times than parenchymal water, and/or from an increased binding and compartmentalization of water protons, and/or from paramagnetic influence of molecules, such as deposited heme, nonheme iron, and deoxyHb.

Fig. 6. Pretreatment (before bevacizumab) mean T2', T2, and ADC values in the volumes of interest (VOIs) of contrast-enhancing tumor (CET), edema, and tumor delineated on T2-w images (tum) compared with the same parameters in the control VOI (ctr). Resulting $P$ values of this comparison are provided above each plot. The left panel represents the responder group, the right panel the nonresponder group, and the line in each plot denotes the mean parameter value in the control VOI.
Alternatively, an observed ADC decrease can be a result of increased cell density due to cell proliferation\(^74,75\) anticipating that sustained high-energy depletion during bevacizumab treatment may promote tumor growth.\(^72\) However, the observed shrinkage of tumor volume by \(\sim 30\%\) can only be explained by a considerable fraction of dying cells. In the case of a sole reduction of interstitial water, the metabolite concentrations should significantly increase. Furthermore, normalization of alkaline tumoral pH\(_i\) indicates reduced malignancy. The maintenance of an alkaline pH\(_i\) in tumor cells has been shown to support cellular proliferation.\(^14,15,27–30\) The alkaline pH\(_i\) was a characteristic finding in previous in vivo \(^31\)P MRS studies of human primary GBMs.\(^76–79\) Increased pH\(_i\) under conditions of anaerobic glycolysis was explained by the enhanced activity of H\(^+\) extruding pathways like the Na\(^+\)/H\(^+\) exchanger or by buffering intracellular protons via the transmembrane carbonic anhydrases, both counteracting the intracellular proton accumulation.\(^14,15,30\) Consequently, the extracellular environment gets more acidic, enhancing the invasiveness of tumor cells and promoting angiogenesis.\(^80,81\) By normalizing these microenvironmental changes, bevacizumab reverses growth and aggressiveness of recurrent GBMs, which may explain its antitumoral effect.

The significant increase in the catabolic membrane lipid GPE in responding tumors points in the same direction because it may indicate therapy-induced membrane degradation in dying cells.\(^82,83\) According to experimental tumor studies, increase of the phosphomonoesters (PME), PEth, and PCho is also related to tumor malignancy,\(^33,34,84\) with ethanolamine-bound phospholipids prevailing in in vivo tumors.\(^31,34,85–87\) The biological effects of preceding radiochemotherapy may explain the absence of significant PEth increase in recurrent GBMs before bevacizumab therapy, whereas in nonresponding tumors, a decrease of the catabolic phosphodiesters (PDE) GPE and GPC may indicate selection of therapy-resistant tumor cells during preceding treatments.

To our knowledge, this is the first in vivo \(^31\)P MRS study discriminating Cho-containing and Eth-containing phospholipid compounds in brain tumors by using \(^1\)H-decoupled \(^31\)P MRS. In previous \(^31\)P MRS studies conducted at 1.5 T without \(^1\)H-decoupling, the PME and PDE signals were broadened by spin-spin coupling. Also, at 1.5 T, the PDE signals are concealed by a broad background peak, which has been assigned to other membrane phospholipids\(^88–91\) and is almost suppressed at 3 T. These technical limitations may explain inconsistent results of previous \(^31\)P MRS studies in brain tumors.\(^35\)

High pH\(_i\) in responders and low PDE in nonresponders before bevacizumab therapy favor the hypothesis that these metabolic changes may be predictive of a short-term therapy response. Even more relevant, increased tumoral T2' values before therapy and a more pronounced T2 decrease upon bevacizumab treatment may predict PFS and OS.

To conclude, this is the first multimodal study combining quantitative MRI with \(^1\)H and \(^31\)P MR spectroscopic imaging, allowing monitoring biological changes in recurrent GBMs 6–8 weeks after initiation.
of treatment with the antiangiogenic antibody bevacizu-
mab. Our results indicate that bevacizumab induces rela-
tive tumor hypoxia with impaired energy homeostasis
and even has antitumoral activity in the responding
GBMs. The real antiangiogenic effect of bevacizumab
is predictive of better outcome.

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