FACULTY OF HEALTH AND MEDICAL SCIENCES
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PhD thesis

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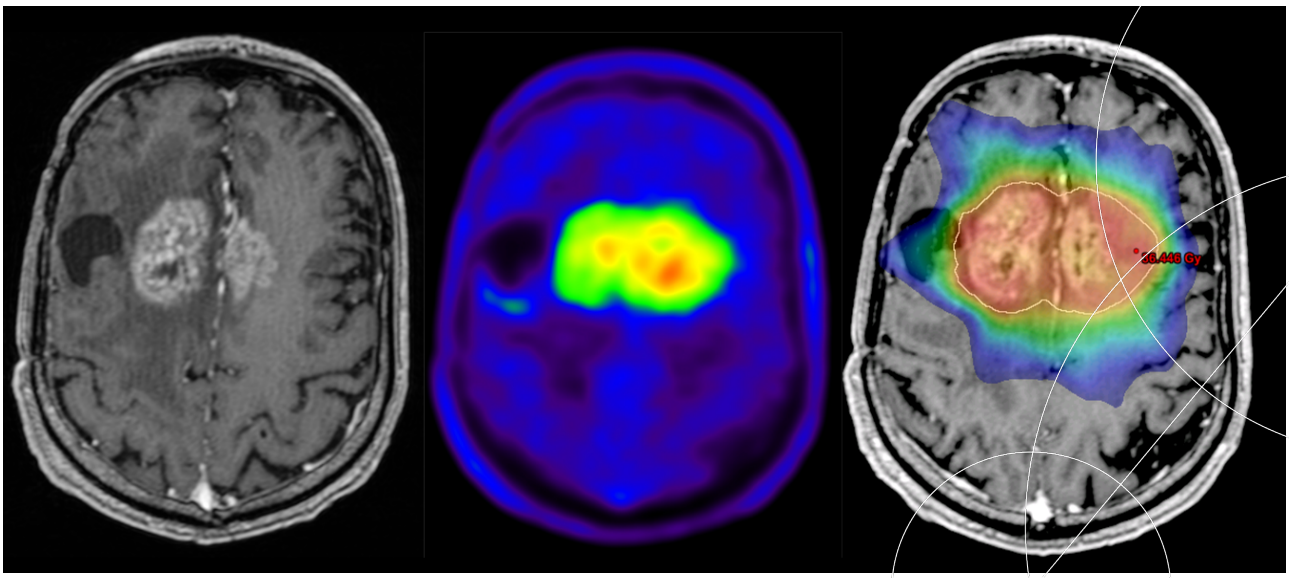
Advanced PET- and MR-imaging in re-irradiation of high-grade glioma



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Contents

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Dansk resumé

Primær hjernekræft (højgradsgliom) er en sjælden men meget alvorlig lidelse. Sygdommen vokser ind det omkringliggende væv og er årsag til neurologiske udfald, epilepsi og svækkede kognitive (intellektuelle) færdigheder. Behandlingen er aggressiv og kan have følgevirkninger, der ligner sygdommens symptomer. Desværre er tilbagefald næsten uundgåelige og målet med behandlingen er således at forlænge livet med acceptabel livskvalitet. Radioterapi udgør en hjørnesten i behandlingen. På mange hospitaler foretages også gentaget strålebehandling ved tilbagefald, men hverken bivirkninger eller effekt er vurderet på systematisk vis med brug af moderne teknologi.

Målet for dette Ph.d. projekt var at evaluere bivirkninger og effekt ved genbestråling af højgradsgliom og at evaluere værdien af positron emissionstomografi (PET) med et aminosyresporstof ved genbestråling. Endvidere at identificere biomarkører i de billeddannende undersøgelser, som kunne forudsige det kliniske forløb efter strålebehandling.

To fremadrettede studier blev udført; et fase I/II studium af genbestråling til patienter med tilbagefald af højgradsgliom og et observationsforsøg med måling af tumors blodgennemstrømning i forløbet af strålebehandling ved brug af magnetisk resonans billeddannelse (MR). Som led i vurderingen af bivirkninger foretog vi kognitiv testning af patienterne i genbestrålingsforsøget.

Forsøgene viste, at bivirkningerne ved genbestråling var acceptable men ikke betydningsløse. Tumors størrelse vurderet ved PET viste sig at være prognostisk for overlevelse efter behandlingen og PET bidrog sandsynligvis med vigtig information til brug for planlægning af radioterapi. Det var muligt at beskrive patienternes kognitive funktion kvantitativt forud for behandlingen og vi har dokumenteret ændringer efter genbestråling, som ikke er beskrevet hidtil. Målingerne af tumors blodgennemstrømning viste overraskende, at denne øgedes tidligt under strålebehandlingen for siden at falde. Omend ændringerne ikke kunne korreleres til det kliniske udfald, kan resultaterne være hypotesegenererende.

English abstract

Primary brain cancer is rare but devastating disease. The cancer infiltrates healthy brain tissue and causes neurological symptoms, seizures and cognitive dysfunction. The treatment is aggressive and may have late adverse effects that mimic the symptoms of the disease. Recurrence is almost inevitable and the goal of all treatment is to prolong life while maintaining quality of life. Radiotherapy is a cornerstone of treatment. In many hospitals repeated irradiation is attempted at recurrence but neither side-effects nor efficacy have been systematically evaluated using modern technology.

The goal of this Ph.D. project was to evaluate the side-effects and efficacy of re-irradiation of high-grade glioma and to determine the value of positron emission tomography (PET) using an amino acid tracer for re-irradiation. Moreover, to identify imaging biomarkers capable of predicting the clinical course following radiotherapy.

Two prospective studies were carried out; a Phase I/II study of re-irradiation to patients with progressive high-grade glioma and an observational study where tumor blood perfusion was measured using magnetic resonance imaging (MRI) in the course of radiotherapy. The patients in the re-irradiation study underwent cognitive testing as a means of assessing side effects.

The studies showed that the side effects of re-irradiation were acceptable but not negligible. Tumor size evaluated by PET was prognostic for survival following radiotherapy and it PET likely contributed valuable information for use in treatment planning. We were able to describe the cognitive function of the patients in a quantitative way and to document changes prospectively, which has not previously been described. Tumor perfusion, surprisingly, was shown to increase significantly during early stages of treatment and later decrease. While these changes did not correlate to outcomes, they may form the basis for generation of hypotheses.

List of papers

In addition to the results presented in this synopsis, this thesis consists of three original manuscripts:

1. Søren Møller, Ian Law, Per Munck af Rosenschold, Junia Costa, Hans Skovgaard Poulsen, Svend Aage Engelholm, Silke Engelholm. Prognostic value of ^{18}F-FET PET imaging in re-irradiation of high-grade glioma: Results of a prospective trial. *Submitted.*

2. Søren Møller, Per Munck af Rosenschöld, Michael Parsons, Silke Engelholm, Hans Skovgaard Poulsen, Svend Aage Engelholm. Cognitive function before and after re-irradiation of high-grade glioma: Results of a prospective trial. *Submitted.*

3. Søren Møller, Michael Lundemann, Ian Law, Hans Skovgaard Poulsen, Henrik BW Larsson, Svend Aage Engelholm. Early changes in tumor perfusion during radiotherapy evaluated by DCE-MRI.

Introduction

Few diseases are as aggressive and devastating as high-grade glioma. It is incurable and all treatment aims to prolong survival while maintaining quality of life. But a minority of patients may survive for several years. During the course of disease, treating clinicians are faced with difficult questions: How to choose the best treatment for this patient? What are the side-effects and are they so severe that treatment is not justified? How to evaluate the effect of treatment?
It is clear that a 'one-size-fits-all' approach is not sufficient and that all treatment benefit must be weighed against the disadvantages. This is the case in all of medicine but nowhere is it more important than in the field of neuro-oncology. Re-irradiation may be a treatment option for some patients at recurrence but neither benefits nor adverse effects have been determined in a systematic fashion.

The aim of this Ph.D. project was to:

- determine the toxicity of re-irradiation for recurrent high-grade glioma
- evaluate positron emission tomography for planning of re-irradiation of high-grade glioma
- identify imaging biomarkers of outcomes in both primary radiotherapy and re-irradiation for high-grade glioma

The results that are presented were generated in two prospective clinical studies. Study 1 was an interventional trial that examined the safety of re-irradiation of recurrent high-grade glioma and the value of positron emission tomography using an amino acid tracer. Study 2 was observational and used dynamic magnetic resonance imaging to examine early changes in tumor perfusion during the course of chemo-radiotherapy for glioblastoma in correlation with treatment outcomes.
The preliminary results for the main endpoints of Study 1 are presented in this thesis, which also contains three original manuscripts. Manuscripts 1 and 2 describe results from the re-irradiation study and manuscript 3 contains the final results of the MRI-perfusion study. In the section *Background,* the motivation for this work is discussed as well as the theory and practice of the two main imaging modalities used. The *Methods* section contains an outline of the protocol for the re-irradiation study. The *Results* section is based primarily on the re-irradiation study whereas all the manuscripts form the basis for the *Discussion* section. In *Conclusions,* the main results of this Ph.D. project are summarized and *Future perspectives* are discussed.

Background

High-grade gliomas are malignant tumors arising from the glial cells of the brain. Histologically and clinically they may be divided into WHO grade III or grade IV, with grade IV being the most common and most aggressive type - also known as glioblastoma (GB).
Typical symptoms include seizures, focal neurological deficits, cognitive impairment and fatigue. The primary treatment is surgery followed by radiation therapy to the residual tumor including a 2 cm margin of the surrounding brain tissue. Concomitant and adjuvant chemotherapy with temozolomide improves overall survival and is well tolerated but the treatment duration is nine months whereas the median survival is just 15 months [1]. Newer studies of elderly patients (> 60-65y) have shown that a shorter course of radiotherapy or monotherapy of temozolomide may be equally effective [2, 3]

However, the disease almost inevitably recurs. Despite the infiltrative nature of the disease, most relapses occur within the previously treated area [4]. Treatment options at recurrence include re-resection, chemotherapy or experimental treatment. A repeated course of radiotherapy may pose a risk of serious neurological toxicity. But developments in imaging- and radiotherapeutic technology have made it possible to define a target and deliver radiation dose with high accuracy. Successful re-irradiation has been described in a number of papers [5–7]. Results have generally been good and low frequencies of adverse effects along with long survival periods have been encouraging. Necrosis accompanied by edema seems to be the most common serious adverse event. But most of the literature available about re-irradiation is based on retrospective series and may be subject to selection bias and incomplete reporting and -follow-up. Different treatment regimes for re-irradiation of HGG have been described but no formal guidelines exist. The effect on neurocognitive function has not been assessed.

Accurate and precise imaging is a prerequisite for precise radiotherapy. MRI is the gold standard for imaging of glioma during planning treatment, evaluation and follow-up [8]. It is sensitive in detecting tumor but lacks specificity due to the various conditions that may cause signal changes or disruption of blood-brain-barrier (e.g., post-operative edema, effects of radiotherapy, ischemia, demyelination, inflammation, seizures etc.). Positron emission tomography (PET) exploits the selective uptake in tumor cells of chemical substances in trace amounts that are labeled with a radioactive positron emitting isotope, often referred to as tracers. The decay of the radioisotope may

then be registered and co-localized with structural images, in the brain preferably from MRI. In 2011, the amino acid analogue 18F-fluoroethyltyrosine became an available tracer at Rigshospitalet. Clinical studies indicate that the 18F-FET-PET/CT technique carries a higher specificity than MRI [9] and an equally high sensitivity.

The following topics are described in further detail below: re-irradiation of HGG, 18F-FET PET imaging, assessment of cognitive functioning in HGG patients and evaluation of tumor perfusion using MRI.

Re-irradiation of high-grade glioma

Outcomes following re-irradiation of relapsed HGG have been described in several articles (summarized in Table A, please see appendix). A similar body of evidence for radiotherapy in combination with systemic therapy (chemotherapy or the anti-VEGF antibody, bevacizumab) and brachytherapy. But for the purpose of comparison and drawing conclusions, we shall focus only on single modality external beam radiotherapy.

Extracting any general information is challenging. The events of 'serious toxicity' and 'radiation necrosis' are not well defined. There is a large variety of treatment regimes (doses, fraction size, treatment volumes), imaging modalities (CT or MRI), size of treated volumes and follow-up (not in table) that may impact both the 'true' incidence- as well as the detection of radiation necrosis. Rates of overall survival and progression-free survival may be confounded by surgical tumor resection prior to radiotherapy and be subject to selection bias.

The recruiting periods predate the wide adaptation of temozolomide in 2005. Temozolomide is a known radio-sensitizer and is believed to increase the rate of pseudoprogression following primary RT. It is conceivable that the addition of temozolomide may decrease the tissue tolerance to repeated irradiation.

The majority of studies are retrospective. As radionecrosis may occur many months after treatment when the patient is receiving salvage treatment (which may even take place in another department) or is beyond active therapy, retrospective studies are likely to underestimate the true incidence of adverse late effects such as radionecrosis.

The studies by Combs et al [5] and Fogh et al [6] found remarkably low rates of toxicity. Combs et al. reported retrospectively the outcomes following 'fractionated stereotactic radiotherapy' (median dose: 36 Gy in 2 Gy fractions) in 101 HGG patients. Overall survival following treatment compared

favorably with historical results (OS: ca. 10 months vs. 6 months)[10] and only one case of radionecrosis was observed. Fogh et al. reported almost identical results in a similar retrospective study of FSRT (median dose: 35 Gy in 3.5 Gy fractions) in 147 HGG patients. In this study, only one patient was reported to suffer any serious adverse event (grade 3 headache). These two studies have been cited widely and have been interpreted as important evidence of the tolerability and efficacy of re-irradiation for relapsed HGG.

Higher incidences of toxicity have also been reported. A prospective dose escalation study from the Royal Marsden Hospital found late toxicity in 36% of patients (45% in actuarial rates at 24 months post treatment) [11]. Radiation dose was concluded to be the most important risk factor, but toxicity was also seen in doses $\leq$ 35 Gy. Although this study differs from those of Combs and Fogh in other ways, it is likely that its' prospective nature and well defined aim of determining toxicity contributes to the much higher rates of toxicity that were found.

Mayer et al. carried out a quantitative analysis of radiotherapy-related risk factors of brain necrosis following re-irradiation for recurrent glioma [12]. Only the cumulative radiotherapy dose was found to be clearly correlated to necrosis. The authors of this study did not address the issue of differences in follow-up mentioned above that are likely to be important: retrospective vs. prospective, imaging modality and study endpoints. Reviewing the literature regarding primary RT also, Lawrence et al. found the irradiated brain volume as well as the fraction size to be important risk factors for necrosis [13]

In conclusion, the existing literature suggests that fractionated re-irradiation to limited volumes of recurrent high-grade glioma may be carried out safely but prospective studies assessing safety and using modern treatment- and imaging modalities are needed. The standard of care has changed since most of the available studies recruited- and treated patients, and this also supports the need for new prospective studies.

Positron emission tomography

Basics

Unstable atoms that undergo β+-decay emit positrons that annihilate into two 511 keV photons heading in opposite directions. By registering simultaneous arrivals at opposite ends, a detector can

count the number of decays within its perimeter and the location of a radioactive isotope can be determined [14] as depicted in the figure below.

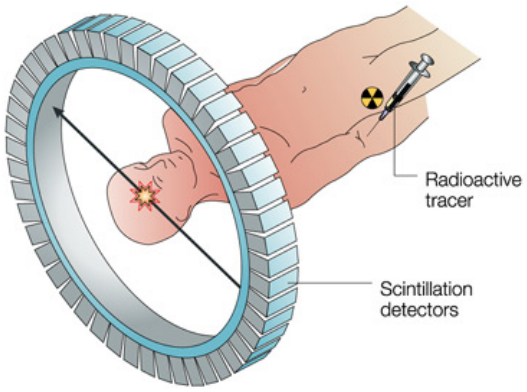


Fig. 1. A schematic illustration of a patient in a PET-scanner. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, copyright 2004 [15] .

Thus, by labeling a molecule with a radioisotope and injecting it into an organism, it is possible to reconstruct an image of the distribution of the molecule. This yields spatial and quantitative information and is the basis of positron emission tomography (PET) [14]. Although the system only registers a small fraction (<1%) of the actual number of decays, it is sensitive enough that only small concentrations of radioactive tracer in the pico- or nanomolar range can be measured, which do not normally influence the physiology of the organ or system being studied. The primary application of PET is currently within clinical oncology. It is routinely used for diagnosing, staging, therapy planning and follow-up of anticancer treatment. The most commonly used isotope is 18F-flourine, which can be used to label glucose molecules as 18F-FDG and amino acid analogues like ethyl-tyrosine as 18F-FET, among others. ^{18}F has a half-life of approximately two hours which is a practical advantage compared to shorter lived isotopes (e.g. ^{11}Carbon, 20 minutes [16]).

18F-FET

An ideal tracer for oncological purposes would be distributed only in viable cancer cells (100 % specificity) and would detect all cancer cells in all patients (100 % sensitivity). FDG, which is the most commonly used tracer in nuclear medicine, clearly is not ideal for visualizing brain tumors, primarily because of the high glucose metabolism of the healthy brain.

18F-FET is better suited for this purpose because the healthy brain only takes up small amounts of amino acid under normal circumstances.

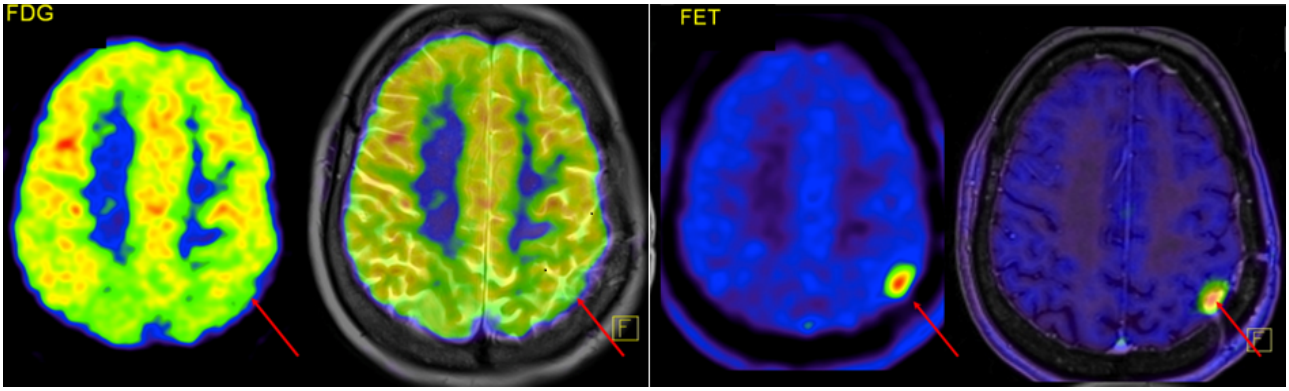


Fig. 2. A small recurrent glioblastoma in the left hemisphere visualized with 18F-FDG PET (left panel) and 18F-FET PET (right panel). *Courtesy of Ian Law.*

When using 18F-FET PET in the primary diagnosis of unselected brain lesions the sensitivity is high (87% for all lesions and 100% for glioblastoma) but the specificity is not (68%) [17]. This is also the case with MRI, which has exquisite sensitivity to detect structural lesions but poor specificity. But a distinction should be made between sensitivity for detecting any disease in a patient and the ability to detect *all* of the disease. The latter is important for local treatment that consists of surgery followed by radiotherapy. Independent reports have shown that the residual volume of 18F-FET PET positive tumor tissue after resection was prognostic for survival whereas the contrast enhancing volume on MRI scan was not [18] [19]. In the case of pretreated high-grade glioma, the main diagnostic challenge is to distinguish between recurrent, progressive tumor growth and treatment related changes which may mimic tumor when evaluated only by MRI [20]. In this setting, a study by Rachinger et al. found 18F-FET PET to have a superior specificity of 93% compared to 50% for MRI [9]. This has important implications when considering re-irradiation as a treatment option for recurrent HGG. Firstly, because 18F-FET PET may aid in establishing the diagnosis of tumor recurrence. Secondly, because precise delineation of tumor is especially important when large margins - and thereby large irradiated volumes - may pose an increased risk of treatment related toxicity [12].

Neurocognitive function in high-grade glioma

The higher functions of the human brain include memory, language, processing of information, creativity, reasoning, learning, abstract thinking, social interaction and many more. These functions are essential for normal life. A majority of patients with high-grade glioma suffer some degree of cognitive dysfunction and it is the most frequent symptom among long-term survivors [21]. Yet the

standard methods of assessing patient status in neuro-oncology (the Macdonald response evaluation [22] and the ECOG performance status [23]) only crudely evaluate dysfunctions in these domains. This is due to the obvious difficulties associated with measuring cognitive dysfunction as opposed to the relative ease with which a visual-field defect, for example, can be diagnosed. Psychometric tools that quantify dysfunctions in cognitive domains have been available for many decades, but the application of these tests in the field of neuro-oncological research has not been widespread until the recent years. In the pivotal study by Walker et al. in 1978 [24], which demonstrated a significant survival benefit from post-operative radiotherapy for HGG, patients were treated with whole brain irradiation to a dose of 60 Gy. Most radiation oncologists today would consider this treatment to be toxic and detrimental to cognitive function, but this was not recognized at the time and there was no better way to treat the patient. Today, cognitive function is evaluated as an integral part of large phase III trials of GB patients [25][26] as it is recognized that quality of life is affected adversely by cognitive dysfunction [27] and as tools to measure this have been validated in glioma patients [28]. In Study 1, the main aim was to evaluate toxicity to re-irradiation using state-of-the-art methods, and we found cognitive testing to be one tool for this purpose, in addition to the imaging modalities and the regular clinical examinations. The test battery that was used is described briefly in the *Methods* section and in further detail in Manuscript 2, where the considerations for choice of tests are also discussed.

Perfusion MRI

Principles of MRI

Magnetic resonance imaging (MRI) is based on the principle of inducing- and measuring changes in magnetization of hydrogen nuclei. In the static magnetic field of an MRI scanner creating a strong B0-field, the hydrogen atoms of a patient will align due to their magnetic nature. When subjected to ‘sideways pushes’ of radio waves of a certain frequency (the resonance frequency), the atoms will start to oscillate. As the pulses continue, the direction of net magnetization is shifted away from the longitudinal axis of the B0 field. When the pulses are discontinued, the atoms will continue to oscillate for a period of time which produces radio waves that now generate voltage in a receiving coil described by the Faraday’s law of induction. The time it takes for magnetic equilibrium to be restored is described by the relaxation times T1 and T2. The loss of the transverse magnetization is measured as the time period T2 and the reestablishment of the longitudinal magnetization is measured as T1. Different tissues display different T1 and T2 times and by adjusting the time

periods and intervals of the transverse impulses and the subsequent measurements, images can be T1 weighted or T2 weighted or just proton weighted and will serve different purposes [29]. In the presence of Gadolinium ions, the 1/T1 and 1/T2 times are increased significantly and are correlated to concentration to the concentration of Gd in a linear fashion. Thus, the presence of the contrast agent improves the ability to discriminate between different tissues and materials [30].

Perfusion MRI

In addition to producing static anatomical images of high quality, so-called dynamic sequences can be generated over a specific time course to measure physiological information. For the purpose of this thesis, we will focus only on perfusion, which is one of the most basic functional parameters of the brain. Perfusion is the volume of blood flowing through a tissue per second. The SI unit is ml/(100g/min). Other related parameters are often analyzed in conjunction with flow. Cerebral blood volume (CBV [ml/100g]), permeability of the BBB (K^{trans} [ml/(100g/min)]) and volume of distribution (CBVd [ml/100g]) are examples of such parameters.

Brain perfusion has clinical interest because a variety of conditions are either caused by altered perfusion (stroke) or may be monitored using perfusion weighted MRI (e.g. multiple sclerosis [31]). Gliomas are characterized by high levels of angiogenesis as well as hypoxia [32] and this makes them an interesting target of research for MRI perfusion. Clinical studies have shown that pre-treatment regional blood volume [33] and early changes in tumor blood volume and regional blood flow were predictive of survival [34] following radio-chemotherapy. These studies have also shown that a heterogeneous pattern of responses within a tumor may occur, with increases in blood volume in some areas and decreases in others.

DSC-MRI vs. DCE-MRI

Almost all studies of brain tumor perfusion have used a method called the dynamic-susceptibility contrast method (DSC-MRI). It is based on tracking a bolus injection of Gadolinium-based indicator from a peripheral vein as it passes through the brain, measuring the transient drop in the T2 and T2* signal[1].

In case of high-grade gliomas, which commonly have areas of deficient blood-brain barrier, leakage of contrast may cause local T1 signal increase that competes with the decrease of T2*-derived signal. This complicates the measurement and requires corrections either before image acquisition

[1] The T2*-time constant is related to the T2 value of a tissue but includes the contribution of magnetic field inhomogeneities in a given tissue [64].

using a preload of contrast agent and/or post processing correction[35]. For similar reasons, the method cannot be used to estimate the permeability of the blood-brain barrier.
Dynamic-contrast enhanced MRI (DCE-MRI) is another method of measuring perfusion by bolus tracking, but it is based on T1 weighted images instead of T2*. Due to the fast transit times of blood through tissue, fast acquisition times are necessary and in order to keep signal-to-noise ratios acceptably high, a high-field strength (e.g. 3T) is needed [36]. Using either assumptions of pharmacokinetic models (e.g. number of compartments) or model-free metrics, DCE MRI allows the estimation of BBB permeability, K^{trans}. Contrast enhancement in glioma is a result of increased permeability and is a hallmark of malignancy. Nonetheless, only few studies have addressed the potential of K^{trans} as a biomarker of prognosis and response. The results have been contradictory, with one group finding high values of K^{trans} to be a negative prognostic factor (though using DCE-CT)[37] and one group, surprisingly, finding the K^{trans} to be positively correlated to survival [38].

Material and methods

Study 1: A phase I/II trial of re-irradiation for recurrent high-grade glioma

The study was carried out at the Departments of Radiation Oncology and -Oncology, University of Copenhagen, Rigshospitalet. Patients could be referred from the other Danish neuro-oncological centers (Odense, Aalborg, Aarhus). The protocol is available in full length at http://www.cirro.dk/assets/files/Protokoller/CIRRO-IP140111-ver5.pdf

Patients

Inclusion criteria: Recurrent high-grade glioma as evidenced by MRI; ECOG performance status 0-2; localized disease; previous focal radiotherapy to the brain completed >6 months prior; no standard treatments available; informed consent; expected life span > 3months; understanding of the Danish language (orally and written); age >18 years. A histological diagnosis of HGG was required but not necessarily at recurrence, although patients were offered stereotactic biopsy prior to re-irradiation as an optional adjunct to this study, the results of which will be reported later.
Exclusion criteria: diffuse or large recurrences (with an expected planning target volume (PTV) larger than 100 cm3 for study groups 1 and 2, which are reported here); early recurrence following primary radiotherapy (≤3 months); infection; wound dehiscence; fistula or other pathologic conditions contraindicating radiotherapy; contraindications to MRI- or 18F-FET PET CT scans

(such as allergy to contrast agents/tracer, insufficient kidney function, claustrophobia, metallic implants)

Study design

A phase I/II study of re-irradiation with dosis escalation (group 2), hypofractionation (group 3) and volume escalation (group 4) in the following 4 groups is currently ongoing (as of 1/1- 2014).

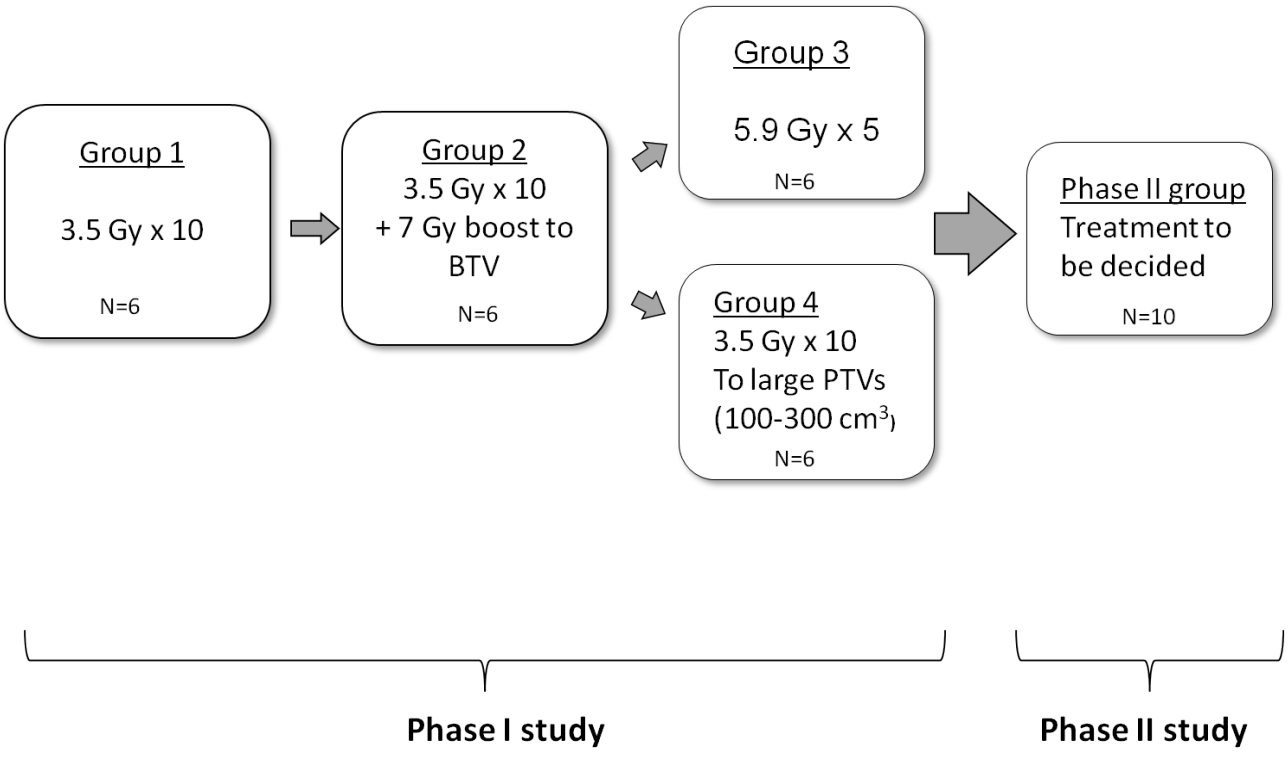


Fig. 3 Diagram showing the sequential design of the re-irradiation study.

Before proceeding to the next study group, a three-month observation time for adverse events was held. During this time, patients could be included and treated according to the current group. When group 4 has completed observation, the phase II part of the study is scheduled to begin. This will include 10 patients. The treatment schedule will be chosen from the phase I study, mainly on the basis of toxicity.

	Dose	PTV	EQD_2 tumor	EQD_2 brain
Group 1	3.5 Gy x 10	<100 cm^3	39.4	45.5
Group 2	3.5 Gy x 10 + 7 Gy boost	<100 cm^3	39.4 49.7	45.5 60.5 (PET pos. volumes)
Group 3	5.9 Gy x 5	<100 cm^3	39.1	52.5
Group 4	3.5 Gy x 10	100-300 cm^3	39.4	45.5

Table 1. Radiotherapy regimes used in the Re-irradiation study. EQD_2-doses were calculated using the linear-quadratic model and assuming $a/b_{tumor} = 10$ and $a/b_{brain} = 3$. Abbreviations: PTV (planning target volume), EQD_2 (2-Gy dose equivalent).

Imaging and radiotherapy

The gross tumor volume (GTV) for treatment was delineated using both MRI and 18F-FET PET. The GTV-MRI was the contrast-enhancing tumor delineated using T1+Gd sequences acquired on a Siemens Magnetom Espree 1.5 T. GTV-PET was contoured automatically using a threshold of 1.6 tumor-to-background. PET-images were acquired (Siemens Biograph mCT). The radiotherapy was stereotactic and highly conformal and only 2 mm margins were added to the gross tumor volume. It was delivered using volumetric modulated arc therapy (Rapid Arc®, Varian) on Novalis Tx accelerators at 6 MV. The organs at risk were the brainstem, optic nerves/chiasm, hippocampi, eyes and healthy brain tissue. The maximum allowed total doses (primary + re-irradiation) were 65.3 Gy and 60 Gy (EQD2, alpha/beta=3) for the brainstem and chiasm, respectively, while the dose to other structures were minimized.

The fasting patient was injected with approximately 200 MBq of 18-FET 20 minutes prior to scanning in a Siemens Biograph mCT scanner. Low dose CT was performed for registration purposes. The acquisition time for the PET scan was 20 minutes. Images were reconstructed using OSEM 3D and then co-registered using a rigid registration to the most recent MRI images using the Siemens TrueD software package. For tumor delineation, a background ROI was manually contoured from contralateral, normal appearing cortex with a 70 % threshold setting. The mean activity in the background was used for normalization of the tumor region of interest. This was contoured by placing an elliptic 3D-shape over the tumor region and applying a threshold for tumor of 1.6 x mean activity in background within this region. The tumor boundaries were thereby semi-automatically contoured. The tumor volume was noted and so was the maximal standard uptake

value (SUV) within the tumor. This was also normalized to the background and was designated the T_{max}/B.

Biopsy from tumor

Patients were offered image-guided biopsy from the tumor when this was feasible. This would serve to confirm the recurrence and allow for estimation of the positive- and negative predictive values of MRI- and 18F-FET-PET/CT, respectively. An optimal two-stage design was used for statistical evaluation [39], in which six patients would initially undergo biopsy. Results from these six would then be evaluated and a confidence interval of the positive- and negative predictive values (PPV and NPV) would be calculated. The study would proceed to obtain a total of 15 biopsies if results of the first stage indicated that it could be shown that the PPV and NPV were larger than 85%. Biopsy was optional for patients as required by the Ethics Committee.

End points

Primary endpoints:

- toxicity to re-irradiation *(phase I)*
- diagnostic accuracy of 18F-FET-PET/CT and MRI evaluated by biopsy *(phase I)*
- time to neurocognitive decline *(phase II)*
- time to progression by RANO criteria *(phase II)*

Secondary endpoints:

- objective response rate evaluated by RANO criteria
- value of 18F-FET-PET in target definition and as a biomarker of response
- biomarkers of hypoxia in biopsy tissue
- radiological changes assessed by advanced MRI

Follow-up

Follow-up was scheduled at the following time points following treatment: 4-, 10-, 16-, 22-, 28-, 34-, 46- and 58- weeks. Evaluation comprised MRI, 18F-FET-PET/CT, clinical evaluation, QOL questionnaires and neurocognitive testing. Patients were scheduled to go off-study after 58 weeks of follow-up or at progression, death or withdrawal of consent.

Evaluation of endpoints

Toxicity was evaluated using the Common Toxicity Criteria of Adverse Events v. 3.0 of neurological symptoms [40]. An event was defined as the occurrence or worsening of a symptom deemed to be a probable result of radiotherapy. In each case, this was decided using patient records (chart, clinical report form and radiotherapy plan). Patients were considered evaluable for a symptom if a baseline score was registered and if seen at for at least one follow-up consultation. Response to treatment (including progression) was evaluated using the RANO criteria [8].

Neurocognitive testing

Patients underwent a brief cognitive test battery at baseline and at each evaluation. The test battery was administered by the study principal investigator (SM). It consisted of 6 individual tests which evaluated the cognitive domains of processing speed, memory, verbal fluency and fine motor coordination. The figure below shows some of these tests, which are described in further detail in Manuscript 2.

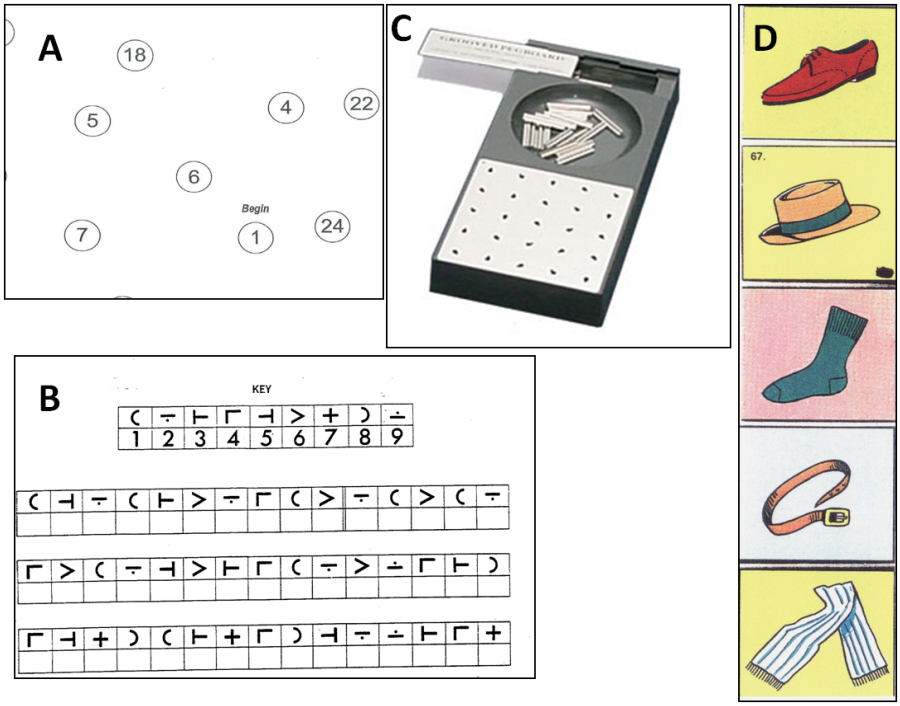


Fig. 4 Depiction of the cognitive test battery. a) an excerpt from the Trail Making Test A, b) an excerpt from the Symbol Digit Modalities Test c) the Grooved Pegboard and d) an excerpt from the MSU memory test.

Study 2: Early changes in tumor perfusion during radiotherapy evaluated by DCE-MRI

A prospective observational study of patients with glioblastoma receiving primary chemo-radiotherapy (RT) at the Dept. of Radiation Oncology, Rigshospitalet was planned. DCE-MRI images were acquired at five time points:

- post-operatively, pre-RT (baseline),
- 1 week into RT
- 6 weeks into RT
- 3 months after RT
- 6 months after RT

Patients

Inclusion criteria were: age 18-70, performance status 0-2, measurable residual tumor on post-operative MRI, signed informed consent. Exclusion criteria were: contraindications to MRI or contrast injection (pacemaker, non-compatible metallic implants, reduced kidney function (GFR<60 ml/min), previous allergic reaction to MRI contrast agent, pregnancy) and claustrophobia.

DCE-MRI

DCE-MRI was performed on a 3-T MR unit (Achieva; Philips Healthcare, Best, The Netherlands) at the Functional Imaging Unit (Dept. of Radiology, Glostrup). We used a saturation-recovery gradient–echo sequence with flip angle 30°, TR=3.9 ms, TE=1.9 ms, centric phase ordering, parallel imaging factor 2, acquired matrix 96×61, field-of-view 230×182 mm^2, five slices, slice thickness/gap 8/1.5 mm. The bolus of contrast (Dotarem 0.1 ml/kg body) was injected using an automatic injector and was followed by 20 ml saline. The bolus tracking used a saturation delay of 120 ms giving a time resolution of 1.25 s for 250 time points in approximately 6 minutes. Image resolution of the perfusion maps was 256 x 256. In case of tumors that were too large to be covered by 4 slices, the procedure was repeated in a second run. Further details about image acquisition can be found in manuscript 3.

Basic kinetic calculations

The regional cerebral blood flow to a tissue of interest, rCBF, is expressed as ml/(100g/min). It can be estimated by the following basic kinetic equation [41]:

$$c_t(t) = f c_a(t) \otimes r(t)$$

where

$c_t(t)$ = tissue concentration at time (t)

$c_a(t)$ = arterial concentration at time (t) (arterial input function)

f = flow

$r(t)$ = impulse residue response function

The arterial input function (AIF) concentration is calculated from the change in relaxation rate within the feeding artery.

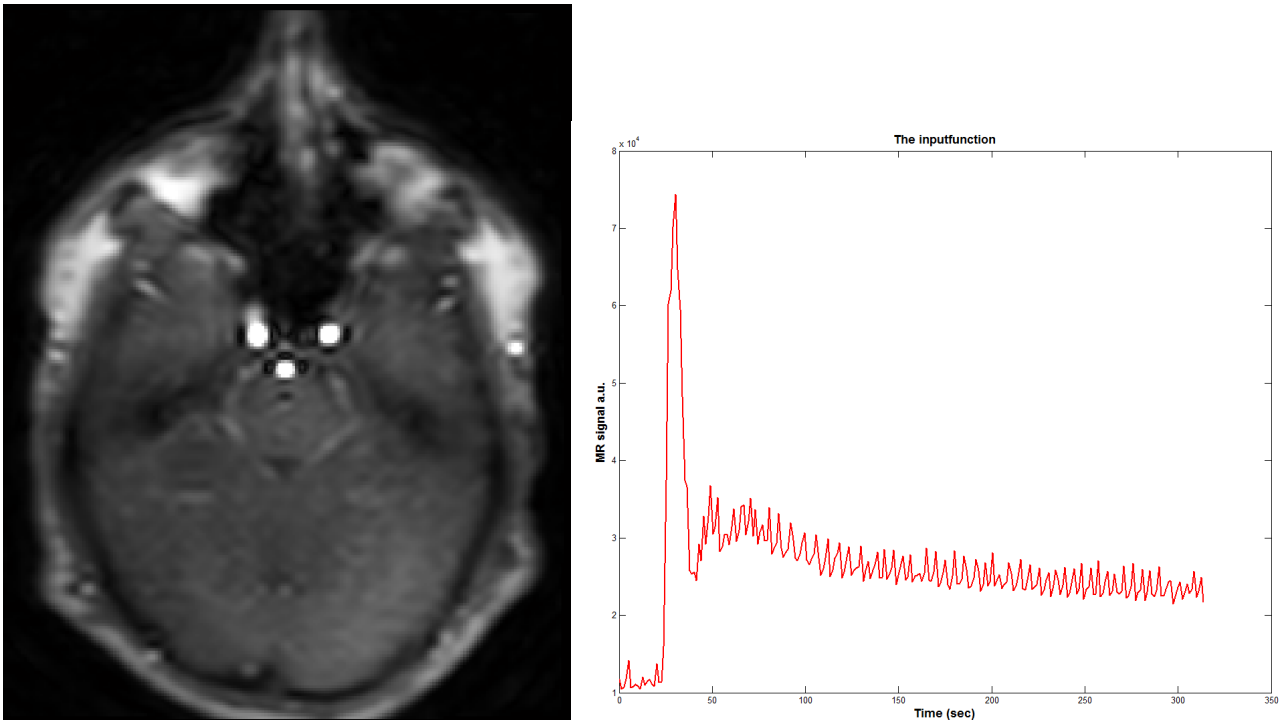


Fig. 5 Generation of the arterial input function. The left panel shows the slice of the dynamic sequence when the contrast bolus passes through the internal carotid arteries. The first peak in the curve of the right panel shows the corresponding MRI signal measured within the artery at this time.

As described in manuscript 3, this AIF is fitted with the corresponding 'output' function measured in the sagittal sinus, to counteract the effect of partial volume due to the small size of the artery.

The impulse residue response function is the (theoretical) fraction of contrast agent remaining in the tissue following an infinitely short input (a so-called 'Delta function'). The convolution equation is solved by using a model independent method described by Tikhonov, that has been used for DSC-MRI [42] and DCE-MRI[43].

The rCBV and $rCBK_i$ can be estimated by a graphical evaluation ('Patlak plot') of the following equation using assumptions of unidirectional flux of a low-permeating substance in a two-compartment system [44] :

$$\frac{C_t(t)}{C_a(t)} = K_i \frac{\int_0^1 C_a(\tau)d\tau}{C_a(t)} + CBV$$

It is apparent that CBK_i is the slope of the fitted line and CBV is the intersection at t=0. Please refer to figure 6.

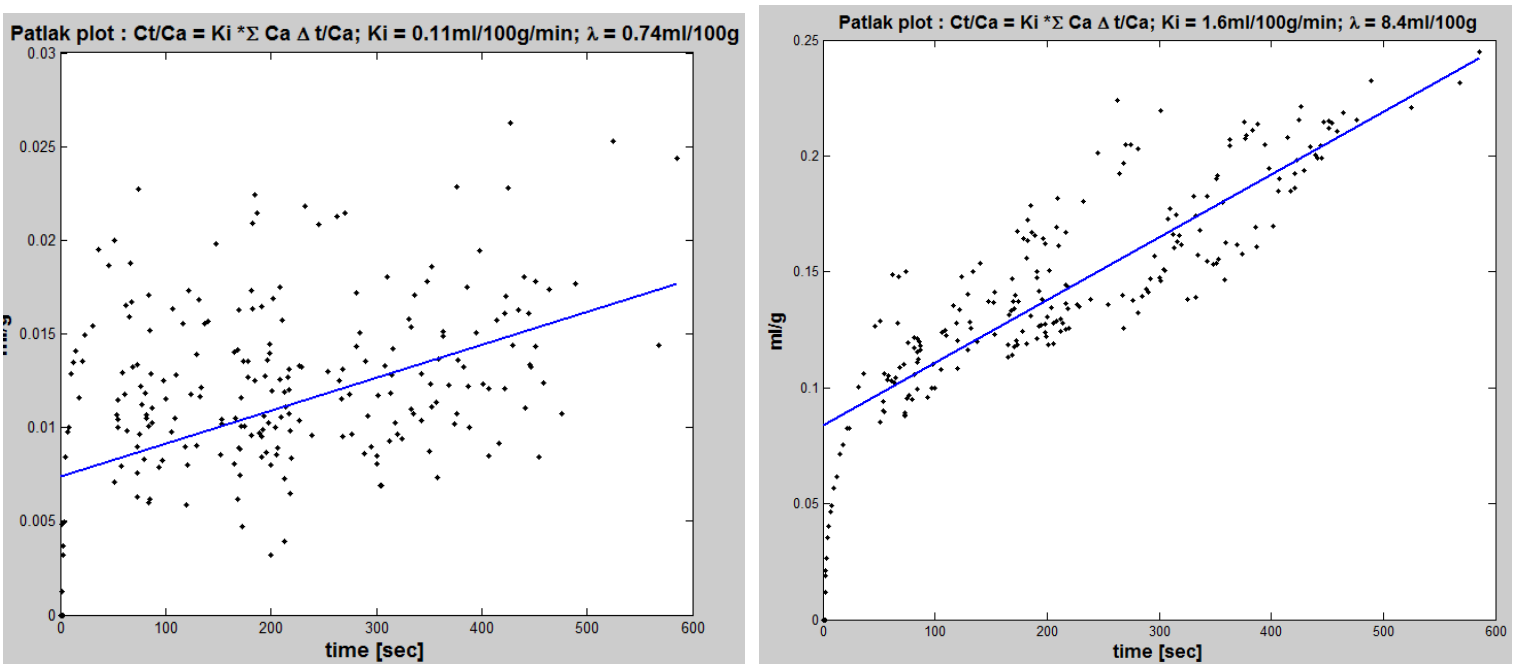


Fig.6 Patlak plot of a ROI in normal appearing white matter (left) and from an area of contrast-enhancing tumor (right). Please notice the significant difference in Ki (0.11 vs. 1.6 ml/100g/min) and CBV (0.74 vs. 8.4 ml/100g).

Results (Re-irradiation study)

Enrollment

The first patient was enrolled on December 14, 2011. Date of censoring follow-up was December 31, 2013. Twenty-eight patients were enrolled. Eighteen patients were referred from Rigshospitalet and ten patients were referred from Aalborg University Hospital.

Number of patients		**n = 28**	
Age, median (range)		54 y.	(30-74 y.)
Performance status	0	36 %	
	1	43 %	
	2	21 %	
Glioblastoma		79 %	
Glioma gr. III		21 %	
Previous RT dose, Gy	60	82 %	
	44 - 45	14 %	
	34	4 %	
Previous temozolomide		100 %	
Previous bevacizumab		61 %	
Surgery prior to re-irradiation		11 %	
Time from diagnosis to study, median		24 mo.	(6-129 mo.)
(range) for glioblastoma only		24 mo.	(6-129 mo.)
Treatment group 1		n=12	
Treatment group 2		n= 9	
Treatment group 3		n= 3	
Treatment group 4		n= 4	

Table 2. Baseline characteristic of patients in the Re-irradiation study and allocation in study groups.

Eighteen patients were referred to the protocol that could not be included in the study. Reasons for non-eligibility were:

- planning target volume larger than 100 cm3 (n=4)
- poor performance status (n=4)
- disseminated disease (n=3)
- patient preferred no treatment (n=3)
- disease involving brainstem (n=2)
- referred to surgery (n=2)

In one of three cases where the patient's disease was deemed too disseminated to enter the study, ^{18}F-FET PET imaging contributed substantially to the staging of disease and to this decision. Please refer to fig. 7. This patient was known to have glioblastoma located in the left parietal region but, in addition, a non-contrast enhancing hyperintense lesion was visible on T2/FLAIR in the left temporal lobe. It was not known whether this represented tumor when assessed only by MRI but the area showed significant ^{18}F-FET uptake. The small lesion visible in the pons was not progressing and was believed to represent Wallerian degeneration.

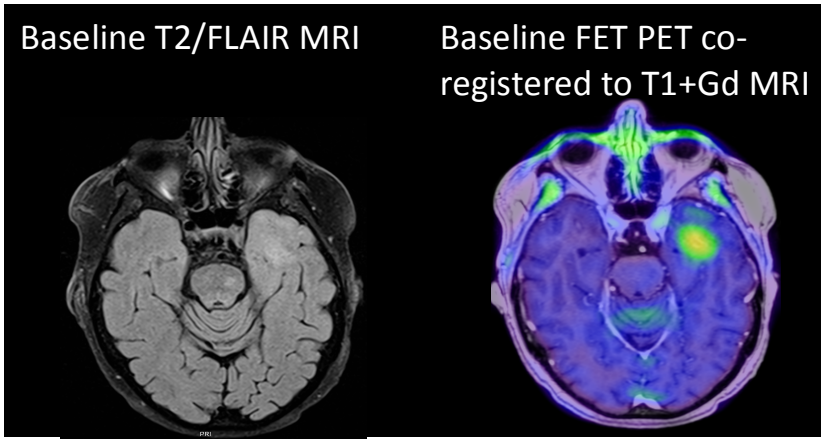


Fig. 7. MRI-and 18F-FET PET/MRI images of a patient who was referred to re-irradiation but was found to have disseminated disease with the aid of the PET-examination.

Biopsies

Fifteen patients were offered stereotactic biopsy from regions of interest in the tumor prior to treatment. Of these, five patients accepted undergoing the procedure (33%). In one of these five cases, the patient could ultimately not receive treatment because of disseminated disease and in another, the surgeon elected to stop the procedure due to unexpected technical difficulties during the operation. Thus, biopsies were successfully obtained from three patients and the results of these are discussed below.

Biopsy patient 1: 52-y male with GB in the left occipital lobe. Tissue samples were obtained from five places along the needle tract. Only the innermost biopsy contained tumor cells. The remaining four contained normal gray matter and normal white matter. It was decided not to reduce the relatively small planning target volume for this patient despite the biopsy findings. Images are not shown.

Biopsy patient 2:57-year old male with centrally located GB. There was a high level of agreement between MRI and 18F-FET PET, but the PET-defined tumor area extended beyond that of MRI in the frontal area of the right hemisphere. The PET-defined tumor (visible as a turquoise line on MRI) was in complete agreement with the biopsy results, whereas tumor extended outside of the area of MRI contrast enhancement. Please refer to figure 8 (Note: left side is on the left - not as

radiological convention). Tumor cells were found in points B and C whereas point A contained only normal brain tissue.

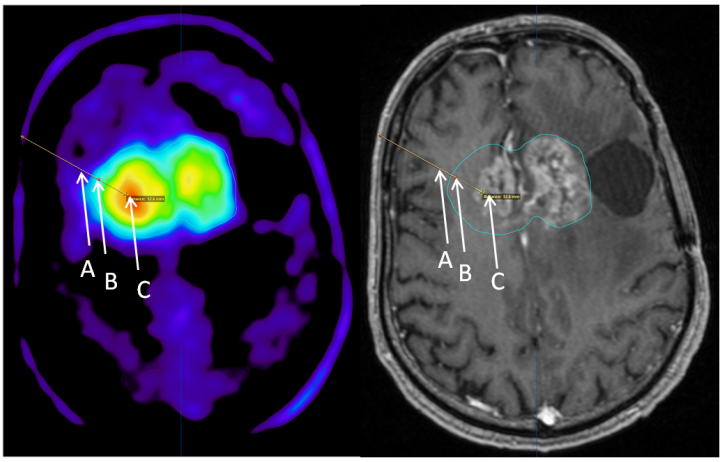


Fig. 8. Biopsy plan of a patient shown on 18F-FET PET (left) and T1W+C MRI. Tumor cells were found in points B and C, but not in point A thus extending beyond the contrast enhancing area but corresponding closely to the tumor area defined by 18F-FET PET.

Biopsy patient 3: 55-year old male with GB. When referred for re-irradiation, the patient had contrast enhancing tumor in the original site in the left temporal lobe as well as non-contrast enhancing changes in the splenium of the corpus callosum and in the basal ganglia of the left hemisphere. All three lesions were PET positive (Tmax/B>1.6). The patient underwent biopsy of the lesions in the temporal lobe and the basal ganglia using one biopsy needle trajectory. Please refer to figure 9 (Note: left side is on the left - not as radiological convention). Five samples were obtained. Only the last biopsy (corresponding to point A) taken from the top of the known tumor area showed tumor cells.

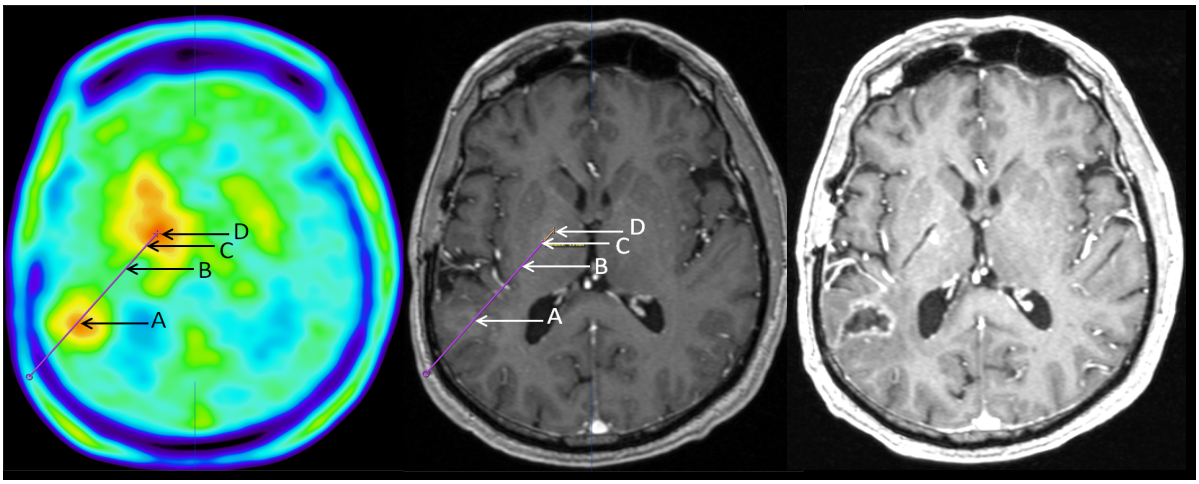


Fig. 9 Biopsy plan of a patient shown on 18F-FET PET (left) and T1W+C MRI (middle). Right image shows T1W+C MRI obtained post-biopsy to verify the location of the needle tract. Only the sample from point A contained tumor cells.

The other areas contained normal gray and white matter with areas of slightly increased cellularity as well as slight reactive changes but no tumor cells. These non-contrast enhancing areas were therefore interpreted as being unspecific sequelae of previous radiotherapy and the patient was treated only in the original tumor area in the temporal lobe. But at the first follow-up one month following radiotherapy, all untreated lesions had progressed on both MRI and PET. Thus, the biopsies from the basal ganglia were false negative.

Radiotherapy planning including imaging

All patients underwent MRI and 18F-FET PET imaging at baseline for planning of radiotherapy. Figure 1 in Manuscript 1 shows an example of a radiotherapy plan for a patient from study group 1. The prognostic impact of baseline imaging parameters as well as early changes are reported in manuscript 1. The results of quantitative and spatial analysis of RT-volumes and imaging are found in table 3.

Value	n	Median	Range
GTV MRI (cm^3)	28	33.7	0-230.0
GTV MRI (minus cavities) (cm^3)	28	26.4	0 – 196.3
GTV PET (cm^3)	28	24.2	0.1 – 214.0
PTV total (cm^3)	28	40.7	7.9 – 265.0
PTV (cm^3)			
group 1-3	24	60.8	16.4 – 119.9
group 4	4	230.5	117.4 – 325.0
Dice's coefficient MRI vs. PET, s^2	27	0.59	0 – 0.85
Max. distance GTV MRI to GTV-PET (mm)	27	10.2	0.6 – 43.0

Table 3. Volumes used for radiotherapy and results of the spatial analysis of overlap between GTV MRI and GTV PET. Abbreviations: GTV (gross tumor volume).

Two patients in groups 1-3 had planning target volumes exceeding 100 cm^3 (115.7 cm^3 and 119.9 cm^3, respectively) which was the upper limit specified by the protocol. Because surgical tumor cavity comprised a significant part of the gross tumor volume in these cases, they remained enrolled in the study and were considered eligible for all subsequent analyses. The inclusion of 18F-FET PET positive volumes in the total gross tumor volume used for radiotherapy increased the size of this volume by a median of 9.7 cm^3 (range: 0 – 76.5 cm^3).

One patient (anaplastic astrocytoma, WHO gr. III) had no contrast enhancing tumor and was therefore not included in the analysis of Dice's coefficient and distances between GTV MRI and GTV PET. These images are shown below on the right. On the left, T1+Gd MRI- and 18F-FET PET images of a patient with a high Dice's coefficient.

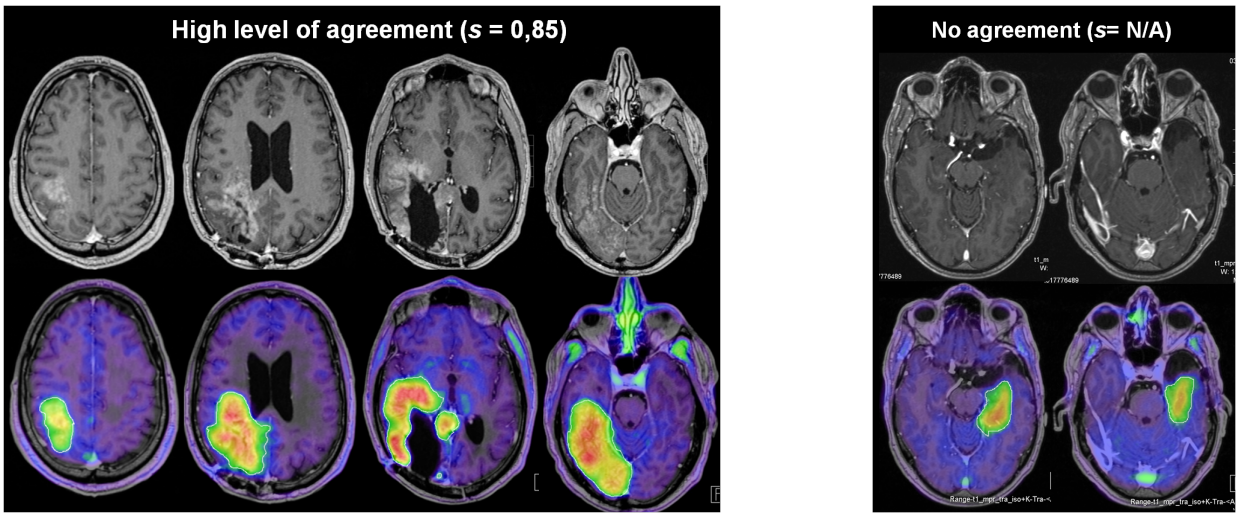


Fig. 10 Examples of tumor visualized by T1W+C MRI (top row) and 18F-FET PET/MRI (bottom row). The left panel show a patient with a high level of agreement. The right panel shows a patient with no contrast enhancing tumor but a significant 18F-FET PET positive tumor volume.

Figure 11 illustrates the results of the analysis of maximum distance from GTV MRI to GTV PET for each individual patient. For this analysis and for the calculation of Dice's coefficient, the GTV MRI minus cavity was used.

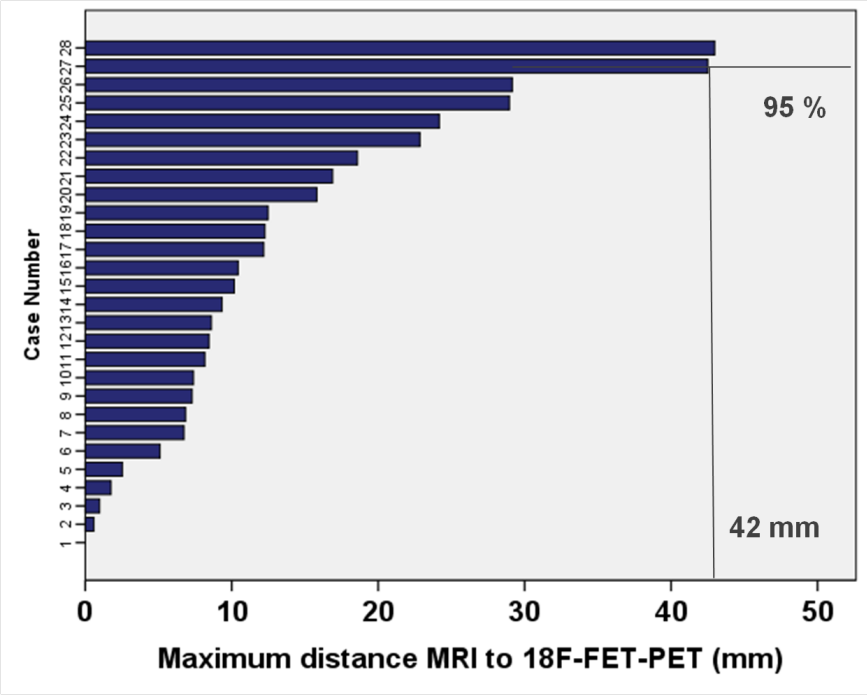


Figure 11. Results of distance analysis measuring the maximum distance from the GTV MRI to GTV PET. Each bar represents one individual patient. The distance encompassing the 95% percentile is shown.

Clinical outcomes

Adverse events

As is evident from table B (please refer to the appendix), the treatment was generally well tolerated. The most commonly reported side effect was headache (9/25 evaluable), which was distributed equally among study groups. No statistical comparison can be made due to the small numbers. Three patients experienced minor (partial) seizures during the treatment period that was attributed to the treatment. For the latter part of the study period, most patients with larger PTVs and preexisting epilepsy were prescribed corticosteroid treatment for the week(s) of radiotherapy and 2-4 weeks after. As was the case with serious late effects, it was challenging to distinguish between treatment- related and tumor-related symptoms, especially for patients with short progression-free survival times.

Seven patients (25%) were progression-free beyond three months after RT and were thus evaluable for late toxicity. Three serious adverse events were observed that were considered wholly attributable to re-irradiation and one that was potentially partly attributable. No cases of brainstem necrosis or radiation induced optic neuritis have been observed. Symptoms from the CTCAE that relate to cognitive function are described separately in manuscript nr. 2 (cognitive disturbance, mental status, memory impairment). In the following, each case of major late events is described.

Late-event patient 1 (group 1): Beginning approximately 4 months after the study treatment, MRI and FET-PET scans concordantly indicated steady tumor growth. On PET, Tmax/B rose significantly from 2.3 to 3.1 and the BTV increased from 9 to 55 cm3. Please refer to figure 12. On MRI, both contrast-enhancing and non-contrast enhancing areas increased. Clinically, the patient did not display any significant deterioration. Re-resection was performed 6 months after treatment. Histopathological examination revealed almost exclusively radionecrosis. Imaging (MRI and FET-PET) three months after surgery indicated increasing lesion size again, but this regressed spontaneously to baseline levels at 6- and 10-months after surgery. The patient has received steroid treatment since the time of re-resection but this has been tapered to a small current dose of 10 mg/day of prednisolone. Grading this event in the CTCAE (category ‘CNS necrosis’) is subject to interpretation, but due to the serious nature of the instituted treatment (neurosurgery) it should be categorized as grade 3-4.

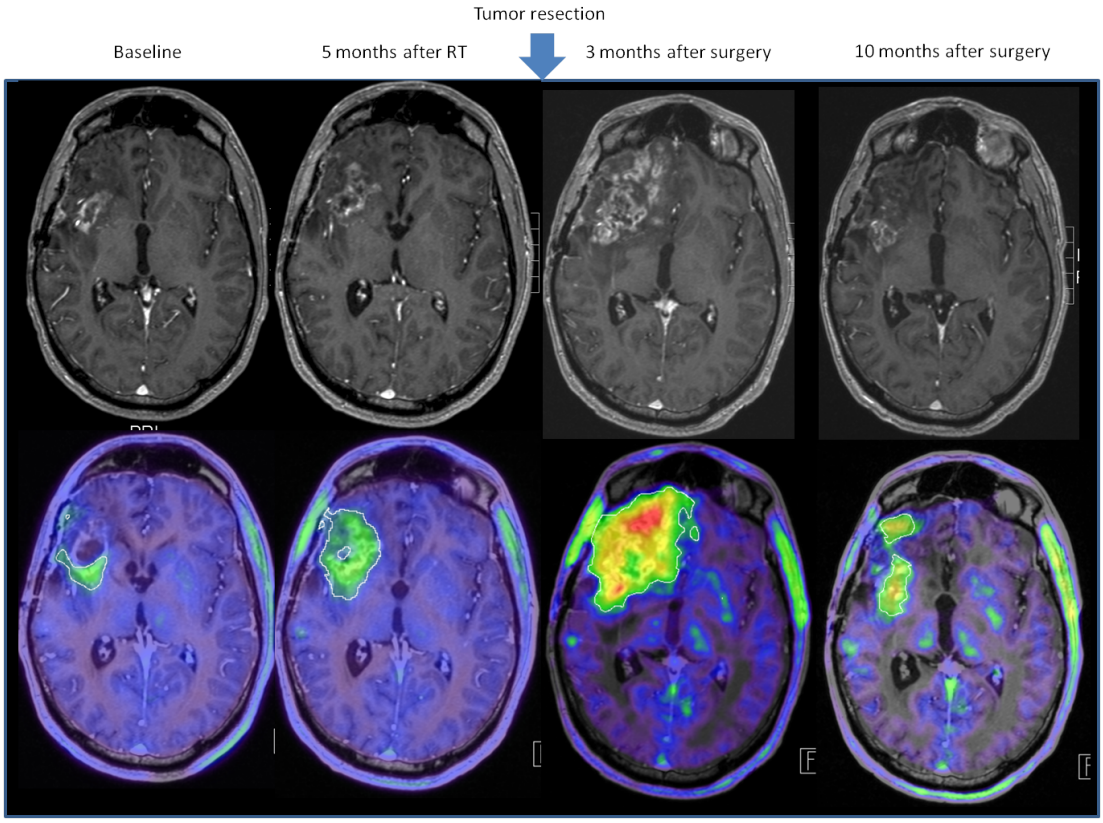


Figure 12. Images at four different time points for a patient who experienced progressive changes on MRI and 18F-FET PET that mimicked tumor progression. Blue arrow denotes time of surgery because of suspected disease progression. Top row: MRI T1W+C. Bottom row: 18F-FET PET/MRI.

Late-event patient 2 (group1): This patient underwent surgical re-resection at disease progression approximately 6 months after treatment. The patient had undergone four previous tumor resections in this area. The dura was described the as 'brittle' and the skin as 'stiff' and 'altered by radiotherapy'. Postoperatively, CSF leakage evolved into a fistula requiring surgery. Re-irradiation was considered a contributing factor. Not counted in table x. of adverse events.

Late-event patient 3 (group 2): Approximately 9 months after re-irradiation, this patient started to complain of balance problems (CTCAE grade 3 – categorized in table x as dizziness), weakened bilateral fine motor coordination and reduced general psychomotor speed. 18F-FET-PET scans showed no change but MRI showed bilateral white matter edema extending backwards from the frontally located lesion. Please refer to figure 13. Good response to steroid treatment, which is ongoing but the future clinical course is unpredictable.

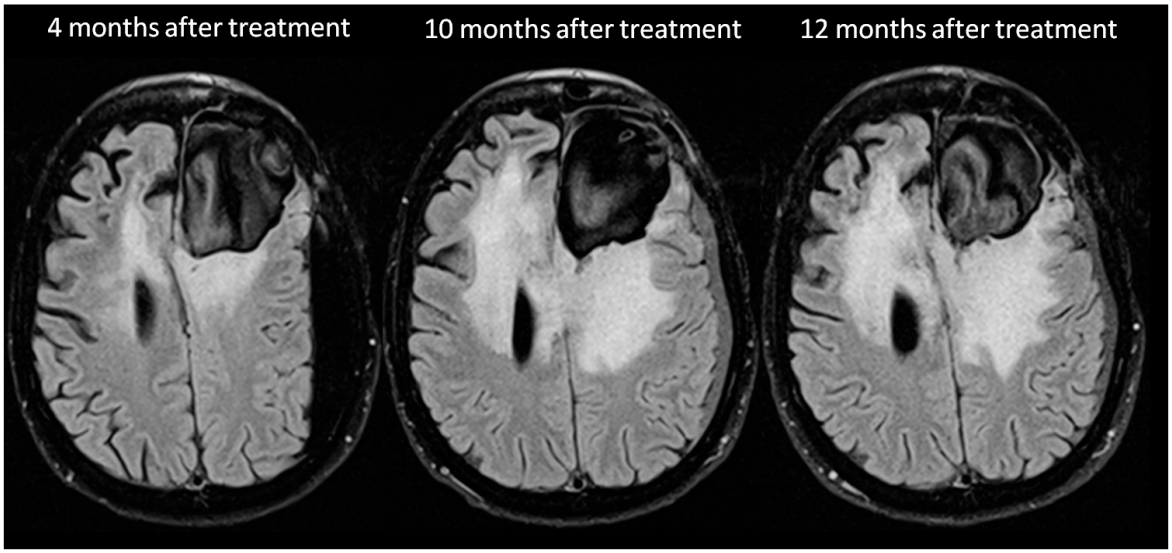


Figure 13. MRI FLAIR showing increasing hyperintense, non-contrast enhancing changes extending backwards in both hemispheres. The contrast enhancing tumor was located posterior to the resection cavity on the left side.

Late event patient 4 (group 2)

Three to four months after treatment, this patient experienced symptoms of elevated intracranial pressure and had increased edema on MRI that was interpreted as being radiotherapy induced. The PET positive volume increased from 1 cm^3 to 12 cm^3. Steroid treatment relieved all symptoms. Lesions regressed on all imaging modalities at 22 weeks following treatment and tumor was not visible on PET. The patient was able to taper steroids and was feeling well. At 34 weeks, MRI showed progression of non-contrast enhancing changes in the white matter and the patient become symptomatic and steroid dependent again. The patient then withdrew from follow-up but progressive disease was later suspected at the referring hospital approximately 12 months after re-irradiation. Please refer to figure 2 in *Manuscript* 2, in which this patient is shown to experience failure on her cognitive test (TMT-A) at the time of the transient progression.

Characteristic	Patient number			Median for study
	1	3	4	
Diagnosis	AA	GB	AA	79% GB
Age (years)	33	37	30	54
Treatment group	1	2	2	Group 1 n=12 (5 evaluable) Group 2 n=9 (2 evaluable)
Time between primary RT and Re-RT (months)	29	108	14	22 months
Previous RT dose (Gy)	60	60	60	N/A
Concomitant TMZ during primary RT	no	no	no	N/A
Previous bevacizumab treatment	yes	yes	no	61% yes
PFS (months)	> 18	> 12	9	3
PTV (cm^3)	53	120	92	66

Table 4. Baseline- and treatment characteristics of patients 1,3 and 4 (experiencing late adverse events) compared to the average characteristics of the study population where applicable. Abbreviations: AA (anaplastic astrocytoma), GB (glioblastoma), TMZ (temozolomide), PFS (progression-free survival), PTV (planning target volume), N/A= not applicable

Besides long PFS, common traits included young age. None had significant medical co-morbidity (e.g., diabetes, hypertension) or smoked tobacco but one patient was a carrier of an asymptomatic factor V ('Leiden') mutation. The planning target volume for patients 3 and 4 were relatively large (92 cm3 and 120 cm3) but both included significant surgical cavities. Grade 3 tumors were also overrepresented (2/3 vs. 6/28) but this may also be a consequence of longer survival times (please refer to table x (prognostic factors) in article 2 – cognitive function). Two patients treated in study group 2 had a PFS larger than three months and they both developed late changes to radiotherapy (patients 3 and 4). When compared with only one in five 'long term survivors' from study group one, there appears to be a higher incidence of late effects in study group 2 (100 % vs. 20%) although this is not supported by statistical calculation (Fisher's exact test: p=0.14).

Radiological outcomes

Twenty-six patients were evaluated at least once using the RANO criteria. Two patients had not yet been evaluated at time of study censoring. There were no objective responses. One patient had a 'minor response' of 49% regression of contrast enhancing tumor area as well as significant regression of non-contrast enhancing area (FLAIR), but this response was very short lived and she had significant tumor progression one month later. Fifty-four percent of the evaluated patients had a best response of stable disease and 46 % had progressive disease. A minority of patients reported some clinical benefit such as improvement of paresis following therapy but these responses were short-lived and not significant enough to warrant a change of score in the Macdonald response evaluation and not accompanied by significant tumor regression evaluated by MRI. Therefore, it was not possible to report these minor responses in a systematic manner. Likewise, in some cases, a visually assessed reduction occurred in the intensity of contrast enhancement in a tumor. Please refer to figure 14.

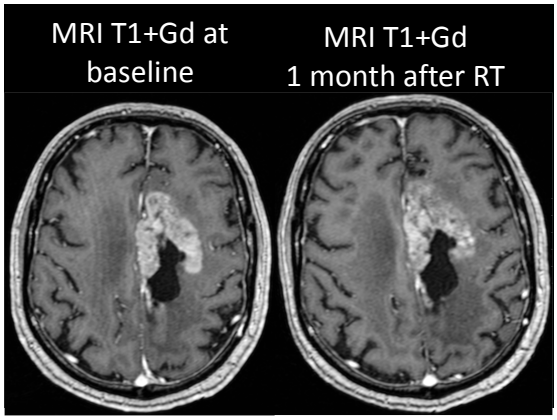


Figure 14. Baseline T1+Gd MRI before (left) and one month after (right) re-irradiation in a patient with GB treated in study group 3 (5.9 Gy x 5). The cross sectional tumor area was unchanged but the contrast enhancement had become less intense in certain regions.

Survival times

Twenty-two patients had progressive disease and 19 had died at time of censoring data collection. One patient died of myocardial infarction after disease progression had been established. All other deaths were due to glioma. The median progression-free survival for the whole population was 2.9 months (95% CI: 2.3-3.6). For GB patients, the PFS was 2.9 months (95% CI:2.2-3.6) and for patients with grade 3 tumors it was 10.6 months (95% CI: 0-29.1). The median overall survival for the whole population following treatment was 8.7 months (95% CI: 5.7-11.8). For GB patients the median OS was 6.9 months (95% CI: 3.2-10.6) while it could not be calculated for grade 3 tumors because only 2/6 were dead at time of censoring. Figure x shows Kaplan-Meier curves of PFS and OS for the whole study population.

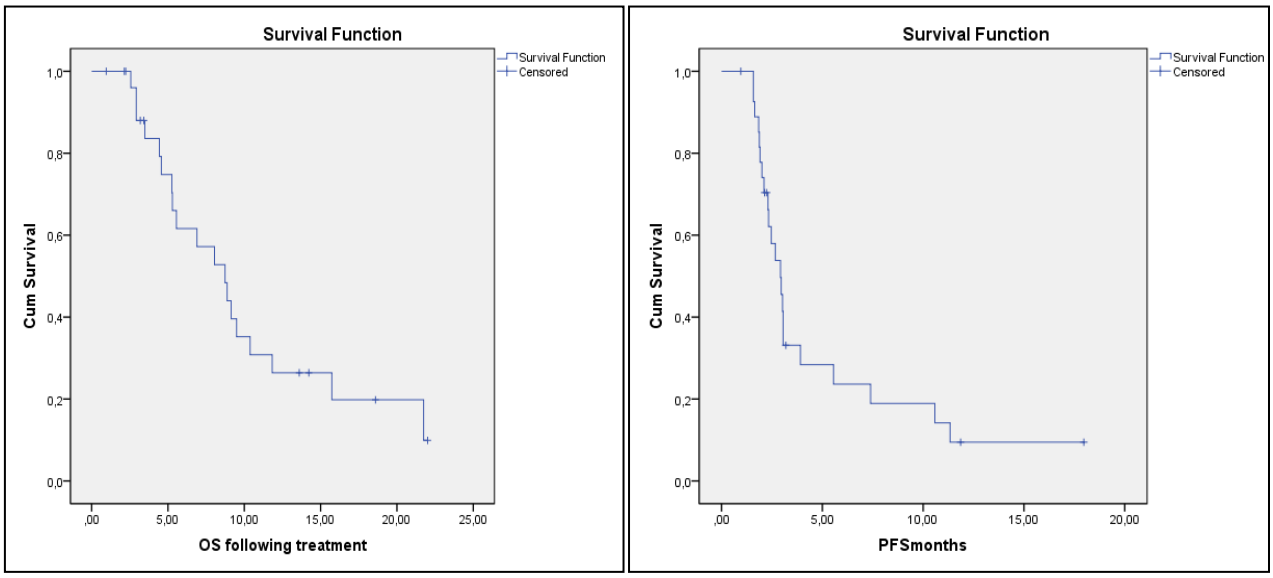
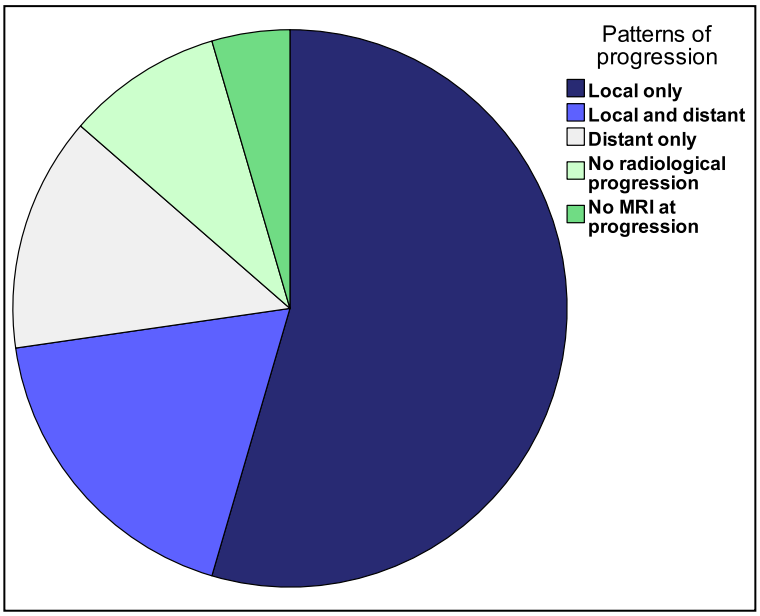


Fig. 15 Kaplan-Meier curves showing overall survival (left) and progression-free survival (right) in months for all patients in the study.

No differences in PFS or OS between patients who had previously received bevacizumab and those who had not could be found. There was no difference in PFS and OS between patients in treatment group 1 and patients in treatment group 2. As listed in table 2, only 4 patients had been treated in groups 3 and 4, respectively, and therefore no meaningful comparison can be made yet. The impact of other potential prognostic factors is also assessed in study 1, where a Cox regression analysis is presented.

Progression patterns



At the time of progression, 19/22 patients (86%) had symptoms of tumor progression. Most tumor regrowths were local (55%) or local *and* distant (18%). In three cases, the diagnosis of progression was based largely on clinical deterioration as the MRI either did not show unequivocal tumor

Fig. 16. Pie chart showing the distribution of the 22 progressions

growth using the RANO criteria (n=2) or was not performed due to poor performance status (n=1). These three patients all suffered early progression (PFS= 58-64 days).

There was no significant difference in the rate of local progression between groups 1 or 2 (p=0.6 by Fisher's exact test). Only 2 recurrences with imaging were available in groups 3 and 4, respectively, and therefore no statistical evaluation has been carried out.
A total of seven patients developed distant metastases within the CNS. In four of these cases, local disease progression was also evident. In 5/7 cases, the metastases were visible on both MRI and 18F-FET PET at the same time point. In one case, it was visible first by MRI and in one case first by PET (please refer to figure 16).

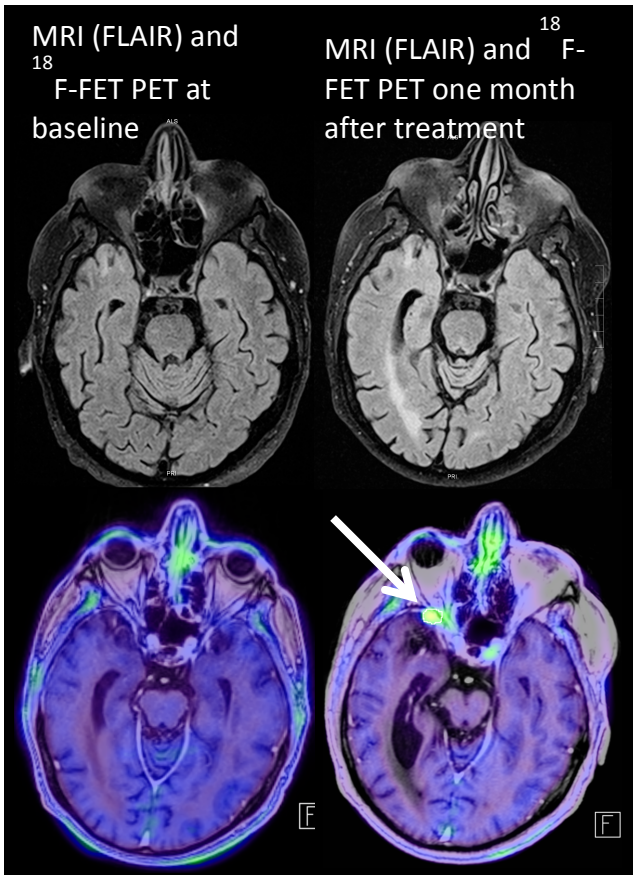


Fig.17 Images showing the appearance of a PET-positive lesion behind the right orbital cavity one month after treatment. The lesion was initially only diagnosed by PET.

Discussion

Recruitment

Re-irradiation study

During planning of the study, it was estimated that 1- 1.5 patients could be accrued/month. The actual rate was 1.1 patient/month over a 2-year period, which was satisfactory. Patients were discussed at a multidisciplinary tumor board prior to entry to ensure that no other treatment options were available or indicated. This also allowed us to screen and accrue a large majority, if not all, patients eligible for the study. The two departments that referred patients treat primary brain tumors from a population of approximately 3.1 million. Extrapolating this number and assuming similar incidence rates, treatment guidelines etc., it is estimated that 24 patients/year in Denmark would be

eligible for re-irradiation if the current inclusion criteria were adapted. This is a small number by most standards but considering that re-irradiation of recurrent HGG is described as a treatment option in guidelines of larger countries (e.g., US, Canada [45] [46] and that the treatment has been well known for +25 years [11] we find this research to be worthwhile and important for a significant population.

DCE-MRI study

We planned to include 15 patients but accrual was very slow despite the considerable number of newly diagnosed GB patients in our department (125-150/year). The main reason for patients to decline participation was the practical challenge of going for scans at another hospital during a period of stress and gravely altered life circumstances. Some declined due to the physical discomfort of the long acquisition times (75-85 minutes) and due to worries about the use of intravenous contrast agents. Another reason for non-eligibility was lack of contrast enhancing tumor ('total' resection). This criterion selects patients with a poorer prognosis. During the study, we experienced a slowing of recruitment that was attributable to an increasing rate of total resections, likely caused by the implementation of 5-ALA for surgery guidance [47].

We had also planned a reproducibility study where a maximum of 8 patients were to undergo two or three DCE-MRI perfusion examinations in the days preceding the radio-chemotherapy course. Henriksen et al. have examined the reproducibility of DCE-MRI perfusion measurements in healthy subjects and found a coefficient of variability (standard deviation/mean) of approximately 15% for both white matter and gray matter [48]. This is considerable and as the variability in tumor had not previously been quantified, we felt that such a study was warranted. Unfortunately, we were not able to recruit patients for the reasons mentioned and it was not completed.

Biopsies acquired in the re-irradiation study

Determining the diagnostic precision of MRI and of 18F-FET PET was a co-primary endpoint of the phase I part of the study. Biopsies from six patients were required for the first stage of this evaluation but only three were obtained successfully. No formal analysis was done of the patients' motivation for not participating. In a few cases, logistical problems could not be solved without delaying radiotherapy unacceptably. These included availability of a surgical bed on short notice. But we believe that fear of complications was a major deterrent for most patients. One of the three patients who underwent biopsy (patient 3) actually did suffer paresis of the right arm and leg immediately postoperatively and only partially regained function. This was reported to the overseeing authority, the Ethics Committee of the Capital Region of Denmark, whereupon we were

required to cease this part of the study until an amended protocol and written patient information with increased emphasis on the risk of complications had been accepted. No patients consented to biopsy after this and we attribute this to the changes in the patient information.

Four patients underwent tumor resection after treatment failure of re-irradiation. These patients were asked whether surgical biopsies from tumor regions of interest could be taken during the procedure. All patients and surgeons accepted this and the samples were successfully collected[2] using neuro-navigational equipment in a routine setting. This indicates that future studies aiming to collect biopsies may accrue more successfully if they target patients who are undergoing surgery for treatment purposes, although this method lacks the high spatial precision of frame-based biopsy due to technical limitations of the navigational equipment and so called 'brain-shift' during surgery. Although we only obtained biopsies from three patients, the results were surprising. In patient one, tumor cells could not be found in the most metabolically active area, which was found to contain normal appearing brain tissue. Only the most central of the five biopsy points contained tumor tissue. This could be explained if the distance calculations were incorrect or if the instruments in the stereotactic head frame had somehow shifted 5-10 mm outwards so that the innermost sample was actually within the central part of the tumor (as opposed to beyond it) but we do not find this likely as the surgeon was very experienced (+25 years of practice) and the procedure part of routine practice. A labeling error of the tissue could also provide an explanation but we do not find this likely either, as the steps of the procedure had been discussed thoroughly prior to being carried out. Results for patient 3 were also surprising. No tumor was found in the metabolically active area in the basal ganglia, but at first evaluation following treatment the lesion had progressed significantly and had become contrast enhancing on MRI, which was unquestionably indicative of tumor. The pathology examination of this biopsied area included standard staining with HE (hematoxylin-eosin), alcian and Gieson. In this case, the correct location of the needle trajectory was verified by an MRI scan performed after the procedure.
Thus, the stereotactic biopsy in the case of patient 3 failed to reveal the true extent of tumor. This was also suspected in the case of patient one, but the 'true' tumor extent at baseline was not revealed as clearly as in case 1.

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[2] This was not a pre-planned part of the study and results are not shown because of the different setting and potential difficulty in generalizing data from re-irradiated patients to other patients.

These findings posed an unexpected methodological challenge for our study because biopsy was considered a reference for the imaging modalities. The most likely explanation is the heterogeneity of (recurrent) glioblastoma in combination with the small size of the tissue samples drawn by needle. In all three cases, the overall diagnosis of recurrent glioblastoma was made but it required several (5-6) anatomically separate biopsies in two of the cases. We aimed to diagnose disease or no disease in *each* of the biopsied regions but the method was not sufficiently accurate in this setting. Increasing the number of samples taken at each point could have improved the sensitivity but at a cost of higher risk of bleeding. The literature describing the diagnostic accuracy of stereotactic biopsy at suspected recurrence is sparse but a study where patients with suspected recurrent HGG were biopsied prior to resection showed a perfect rate of correlation (10/10) for non-enhancing lesions but a poor correlation for enhancing lesions (8/13) [49].

Taken together with our results, we found that stereotactic biopsy was not an optimal method for evaluating imaging modalities in recurrent HGG. Open surgery, which also has a potential therapeutic benefit, is likely to yield more accurate results and should be considered whenever possible. But re-resection is a routine treatment at our department and surgery was always considered prior to referral to our study. The reasons for not operating have not been formally analyzed but tumor proximity to eloquent areas is a common explanation. It is likely that biopsy in such an area may entail greater risk than normally found in the literature, and indeed one patient suffered a serious complication to the procedure.

Toxicity

With 22 progression events at time of censoring, only 25% of patients (n=7) had achieved progression-free survival beyond three months. Only these seven patients were actually evaluable for late toxicity. Three of these patients (patients 1, 3 and 4) experienced symptoms that were likely due to radiotherapy and one patient (patient 2) had post-operative complications where an adverse effect of radiotherapy (primary RT or re-irradiation) was a potentially contributing factor.

Patient 1 had apparent tumor progression on both MRI and 18F-FET PET without clinical symptoms. There are a number of troublesome issues with this case. First, the patient underwent a neurosurgical procedure that was of no apparent therapeutic benefit. Second, it demonstrated that 18F-FET PET uptake is not completely specific for tumor. Third, there is a risk that we will be faced with a similarly challenging patient in the future. It is encouraging that he had no clinical symptoms, but this may be related to the frontal location of the lesion as well as the pathological phenomenon underlying the images. In the case of patients 3 and 4, the suspected toxicity was

treatable with long-term steroids administration. Although this treatment has numerous side-effects, we find it to be an acceptable cost of treatment, assuming that RT also had beneficial effects and that the long PFS was due to RT.

Counting these three patients (1, 3 and 4) among those who are evaluable, we find a frequency of serious late adverse events of 43%. This corresponds well with the rate of 36% reported by Shepherd et al. from the Royal Marsden Hospital in another prospective study of single modality fractionated stereotactic re-irradiation of recurrent HGG [11]. The authors concluded, likewise, that toxicity was manageable and acceptable given the setting. The discrepancy between the results of these two prospective studies and the large retrospective series mentioned in the introduction (which found almost no toxicity) is remarkable. This cannot be ascribed to dose as Shepherd et al. found toxicity even at the lowest doses used (30-35 Gy). It is possible that larger fraction sizes (5 Gy/f) may have resulted in a higher risk toxicity. But given our results, where one patient in group 1 developed necrosis following a dose of 35 Gy, we find it more likely that the frequency of adverse events has been grossly under diagnosed in the two large retrospective series.

Manuscript 2 describes the first documented attempt to evaluate the cognitive performance of patients before and after re-irradiation of recurrent HGG. The simple checkboxes of the Macdonald criteria have the advantage of being reproducible and widely used but we believed that this method would not have sufficient sensitivity to detect dysfunction of the brain's higher domains. For this reason, neurocognitive testing was an integral part of the study protocol as a mandatory examination at baseline and at every subsequent evaluation. We aimed to use tests that were easily administered by non-neuropsychologists and had been previously applied in neuro-oncological studies. Manuscript 2 documents the feasibility of the approach, the level of dysfunction at baseline as well as the results for the longer term survivors. The patients were too few and too heterogeneous for us to make any statistically valid conclusions but we believe to have identified one case of radiation-induced decline in cognitive function. In future studies, other thresholds for reliable change may be used, but we have now documented the initial experience of testing a group of heavily pretreated patients that will enable researchers to make more qualified choices and this is in line with the phase I concept of this trial. Some readers may find that the survival of these patients was too poor warrant efforts to describe- and preserve the patients' cognitive function. We would disagree with this view and will argue that any non-curative treatment must have very thorough documentation regarding side effects that can affect quality of life adversely.

Comments on radiotherapy dose-levels

In this study we aimed to learn whether re-irradiation could be carried out safely and, if possible, to determine an optimal dose regimen based primarily on toxicity and secondarily on efficacy and feasibility. Please refer to *Materials and Methods* for a description of the study groups. Dose level 1 had been described previously [6] and we did not expect significant toxicity. Dose level 2 was novel and represents a 20 % increase in dose that was deemed necessary to obtain a reasonable separation between groups 1 and 2. Dose level 3 was novel and aimed to test a possible effect of fraction size by delivering an equal EQD_2-dose as level 1 (39.4 Gy) in 5- instead of 10 fractions. Dose level 4 was included to test the feasibility and toxicity of treating larger tumors. Recruitment and follow-up for groups 3 and 4 is still ongoing and no clear conclusions regarding the effect of either fraction-size or volume can be made yet. However, the results of paper 1 indicate that survival times are inversely correlated to the PET-positive tumor volume. Re-irradiation to large volumes may therefore not be clinically indicated at all, but this remains to be confirmed in multivariate analysis before clear conclusions can be made.

For calculations of EQD_2 doses, we assumed $a/b_{tumor} = 10$ and $a/b_{brain} = 3$ [50]. From this assumption, it is clear that using smaller fraction sizes (e.g. 2 Gy) in principle would have entailed a smaller risk of late toxicity. This has been demonstrated in studies of whole-brain irradiation But in this study, larger fraction sizes were used. This was motivated by three factors. Firstly, empirical data indicated low toxicity using dose level 1 [6]. Secondly, the radiotherapy was highly conformal and no margin of healthy tissue was added to the GTV, aside from 2 mm to account for technical insecurities. The method of delivery, VMAT, makes very steep dose gradients possible and therefore only small volumes of healthy tissue would be exposed to the possible detrimental effects of large fraction sizes. Lastly, we considered the overall treatment time to be an important issue given the poor prognosis.

Progression-free survival and overall survival

The patients generally fared poorly in this study. We found that the exact time of progression was a challenge to determine due to the experimental nature of the treatment, the early timing of the first two evaluations (4- and 10 weeks after treatment) and the high degree of pretreatment. No objective responses as defined by the RANO criteria were observed and only 5 patients have currently achieved a progression-free survival of more than 6 months (18%). The median progression-free survival was 2.9 months (95% CI: 2.3-3.6). Combs et al. found a longer progression free survival following re-irradiation of 5 months for 59 patients with GBM [5]. But it is likely that their patients

had received less pretreatment as evidenced by the median times from end of primary RT to re-irradiation which was 10 months as compared to 22 months in our study. In addition, our study was prospective and required very frequent evaluations and may therefore have been more sensitive to progression. While there seems to be general consensus that only localized tumor recurrences should be re-treated, the definition of 'localized' may be subject to individual interpretation at different treating centers. All in all, many reasons exist why comparisons of OS and, in particular PFS, between studies is difficult and perhaps not meaningful. Only randomized, controlled trials are capable of determining if this treatment can prolong survival.

Carrying out an independent radiological review may sometimes alter the interpretation of a study [51]. It is likely that a radiological review could alter the estimated progression time in some cases, but we do not find it likely that this would alter the conclusion and it is not planned at this time.

Response rate

The lack of objective responses was perhaps not surprising given the low rate of response for glioblastoma undergoing primary concomitant radio-chemotherapy (10.3% in the database of the Dept. of Radiation Biology, n=272 [*Hans Skovgaard Poulsen, personal correspondence*]). Tumors are generally considered to be less sensitive to treatment at recurrence. The minor responses we observed are not quantifiable by standard MRI sequences and correlations with outcome have not been explored. It is conceivable that measurements of perfusion using DCE-MRI could have contributed information in such cases.

Imaging aspects

Tumor volumes at baseline and implications for radiotherapy planning

In article 1, we demonstrated that the size of the PET-positive tumor volume at baseline was highly prognostic for PFS and OS for treatment groups 1 and 2. Repeating the calculation of the hazard ratio to include the whole current study population also yields significant values (HR=1.4 95%CI: 1.1-1.7 p=0.003) and we find this unlikely to change even as follow-up becomes longer and more patients are included. As discussed in the article, a multivariate analysis could not be performed but when adjusting for the only other parameter that was significantly prognostic (steroid use at baseline), it remained significant. Thus, the baseline BTV is an expression of tumor biology and disease stage that was not obtainable by MRI. Taken together with the results of the biopsies obtained and the available literature about the specificity of the tracer in recurrent HGG, we find that 18F-FET PET contributes important information for tumor delineation in re-irradiation. Figure

11 shows the maximal distance from MRI-contrast enhancing tumor to the PET-defined tumor. It is apparent that the margin needed to cover all the PET-positive volume for 95% patients would be 42 mm. This is in good agreement with the finding of Grosu et al., who examined the use of 11C-MET PET for planning of primary RT [52]. The authors found the PET positive areas to extend outside of the contrast enhancing areas in 74% of cases, with 11C-MET uptake up to 45 mm from the contrast enhancement. Ultimately, we cannot be sure that 18F-FET PET positive volumes should to be in the radiation field since the specificity of the examination is not 100%. But the same can be said of MRI and the available biopsy studies indicate a higher specificity of 18F-FET PET than MRI.
For the sake of example, a margin of 42 mm was added to the GTV MRI of patient 27 in figure x. The resulting radiotherapy plan is shown below together with the actual plan, based on MRI and 18F-FET PET. The PTV was 548 cm^3, which is an unfeasibly large volume for hypofractionated re-irradiation, and this would most likely be the case for all patients. In contrast, the inclusion of the 18F-FET PET positive volume to the GTV only increased the resulting total GTV by a median of approximately 10 cm^3, which is acceptable.

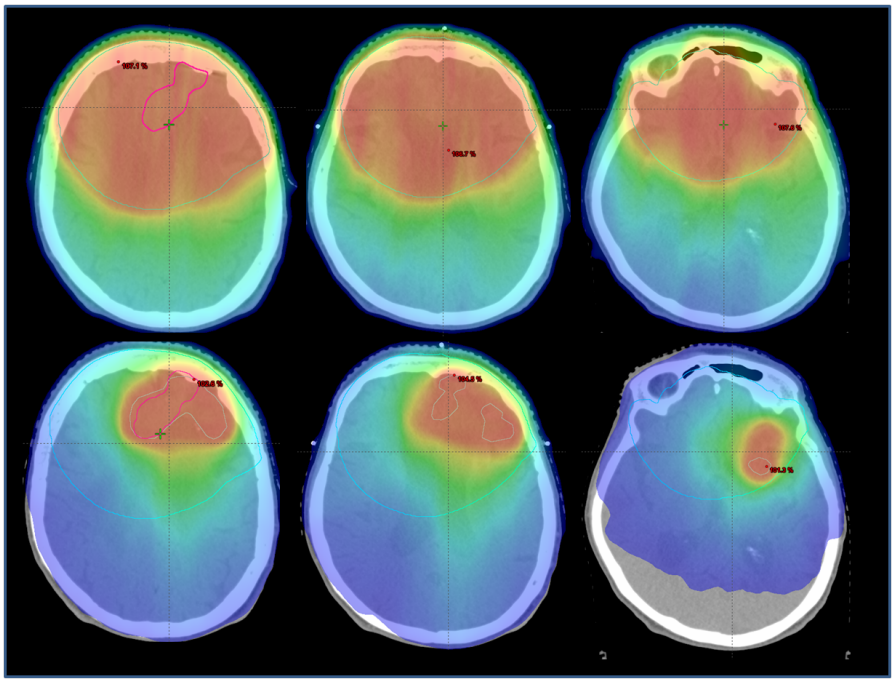

Fig. 18. Radiotherapy plans for a patient with a frontally located tumor. Top row: a margin of 42 mm was added to the GTV MRI to demonstrate the impact on the size of the radiation field if all PET positive areas should be covered without availability of 18F-FET PET imaging. Bottom row: the radiotherapy plan used to treat the patient.

As with imaging at follow-up, inter-individual differences in contouring may exist and radiological review could yield differently contoured GTVs but this has not been performed and is not planned. In designing this study and defining how the target for RT should be delineated, we chose to rely on T1W-contrast-enhancing tumor and the 18F-FET PET scan, despite the limitations they may have. No international consensus for tumor delineation in re-irradiation exists and any treatment regime

will contain compromises. As discussed in article 1, the threshold of 1.6 tumor-to-brain used in tumor delineation on 18F-FET PET is lower than some authors suggest should be used in previously irradiated patients [53]. The purpose of the small stereotactic margin of 2 mm from GTV total to PTV that was used in the study is only to compensate for technical insecurities in positioning etc. We therefore relied on the imaging to display the 'whole' tumor including infiltrating tumor cells in areas of gliosis. Adding a larger margin to compensate for limitations in imaging technology and micro-invasion into surrounding tissue (a so-called clinical target volume, CTV) is used in most branches of radiotherapy, including primary treatment of HGG. The addition of a CTV was considered (e.g. 5 mm or 10 mm) but this would have increased the total irradiated volumes considerably and likely have increased the risk of toxicity and decreased the number of eligible patients. Thus an anisotropic 'biological' margin was used instead of a static margin.

T2/FLAIR

T2/ FLAIR MRI sequences are recognized to be more sensitive than T1 weighted sequences and may help visualize the full extent of glioma invasion[54]. T2/FLAIR is recommended for use in the current response assessment criteria [8]. But the specificity is low and there are currently no guidelines on how to measure these lesions or how to classify them as progressing. In the current study, the T2 and FLAIR sequences were acquired at all time points, but only the FLAIR sequence was evaluated in a systematic manner. It was not used for target definition but for follow-up. Unidirectional measurements were made in all cases on axial slices and, when possible, bi-directional measurements. We found the median length of FLAIR lesions at baseline to be 7.0 cm (range: 3.2-12.5 cm) and it is likely that encompassing all FLAIR lesions in the field would result in much higher rates of toxicity.

Imaging at follow-up

Distinguishing necrosis from tumor growth

Evaluating the response to re-irradiation was challenging. The treatment had only rarely been carried previously at our institution. For each patient, the first three evaluations were carried out within a three-month period following treatment. As described in the chapter *Background,* increasing size of the contrast-enhancing lesion in the first months after radiotherapy may represent unspecific changes ('pseudoprogression'). The incidence of pseudoprogression following re-irradiation is not described in the literature. In this study, two cases of reversible lesion growth were identified (patients 1 and 4 in *Results*). But due to the limitations of the imaging technologies, it

cannot be ruled out that a greater number of patients with growing lesions and clinical deterioration actually had progressive symptomatic radiation necrosis. This diagnosis was considered in all cases and the location of the lesion was considered in correlation with the radiotherapy plan, results of the PET-scan and the time course. In no cases did we find evidence of this but this cannot be ruled out completely. It is also conceivable that tumor progression and progressive radionecrosis could occur simultaneously, but this is also difficult to answer due to the low number of re-resections performed after study treatment. It is unknown whether perfusion weighted imaging could have contributed information at follow-up. Using DCE-MRI in patients that had received less pretreatment, Larsen et al. have recently shown that CBV reliably distinguishes recurrent glioma from treatment related changes[55]. This should be evaluated in future studies of re-irradiation, and preferably in a larger study population than the eleven patients included in our study . However, the methods used to derive quantitative values of perfusion in the DCE-MRI study were very time-consuming. For generation of a perfusion map from one scan (with 2 runs), approximately one hour of work and 8 hours of computer processing were required. Subsequent image registration, contouring, quality assurance and statistical analysis all make the procedure unfit for use in clinical routine in its' current form. It is possible to generate the maps in a quicker fashion but without registration to regions of interest, only visual inspection is possible. As no significant predictors of outcome were identified, we cannot currently recommend implementation of this procedure outside of clinical trials.

Value of 18F-FET PET in follow-up

In manuscript 1, it was demonstrated that an automated evaluation of PET images using only Tmax/B and tumor volume does not yield sufficient information to be of use for follow-up. Changes in the spatial configuration of the tumor need to be considered. But in order to determine the actual value of 18F-FET PET for follow-up it would have been necessary for the describing nuclear medicine specialist to clearly state the result of the examination in standardized terms such as those used in the RANO criteria or in RECIST (progressive disease, stable disease, partial response, complete response). In many cases, somewhat unspecific terms such as 'the metabolically active volume' were used in place of 'tumor' and graduated descriptions like 'slight progression' were seen. This was probably due to both a lack of standardized cut-offs for PET response evaluation as well as the fact that the technique was implemented at our institution only weeks before the first patient was included. Thus, it would not have been possible to define strict

guidelines prior to study start. In addition, since we had limited experience with evaluating re-irradiated tumors, it was not possible to use 18F-FET PET as a single modality in follow-up. Lastly, as PET in many cases aided in the interpretation of clinical situations, an analysis of PET vs. MRI in determining time of progression would have been critically biased. For the seven patients who developed distant metastases during follow-up, MRI and 18F-FET PET proved equally sensitive in detecting new lesions. But while it is possible to carry out a blinded description of an MRI scan (with no knowledge of PET results), this is not entirely the case for PET scans, which are co-registered to the most recent MRI scans. The analyzing nuclear medicine specialist will thus have access to the MRI and will look through it in a more or less systematic fashion, as the procedure in our institution calls for. MRI may therefore be at a disadvantage in a head-to-head comparison. Naturally, a study could be designed that addresses this issue (using only registration to CT, for example) but this was not highly prioritized. In one case, the PET scan detected a distant metastasis just posterior to the orbital cavity of a GBM patient that was not seen on MRI until six weeks later. In cases where further treatment is warranted, high sensitivity to dissemination is important. Similarly, in one of the three cases where a patient could not enter the study because of disseminated disease (please refer to *results*), this was diagnosed with the aid of the PET-scan. It is somewhat difficult to quantify the significance of these two cases but we believe that they demonstrate the utility of 18F-FET PET in the staging of recurrent HGG. MRI also performed well for this purpose but the sensitivity seems to be improved by combining the modalities as T2W/FLAIR signal abnormalities may have many different causes.

Early responses to radiotherapy

In manuscripts 1 and 3, we have demonstrated an early response to radiotherapy (re-irradiation and primary RT) using 18F-FET PET and perfusion weighted MRI. Possible mechanisms for increased perfusion (e.g., increased VEGF release by tumor cells as a stress response [56]) are discussed in manuscript 3. With 18F-FET PET, we observed a statistically significant increase in tumor volume but a trend ($p=0.06$) towards decreasing T_{max}/B during RT. There was also a non-significant trend for large decreases of T_{max}/B to be correlated to poor outcomes. As discussed, we consider this to be an indirect phenomenon caused by a correlation between high T_{max}/B and large tumor volume (BTV). The finding contradicts those of Galldiks et al., who found decreasing T_{max}/B to be prognostic of good outcome following primary RT of glioblastoma [57] but it is likely that newly diagnosed tumors are more susceptible to the effects of radiotherapy so that those results to a higher degree reflect the killing of tumor cells, whereas the significant decrease that we observed could be

due to infiltration of reactive immune cells which may have lower 18F-FET uptake than glioma cells, leading to decreased density of highly active cells. This explanation, which was initially offered by Spence et al.[58], could also explain the early growth in tumor volume (BTV). In general however, the associations that we have shown were not strong enough to provide a clear explanation for these phenomena. The limited number of patients and the generally poor separation between responding individuals and non-responding individuals is also a limitation of our studies.

Conclusions

Manuscripts 1, 2 and 3 and the results of the Re-irradiation study presented here warrant the following conclusions:

1. *The 18F-FET PET-positive tumor volume at baseline was prognostic for PFS and OS.*
   This indicates that patients with large tumors, where size is defined by the metabolically active tumor volume, are not likely to benefit from re-irradiation. This information was not obtainable by the contrast enhancing MRI volume. However, the Cox regression analysis was only carried out with two covariates and should be confirmed in a complete model containing other well-known prognostic factors when possible.
2. *Toxicity to re-irradiation was acceptable but not negligible*.
   All patients could complete treatment but due to early progression in many cases, only a minority was evaluable for late toxicity. This included asymptomatic necrosis and symptomatic white matter changes that responded to steroid therapy. Early results do not support further use of treatment regime 2 (3.5 Gy x 10 + 7 Gy boost) but the study should complete accrual and follow-up before final recommendations are made.
3. *An early reaction to radiotherapy was demonstrated by DCE-MRI during primary RT and by 18F-FET PET during re-irradiation.*
   This may be caused by a poorly described tissue reaction to radiotherapy or simply tumor growth preceding the response to therapy.
4. *Cognitive testing was feasible in clinical practice*.
   Short survival times and patient heterogeneity did not allow for any definitive conclusions regarding the impact of re-irradiation on cognitive function. But testing was feasible in this poorly performing patient group and showed potential as an aid for assessment of response and cognitive deterioration.

5. *18F-FET PET likely contributed important information for radiotherapy planning*.
   Staging of disease was improved with PET. Areas of 18F-FET uptake extended up to 42 mm from T1W+C MRI defined tumor. 18F-FET PET has been shown to be more specific that MRI at recurrence and it is highly likely that PET-positive areas should be included in the radiation fields.
6. *The efficacy of re-irradiation was moderate at best.*.
   No objective responses were observed but 18% of patients were progression-free at six months. It is unknown whether this was an effect of treatment or of selection of patients with indolent disease.
7. *The diagnostic accuracy of 18F-FET PET could not be determined by the study's optional biopsy part.*
   Recruitment was insufficient and no definitive conclusions can be made. But the few results obtained were surprising and suggested that needle biopsy was not sufficient for this purpose.
8. *The role of 18F-FET PET in follow-up after re-irradiation remains to be established.*
   Early changes in quantitative parameters of the 18F-FET PET scans following radiotherapy did not correlate with outcomes. The but when interpreted by a nuclear medicine specialist, the studies aided in assessing response to treatment, which was challenging.

Future perspectives

In this study, we were not able to contribute with information regarding the diagnostic accuracy of 18F-FET PET using biopsy controls. Therefore, we cannot make any clear recommendations about the use of margins for future re-irradiation. However, more detailed analyses of patterns of recurrence may provide information about the adequacy of the margins used. As described, most recurrences occurred locally, and the area with the highest 18F-FET uptake generally did not shift from within the irradiated area to areas outside. We have interpreted this as a failure of RT in achieving adequate tumor cell kill, but it cannot be ruled out that progressive tumor grew from the edge of the radiation field due to inadequate margins. This analysis is planned upon completion of the phase I study.

As of February 2014, five more patients must be included in the trial to complete accrual in the phase 1 part of the study. The poor survival times observed in this study highlight the need for tools to improve selection of patients that may potentially benefit from re-irradiation. Eighteen percent of

the patients were progression-free at 6 months but no objective responses have been observed at this time. When the phase I-part of the study has finished accrual and follow-up (expected in 2014/2015) it will be possible to carry out a multivariate analysis of prognostic factors for PFS and OS and generate a prognostic model that may aid in predicting which patients are likely to live long enough to possibly benefit from treatment. It will be possible to apply (elements of) this risk-model to the planned phase II part of the study which aims to assess responses and progression-free survival.

At the end of the entire study, the efficacy of re-irradiation will be evaluated in conjunction with toxicity. It will then be decided whether the results warrant further investigation in the form of a controlled trial (phase III). Such a trial would face a number of challenges: a very small and selected patient group, availability of ^{18}F-FET PET, competing future protocols, choice of comparator (e.g. placebo, surgery), advances in surgical technique, funding etc.

Given these obstacles and modest efficacy observed so far, it may be more productive to search for other treatment modalities that re-irradiation may be combined with. Possible candidates include the VEGF-antibody, bevacizumab, which seems to improve efficacy of re-irradiation[59] despite the recent disappointing results of up-front treatment [25, 26]. Immunotherapy has not been successfully used in the treatment of glioblastoma, but the field seems to be experiencing a revival in these years. There is experimental evidence to suggest a role of radiotherapy as an adjuvant to systemic immunotherapy due to local effects (e.g., increased exposure of tumor antigens by irradiated cancer cells) [60]but this remains to be established in clinical trials. The effects of immunotherapy can be challenging to evaluate with current imaging evaluation criteria [61] and it is conceivable that PET and dynamic MRI may offer valuable information in this setting.

In this study, 18F-FET PET was used for tumor delineation and the prognostic significance of the metabolic volume supported this function. Novel tools of molecular imaging that probe other aspects of tumor biology and aid in establishing the physiological tumor phenotype, will be available for human use soon; these include the hypoxia tracers, e.g., Cu-ATSM[62], or dynamic nuclear polarization (DNP) using 13C magnetic resonance spectroscopy[63]. These also have the potential to contribute information in the planning or follow-up of (re-)irradiation of glioma.

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Appendix

Table A: Results following re-irradiation of high-grade glioma (monotherapy)

Recruiting period	n=	Glioma grade (WHO)	Regime: Total dose/no. of fractions/time (median)	Mean tumor volume and range (ccm)	Radiation necrosis (%)	Median survival all high-grade gliomas (months)	Median survival by WHO grade (months)	Response	Reference
1988-1991 Retrospective	7 7	III IV	36 Gy / 20	not specified	not specified	not specified	7	Radiographic: SD or 'regression' 68%	Kim 1997 Am J Clin Oncol
1989-1994 Prospective	32	III/IV **	20 – 50 Gy dose escalation / 5 fractions	24 (3-93)	36 (associated with dosis >40 Gy)	11	not specified	not specified	Shepherd 1997 Int. J. Radiation Oncology Biol. Phys.
1994-1996 Prospective	1 19	III IV	24 Gy / 30 Gy / 35 Gy total. Hypofract. dose escalation	13 (1-48)	N/A but no re-operations needed.	10,5		22% minor response (25% regression), Response correlated with higher dose	Hudes 1999 Int. J. Radiation Oncology Biol. Phys.
1991-1998 Retrospective	46 25	III/IV III/IV	Single dose 17 Gy Fractionated 37,5 Gy/15	10 (1-54) PTV:30 (3-125) 25 (4-115) PTV:74 (10-200)	30 8 (p<0,05)	11 vs. 12 (NS)	III 14,7** IV 7,1	not specified	Cho 1999 Int. J. Radiation Oncology Biol. Phys.
1995-2000 Retrospective	10	III/IV	Stereotactic IMRT 30 Gy 5 Gy/F 6 F/W	35 (4-75)	60*	10,1	not specified	not specified	Voynov 2002 Am J Clin Oncol
1997-2003 Retrospective	5 14	III IV (74%)	30 Gy / 6	PTV: 15 (4-70)	not specified	9,3	15,4 7,9	not specified	Vordermark 2005 BMC Cancer

1990-2004 Retrospective	42 59	III IV	36 Gy total 2 Gy/F 5 F/W (median)	PTV: 49,3 (2,5-636)	0,5 %	not specified	16 8	not specified	Combs 2005 J Clin Oncol
2003-2005 Prospective	4 11	III IV (73%)	35 Gy 5 Gy/F 3 F/W	6 (1-22) PTV: 22 (4-87)	27 % (no reop. but edema, steroid use)	12	not specified	Radiographic: PR=27% SD=33% PD=40%	Ernst-Stecken 2007 J Neurooncol
1994-2007 Retrospective	2 29	III IV	20 Gy (median)		No severe tox.		10,2		Hencke 2009 Stralenther Onkol
1998-2008 Retrospective	53		30 Gy median (20-60 Gy)	Tumor volume median =35 ml	0 grade 3 or more		9 months		Fokas 2009 Stralenther Onkol
1994-2008 Retrospective	147	III/IV but not specified further	35 Gy median 3,5 Gy x 10	Median =22 (0,6-104ml)	0,6% (grade III headache)	11 months		Minmal response: 10% SD: 60 % PD: 30 %	Fogh 2010 J Clin Oncol

* Evaluated by imaging. Clinical performance not discussed.

** Results of both treatment groups (SRS vs. FSRT) combined

¤ 6 patients received whole brain RT, 13 hemi-brain RT and 12 limited field RT.

	Group 1		Group 2		Group 3		Group 4		Total	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Early and late symptoms										
No. of evaluable patients	**12**		**9**		**3**		**4**		**28**	
Seizure	8%	0	11%	11%	0	0	0	0	8%	4 %
Headache	25%	0	38%	13 %	33%	0	0	0	32 %	4 %
Fatigue	25%	0	25%	0	0	0	0	0	20 %	0
Alopecia	100%	0	100%	0	100%	0	100%	0	100 %[1]	N/A
Skin reaction	8%	0	13%	0	0	0	50%	0	12 %[2]	0
Conjunctivitis	0	0	0	0	0	0	0	0	0	0
Nausea	0	0	13%	13%	0	0	0	0	5 %	5 %
Dizziness	17%	0	14%	0	0	0	0	0	8 %	4 %
Increased steroid use	8%	0	50%	0	0	0	0	0	19%	N/A
Major late events										
No. of evaluable patients	**5**		**2**		**0**		**0**		**7**	
Radio-necrosis	N/A	20%	N/A	0	N/A	0	N/A	0	N/A	14%
Progressive white matter changes	N/A	0	N/A	100%	N/A	0	N/A	0	N/A	29%

1.In 7/8 cases (88%) where a follow-up of 16 weeks was achieved, alopecia was reversible.

2. Only transient grade 1 events were recorded (no intervention indicated).

Table B. Toxicity to re-irradiation for each study group. Grades refer to CTCAE v. 3 [39]

Manuscript 1

Title Page

Title

Prognostic value of ^{18}F-FET PET imaging in re-irradiation of high-grade glioma: Results of a prospective trial.

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Abstract

Background and Purpose: Positron emission tomography (PET) provides quantitative metabolic information that may include biomarkers of treatment outcome. Our aim was to determine the prognostic value of early ^{18}F-fluoroethyl-tyrosine (^{18}F-FET) PET scans acquired in the course of re-irradiation for recurrent high-grade glioma (HGG).

Material and Methods: Two dose groups in a phase I/II trial of re-irradiation of HGG have completed treatment (total doses: 35 Gy and 42 Gy). MRI and ^{18}F-FET PET was used for target delineation and follow-up. Images were acquired at baseline, during radiotherapy and 4 weeks post-treatment and compared by measuring the metabolically active tumor volume and maximal activity (T_{max}/B). Correlations with outcomes were assessed by Cox regression analysis.

Results: Twenty-one patients with HGG were included. Baseline tumor volume defined by ^{18}F-FET PET, but not MRI, was prognostic for overall survival (OS) (HR= 1.8 per increase of 10 cm^3, p=0.003) and progression-free survival (PFS) (HR=1.40, p=0.02) following re-irradiation. Changes in tumor volume or T_{max}/B at these time points did not correlate significantly with outcome.

Conclusions: Baseline tumor volume defined by ^{18}F-FET PET, but not MRI, was prognostic for OS and PFS. Changes in tumor volume or T_{max}/B during- and after treatment were not correlated with outcomes.

Keywords

High-grade glioma; positron emission tomography; re-irradiation; ^{18}F-FET PET; imaging biomarker.

Introduction

High-grade glioma (HGG) is a devastating and lethal cancer of the brain. There is international consensus regarding the primary treatment[1] but the disease invariably recurs. Recurrences are most often local, even after salvage treatment[2], which suggests that achievement of local disease control is a worthwhile treatment goal.

Re-irradiation to localized recurrences using modern imaging- and delivery techniques (magnetic resonance imaging (MRI), 3-dimensional conformal radiotherapy, intensity modulated radiotherapy (IMRT)) has been shown to be well tolerated and safe[3]. The risk of serious toxicity has been shown to depend on dose, fraction size and target volume [4] [5], and therefore precise target delineation is important. Although MRI is currently the standard imaging modality for HGG[6], it has limited specificity in the setting of recurrent disease[7]. The amino acid analogue, ^{18}F-fluoroethyl-tyrosine (^{18}F-FET) is a PET tracer with higher specificity than MRI in previously treated patients[7]. In a study where PET imaging using another amino acid tracer, ^{11}C-methionine, was used for target delineation, a survival benefit was observed when compared with MRI-based planning alone, but this remains to be confirmed[8]. In addition, PET has potential as an imaging biomarker of outcome following treatment because it yields quantitative information about tumor metabolism.

A prospective phase I/II study of re-irradiation for recurrent HGG using ^{18}F-FET PET and MRI for target delineation and follow-up is currently being carried out. In this article, the data obtained by ^{18}F -FET PET/MRI scans before, during and after radiotherapy of 21 patients

comprising the first two of four study groups are analyzed in correlation with clinical outcomes. The aim is to evaluate the possible prognostic value of ^{18}F-FET PET imaging in the course of re-irradiation for recurrent HGG.

Materials and methods

A clinical phase I/II dose-volume escalation study was planned consisting of four sequential treatment groups that were to receive different radiotherapy doses. The study was carried out in accordance with the Helsinki II Declaration and approved by the Ethics Board of the Capital Region of Denmark (protocol: H-2-2011-092). It is registered in the ClinicalTrials.gov database (NCT02025231). Written informed consent was required for entry into the study.

Patients

Inclusion criteria: Recurrent high-grade glioma as defined by the RANO criteria[6]; ECOG (Eastern Cooperative Oncology Group) performance status 0-2; localized disease; previous radiotherapy completed >6 months prior; no standard treatments available; expected life span > 3 months; age >18 years. A histological diagnosis of HGG was required but not necessarily at recurrence.

Exclusion criteria: Diffuse/large recurrences (planning target volume (PTV) > 100 cm^3); early recurrence following primary radiotherapy (≤3 months); fistula or other local pathologic conditions; contraindications to MRI- or ^{18}F-FET PET CT.

Imaging and treatment

The gross tumor volume (GTV) for radiotherapy was defined using MRI and ^{18}F-FET PET imaging. Both imaging modalities were used for each evaluation at follow-up.

Magnetic resonance imaging

MRI for treatment planning and all subsequent scans was carried out on a Siemens Magnetom Espree 1.5 T scanner. Standard clinical sequences (T1 pre-and post Gadolinium (Gd)-contrast (Gadovist ®, 0.1 ml/kg) and T2/T2 fluid attenuation inversion recovery (FLAIR)) were acquired at each time point. GTV MRI was contoured by a radiologist using a T1-post Gd-contrast MRI sequence. Only contrast-enhancing tumor was contoured.

^{18}F-FET PET /CT imaging

^{18}F-FET PET and planning CT scanning was performed in a single session with an integrated 64 CT slice hybrid PET/CT system (Siemens Biograph mCT scanner). A single static ^{18}F-FET PET frame of the entire brain was acquired at 20 to 40 minutes after i.v. injection of 200 MBq ^{18}F-FET. For all images, default random, scatter, and dead time correction and CT-based attenuation correction was applied. Image reconstruction was performed using OSEM 3D (4 iterations, 16 subsets with a matrix size of 336x336x74 (0.8x0.8x1 mm voxel size)). Images were filtered with a 5 mm FWHM Gaussian filter. The ^{18}F-FET PET image was co-registered to T1-post Gd-contrast MRI. A 3D crescent shaped background (B) region of interest (ROI) encompassing the activity > 70 % of maximum was delineated in healthy appearing gray and white matter above the insula in the contralateral hemisphere. The GTV-PET was auto-contoured in 3D defining tumor tissue at a threshold of above 1.6 of mean SUV (standardized uptake value) in the background ROI (Syngo-TrueD, Siemens)[9]. The maximal tumor uptake normalized to background brain tissue (T_{max}/B) was calculated from the maximal tumor activity (SUV_{max}) and B.

Radiotherapy

The planning target volume (PTV) equalled the union of the GTV-MRI and the GTV-PET plus a 2 mm margin. A thermoplastic mask was used for fixation. The prescribed dose to the PTV was 35 Gy (group 1) or the GTV-PET was prescribed 42 Gy and the PTV 35 Gy (group 2), respectively, in 10 fractions and 5 fractions/week. Stereotactic radiotherapy was delivered using Volumetric Modulated Arc Therapy (VMAT), typically using 2 arcs with 360 degrees rotation (Novalis Tx accelerator, RapidArc, Varian Medical Systems). The permitted dose variation was 97-105% of the prescribed dose. Organs at risk were the brainstem, optic nerves/chiasm, hippocampi, eyes and healthy brain tissue. Maximum allowed total doses for all RT were 65.3 Gy and 60 Gy (EQD2, alpha/beta=3) for the brainstem and chiasm, respectively, while the dose to other structures were minimized. Daily stereoscopic imaging was performed to ensure accurate patient positioning using 6-degrees of freedom (6D Robotics Couch® and ExacTrac®, BrainLab AG), using a 1 mm tolerance.

Endpoints, Follow-up and Statistics

The primary study endpoint was early and late toxicity grade 3-5 (CTC-AE version 3.0)[10]. Treatment response was evaluated using the RANO (Response Assessment in Neuro-Oncology) criteria [6]. Follow-up scans and clinical evaluations (MRI and ^{18}F-FET PET) were carried out at the following time-points: during the 2nd week of RT and 4, 10, 16, 22, 28, 34, 46 and 58 weeks after RT.

Survival times following radiotherapy were calculated from the date of inclusion. Relationships between variables were evaluated using the Spearman's rank correlation coefficient, r_S. Paired data were compared using the Wilcoxon signed rank test. Unpaired data were compared using one-way ANOVA. Cox's proportional hazards regression analysis was

used to assess the effects of covariates on survival times. The linearity of continuous covariates was assessed using cumulated Martingales residuals. The landmark method was used to calculate survival times from scans performed after the inclusion date. SPSS 19 was used for statistical calculations.

Results

Twenty-one patients were recruited to study groups 1 and 2 between December 2011 and February 2013. Study groups 3 and 4 are currently accruing. Patients' baseline characteristics and details regarding the treatment are listed in table 1. Figure 1 shows an example of MRI- and ^{18}F-FET PET imaging as well as a treatment plan for one patient. All patients completed treatment as planned. MRI and ^{18}F-FET PET scans were acquired at baseline for all patients, during radiotherapy for 19/21 patients (90%), and for all patients 4 weeks post radiotherapy. The median radiotherapy dose received at the time of the second scan was 31.5 Gy.

In most cases (18/21), the ^{18}F-FET PET scan supported the diagnosis of recurrence, but in three cases the baseline biological tumor volume (BTV) was less than 1 cm^3. In two of these cases, the PET-scan at baseline did not unequivocally support the diagnosis of recurrent tumor (one anaplastic astrocytoma, one GBM).

Quantitative ^{18}F-FET PET data

The BTV, ranged from 0.1 cm^3 to 60.0 cm^3. The median change in BTV at scan 2 (ΔBTV2) was a 6 cm^3 increase and this was statistically significant ($p<0.001$). At scan 3, the median ΔBTV3 was 4.4 cm^3. (Figure 1B).

T_{max}/B was slightly lower at scan 2 compared to baseline, which indicates an early response to therapy in the most active tumor volume. At scan 3, the reduction was significant (median change = -0.20, range: -2.0, +1.0 $p<0.05$) and a correlation was found between early response and late response (Figure 1C). There were no detectable differences between ΔBTV, Δ T_{max}/B and Δ T_{mean}/B for treatment groups 1 and 2.

Clinical outcomes and prognostic factors

At the time of censoring, 18 patients had experienced disease progression and 16 patients had died. The median PFS was 2.7 months (95% CI: 1.7-3.6). The OS following re-irradiation was 9.1 months (95% CI: 7.1-11.2). PFS was significantly prognostic for OS with a hazard ratio of 0.8 per 10 days (95% CI: 0.7-1.0 p=0.02).

To test the potential prognostic significance of values derived from ^{18}F-FET PET scans and of well known clinical characteristics, univariate Cox regression analyses were carried out. The number of events for both PFS and OS was considered insufficient for multivariate analysis. Results are shown in Table 2. The BTV at baseline and steroid use at baseline were significantly prognostic for OS. When adjusted for the effect of steroid treatment, the BTV at baseline remained statistically significant (HR=1.7 95%CI: 1.2-2.4 p=0.005). When the GBM patients (n=16) were analysed separately, the baseline BTV was also prognostic (HR=1.5 95%CI: 1.1-2.2 p=.02).

ΔBTV at scan 2 or at scan 3 were not correlated to PFS or OS outcomes. Δ T_{max}/B at scan 2 and 3 were not significantly correlated to outcomes but there appears to be a tendency for responders (that is, with a decrease in value) to have a higher hazard ratio for progression and death. This trend was evident for changes expressed both as absolute- and relative values and when setting

changes of less than 5 % to zero [11]. A positive correlation was found between T_{max}/B at baseline and Δ T_{max}/B at scans 2 and 3 (r_S= -0.52 and -0.69 and p=0.02 and p<0.01, respectively). T_{max}/B at baseline also showed a non-significant trend towards being prognostic for both PFS and OS and was correlated to ^{18}F-FET PET tumor volume at baseline (r_S=0.78 p<0.01).

Discussion

^{18}F-FET uptake is mediated through an over expression of the L-amino acid transporter 2 (LAT2)[12] and is correlated to vascular- and cell density in glioma[13]. ^{18}F-FET PET imaging can efficiently define both solid and infiltrating tumor components[9]. Tumor cell death from ionizing radiation could therefore be expected to decrease parameters of ^{18}F-FET-uptake resulting in lower tumor volumes as well as lower values of maximal intensity (T_{max}/B). We hypothesized that early ^{18}F-FET PET scans following re-irradiation of HGG could provide quantitative information about the anti-tumor effect which would ultimately predict the duration of response as well as survival following treatment.

Mechanisms of ^{18}F-FET PET changes during radiotherapy

The data above suggest that the changes in the ^{18}F-FET PET positive volume (BTV) and T_{max}/B observed following re-irradiation are modest. A slight but significant increase in BTV during treatment was noted but this decreased at scan 3. This increase may reflect either tumor growth in the period between baseline scan and response to radiotherapy or unspecific ^{18}F-FET-uptake caused by an early tissue reaction to radiotherapy. Recent animal experiments have shown that reactive astrogliosis may be abundant following radiotherapy[14]. While the authors found this tissue to have significantly lower activity than tumor cells, it could potentially influence the

delineation when using a threshold value of 1.6 (tumor-to-brain) as was the case in our study. Using a higher threshold value than 1.6 for tumor delineation during follow-up (e.g. 2.0[15]) could potentially increase the specificity of the examination but would invariably compromise the sensitivity to detect infiltrating tumor. As the radiotherapy in our study was delivered using a 'stereotactic' margin (2 mm) adequate tumor delineation was considered critical. Furthermore, a 2.0 tumor-to-brain threshold might induce errors because of considerable partial volume effects in the measurements of small volumes (< 2 cm^3) that could affect results adversely. In a study similar to ours[11], the threshold of 2.0 was evaluated but discarded for a lack of sensitivity to changes of treatment effects [*personal correspondence KJ Langen*].

BTV is a crude measurement that contains no spatial information about the disease. A tumor may respond to RT centrally but progress at the margins (or by distant metastases) with no net change of the overall volume. Such a pattern of failure could potentially explain the lack of association between tumor shrinkage and PFS and OS. However, by registering the baseline BTV to the subsequent scans, it was evident that the point of maximal activity did not shift from within the treated BTV to areas outside the radiation field in 18 of 21 cases. The three patients whose T_{max}/B shifted outside of the irradiated volume had developed distant metastases 4 weeks post treatment. This indicates that RT generally failed to extinguish the most active tumor as measured by ^{18}F-FET PET within the measured time period. Lastly, in 16/21 cases, the mean activity in the treated BTV decreased or was unchanged. This was not statistically significant ($p=0.1$), but taken together with the significant decrease of the T_{max}/B, the results offer some encouragement that the treatment at least had a measurable biological effect, even despite the failure to correlate this to clinical outcomes.

Prognostic value of MRI

The baseline tumor volume defined by MRI was not found to have prognostic value in this study. The specificity of MRI for identifying recurrence has been found to be in the order of 50 % and it may also fail to identify infiltrating tumor tissue[7]. Thus, contrast enhancement caused by the effect of surgery and radiotherapy may be misinterpreted as tumor tissue while underestimating the extent of tumor infiltration in the periphery. Although surgical cavities were not routinely included in the gross tumor volume (GTV), we found measurable volumes of cavity (> 1 cm^3) in the GTV in 9 cases. In a post-hoc attempt to determine the true contrast-enhancing tumor volume within the GTV, these cavities were contoured and subtracted from the GTV. A Cox regression analysis with these corrected volumes was then carried out, but no significant correlation to OS (HR= 1.1 95%CI: 0.8-1.4) or PFS (HR= 0.9 95% CI: 0.7-1.2) could be found.

Considering the limited specificity of MRI, it is a weakness of our study that histological verification of recurrent tumor was available in only 5 cases. Although the ^{18}F-FET PET scan supported the diagnosis of recurrence in a large majority of cases, most often there was no prior ^{18}F-FET PET scan available for comparison. The time elapsed from primary radiotherapy was generally long (median=26 months, range: 12-108 months) and the prevalence of early treatment related changes (e.g., pseudoprogression) was therefore likely to be low. Thus, while we cannot rule out that a minority of patients indeed did not have recurrent tumor, we believe that the conclusions of the study would be unaffected.

Prognostic value of ^{18}F-FET PET

Biological tumor volume

Although it was not possible to carry out a multivariate proportional hazards analysis, there was a strong positive correlation observed between the BTV at baseline and OS, even when adjusting for the only other variable that was significantly prognostic (steroid use at baseline). This indicates a biological significance of the baseline BTV. This has been reported previously in the setting of primary radio-chemotherapy for HGG[11,16], but not in re-irradiation for recurrent disease as far as we are aware. In a retrospective study of 56 patients that had received re-irradiation[17], the baseline BTV was not found to be prognostic but that study differed from the current in significant ways (e.g., concurrent treatment, patients' level of pretreatment and delineation of tumor).

Changes in T_{max}/B

Galldiks et al. performed early ^{18}F-FET PET scans following primary RT in a prospective study and found that a decrease in T_{max}/B was predictive of favorable outcome[11]. This is intuitively comprehendible but in direct opposition to our findings. We find it most likely that the inverse correlation that seems to exist in our study is an epiphenomenon caused by the fact that larger tumors had higher T_{max}/B values and were more likely to respond to radiotherapy, but these responses were not significant enough to outweigh the baseline prognostic disadvantage of tumor size. However, both studies are small (n=25 and n=21) and the results should not be over interpreted, especially as the correlations to PFS and OS in the current study were not statistically significant.

To conclude, we found the baseline biological tumor volume, BTV, to be of prognostic value in this prospective study of re-irradiation in patients with recurrent high-grade glioma. The tumor volume defined by MRI was not prognostic. The changes observed in the quantitative ^{18}F-FET

PET parameters of BTV and T_{max}/B were modest following re-irradiation, and they were not significantly correlated to outcomes. These findings may help identify those patients whose life expectancy is too short to warrant a course of re-irradiation.

Acknowledgements

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Conflict of Interest Statement

None.

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Figure captions

Figure 1. Baseline co-registered MRI and ^{18}F-FET PET images and radiotherapy plan of a 54-year old male patient with recurrent GBM in the corpus callosum. The white line on the treatment plan marks the planning target volume, which was treated to a total dose of 35 Gy (study group 1). The patient had a large BTV of 54 cm^3 and died approximately 3 months after being included in the study.

Figure 2. Changes in tumor volume defined by ^{18}F-FET PET (BTV) and maximal activity (T_{max}/B) during- and after re-irradiation. (A) Change in BTV at scan 2 and scan 3 for individuals. (B) Mean BTV before, during- and after re-irradiation. (C) Scatter plot of correlation between change in BTV at scan 2 and at scan 3. (D) Change in T_{max}/B at scan 2 and scan 3 for individuals. (E) Mean T_{max}/B before, during- and after re-irradiation. (F) Scatter plot of correlation between change in T_{max}/B at scan 2 and at scan 3.

Patients	**n = 21**		
Age, years, median (range)	53		(30-66)
Performance status			
0	9	(43 %)	
1	9	(43 %)	
2	3	(14 %)	
Diagnosis			
Glioblastoma	16	(76 %)	
Glioma WHO gr. III	5	(24 %)	
Previous treatment			
Radiotherapy 60 Gy	16	(76 %)	
Radiotherapy 44 - 45 Gy	5	(24 %)	
Temozolomide	21	(100 %)	
Bevacizumab	12	(57 %)	
Surgery prior to reirradiation	2	(9 %)	
Months since diagnosis, median (range)	23		(7-129)
Treatment allocation in study			
Group 1	12	(57 %)	
Group 2	9	(43 %)	
Target volumes for radiotherapy, median (cm^3)			
Tumor volume by MRI,	29.2		(7.9-81.8)
Tumor volume by FET-PET	22.0		(0.1-60.0)
Planning target volume	57.5		(16.4 – 119.9)

Table 1. Baseline patient characteristics

Covariate	Level	Hazard Ratio (95 % CI)		p-value	Hazard Ratio (95 % CI)		p-value
		PFS			OS		
BTV baseline	per 10 cm^3	1.4	(1.1 - 1.9)	0.02	1.8	(1.2 – 2.6)	0.003
Steroid T_x baseline	yes vs. no	4.0	(1.2 - 12.9)	0.02	3.5	(1.2 - 10.2)	0.02
Age	per 10 years	1.5	(1.0 - 2.4)	0.05	1.4	(0.9 - 2.1)	0.12
Performance status	1-2 vs. 0	3.0	(1.0 – 8.6)	0.05	2.6	(0.8 – 8.2)	0.10
Diagnosis	grade IV vs. grade III	2.6	(0.7 – 9.5)	0.14	4.2	(0.9 – 18.7)	0.06
MRI vol. baseline	per 10 cm^3	0.8	(0.7 - 1.1)	0.13	0.8	(0.7 - 1.1)	0.13
Tmax/B baseline	continuous	1.5	(0.9 - 2.5)	0.09	1.5	(0.9 - 2.5)	0.09
Δ BTV scan 2	per decrease of 10 cm^3	0.8	(0.6 – 1.1)	0.25	0.8	(0.6 – 1.1)	0.18
Δ BTV scan 3	per decrease of 10 cm^3	0.9	(0.8 - 1.1)	0.45	1.0	(0.8 - 1.1)	0.68
Δ Tmax/B scan 2	per increase of 1 (absolute values)	0.6	(0.2 – 1.6)	0.28	0.4	(0.1 – 1.2)	0.11
Δ Tmax/B scan 2	per increase of 10 % (relative to baseline)	0.8	(0.5 – 1.2)	0.34	0.7	(0.4 – 1.2)	0.20
Δ Tmax/B scan 3	per increase of 1 (absolute values)	0.6	(0.3 – 1.2)	0.13	0.6	(0.3 – 1.0)	0.07
Δ Tmax/B scan 3	per increase of 10 % (relative to baseline)	0.8	(0.6 – 1.1)	0.19	0.8	(0.6 – 1.0)	0.07

Table 2. Univariate analysis of prognostic factors for PFS and OS.
Abbreviations: CI, confidence interval; PFS, progression-free survial; OS, overall survival following treatment; BTV, biological tumor volume(defined by 18F-FET PET); T_{max}/B, maximal activity;

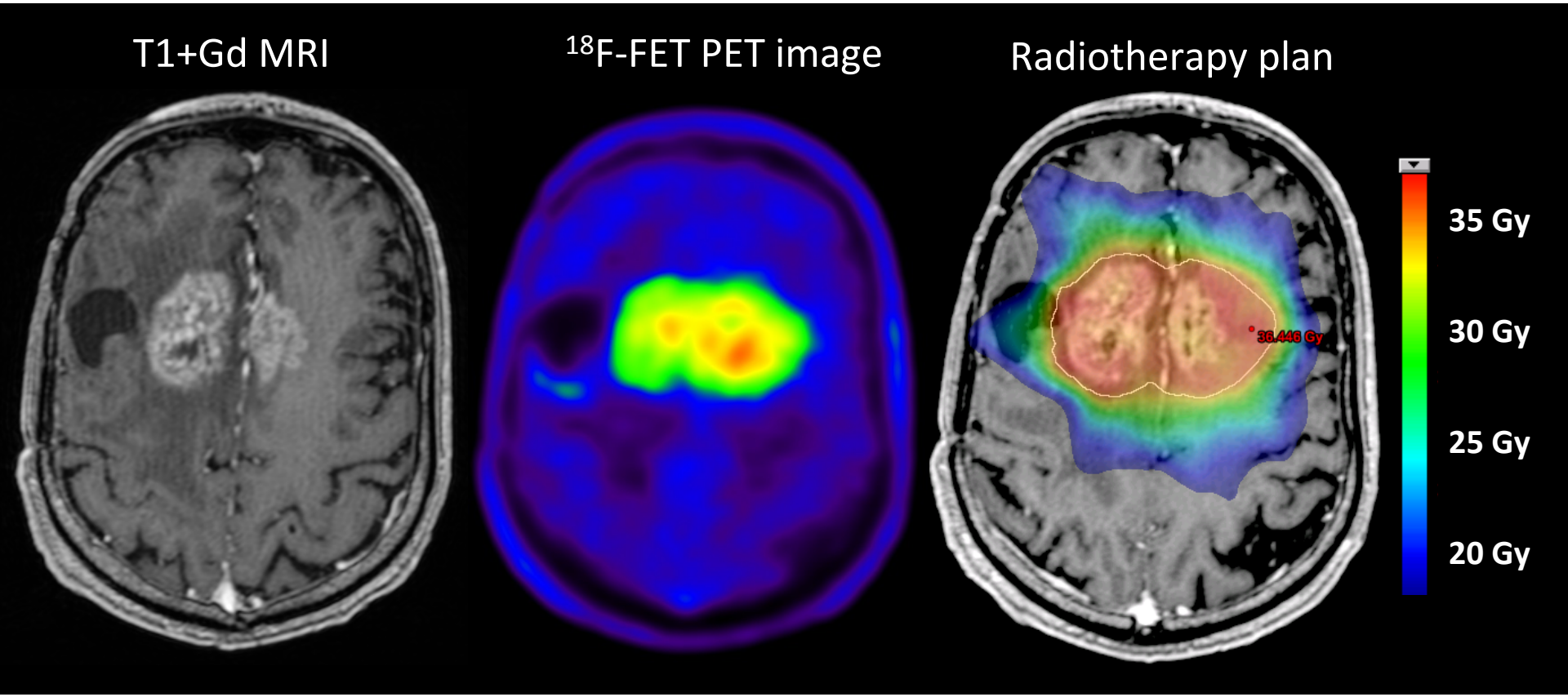

Figure 1. Baseline co-registered MRI and ^{18}F-FET PET images and radiotherapy plan of a 54-year old male patient with recurrent GBM in the corpus callosum. The white line on the treatment plan marks the planning target volume, which was treated to a total dose of 35 Gy (study group 1). The patient had a large BTV of 54 cm^3 and died approximately 3 months after being included in the study.

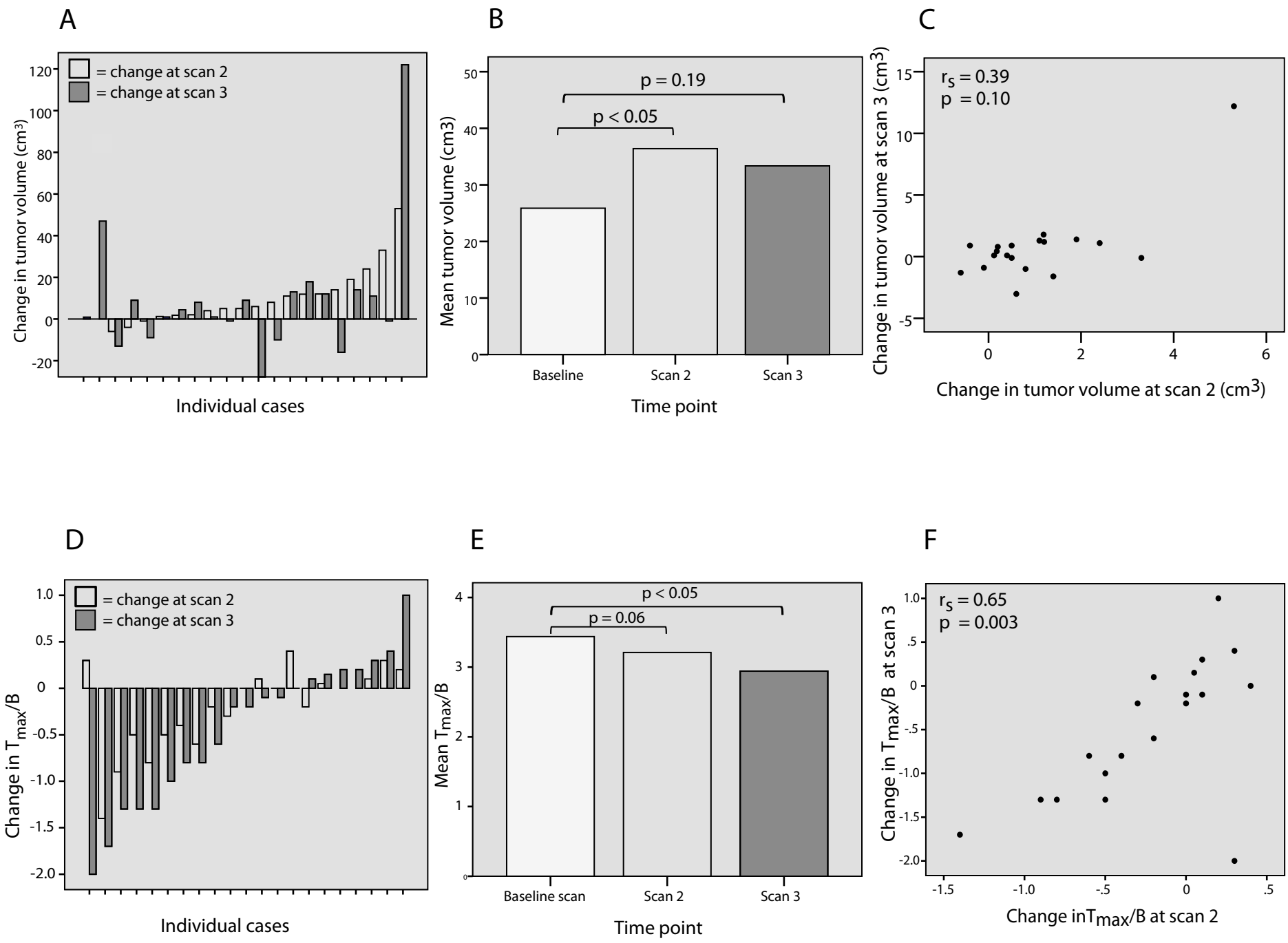

Figure 2. Changes in tumor volume defined by 18F-FET PET (BTV) and maximal activity (Tmax/B) during- and after re-irradiation. (A) Change in BTV at scan 2 and scan 3 for individuals. (B) Mean BTV before, during- and after re-irradiation. (C) Scatter plot of correlation between change in BTV at scan 2 and at scan 3. (D) Change in Tmax/B at scan 2 and scan 3 for individuals. (E) Mean Tmax/B before, during- and after re-irradiation.

Manuscript 2

Title

Cognitive function before and after re-irradiation of high-grade glioma: Results of a prospective trial.

Authors and affiliations

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Abstract

BACKGROUND
Re-irradiation of recurrent high-grade glioma is generally regarded as a safe treatment option for a subgroup of patients. But it is unknown whether re-irradiation affects cognitive function adversely and whether this is a relevant concern given the poor prognosis.
METHODS
A prospective phase I/II study of hypofractionated stereotactic re-irradiation to patients with recurrent high-grade glioma is ongoing. The patients were given a brief neuropsychological test battery before radiotherapy and at all follow-up evaluations. The battery assessed four major cognitive domains: information processing speed, memory, verbal fluency and motor coordination.
RESULTS
Twenty-eight patients were included and all underwent cognitive testing. Patients were tested a median of three times (range:1 – 9). The group was generally heavily pretreated and 57% were severely impaired in the information processing speed domain at baseline. Standard (Z) scores and changes in these were prognostic for progression-free survival (PFS) and overall survival (OS) in univariate Cox regression analysis. Median PFS was 3.0 months and median OS was 8.7 months. A subgroup of patients with long survival times was analyzed at the single patient level. Using a reliable change index, we found temporary impaired cognitive performance in one patient which corresponded with the clinical finding of late radiation induced edema.
CONCLUSION
Longitudinal cognitive testing in this prospective trial was feasible and yielded potentially valuable prognostic information. We were able to identify one case of late adverse effects. For the subset of patients with long survival, loss of cognitive function may be an important issue and further investigation is warranted.

Keywords

Re-irradiation, cognitive function, high-grade glioma, clinical trial

Introduction

High-grade glioma (HGG) is a devastating primary cancer of the brain for which no curative treatment exists. But long-term survival is becoming more common[1] due to advances in surgical techniques, chemotherapy and radiotherapy. Maintaining quality of life for the patient despite aggressive interventions is an important goal of treatment. One aspect of quality of life is cognitive function. This is increasingly recognized by the neuro-oncology community, and recent multicenter phase III trials with glioblastoma patients have included neurocognitive endpoints [2, 3].
Radiotherapy has been a mainstay of treatment for HGG since a clear survival benefit was demonstrated [4]. Re-irradiation at recurrence is not a new concept but much of the literature available is based on retrospective patient series. Technology has improved both the ability to image tumors and to deliver precision radiotherapy. Questions about the adverse effects of re-irradiation remain, however, and one aspect of this is the potential impact on cognitive function. This has to our knowledge not been investigated previously and, generally, only a few studies have evaluated cognitive function in patients with recurrent HGG[5–7]

A prospective clinical study of re-irradiation of HGG is ongoing. The participants completed a cognitive test battery at baseline and at each evaluation following radiotherapy. In this work, the initial results from the first 28 patients are reported. We report the results of baseline testing and their correlation to outcomes as well as changes in cognitive function following re-irradiation.

Materials and methods

Study details
A phase I/II study of re-irradiation for patients with recurrent high-grade glioma is currently ongoing. The gross tumor volume (GTV)to be treated using external beam radiotherapy was delineated using both magnetic resonance imaging (MRI) (Siemens Magnetom Espree 1.5 T) and ^{18}F-fluoro-ethyl-tyrosine positron emission tomography (^{18}F-FET PET) (Siemens Biograph mCT). The radiotherapy was stereotactic and highly conformal and only 2 mm margins were added to the gross tumor volume. It was delivered using volumetric modulated arc therapy (Rapid Arc®, Varian) on Novalis Tx accelerators at 6 MV.
The study examines three different dose levels of radiotherapy: 3.5 Gy x 10 (group 1), 3.5 Gy x 10 + a 7 Gy boost to PET-positive areas of tumor (group 2) and 5.9 Gy x 5 (group 3). For these three dose levels the maximum allowed planning target volume for radiotherapy (PTV) was 100 cm^3 . A fourth group was treated with 3.5 Gy x 10 to PTVs of 100-300 cm^3 (larger tumors).
Treatment response (including progression) was evaluated using the Response Assessment in Neuro-Oncology (RANO) criteria[8]. The primary endpoint of the phase I part of the study was early and late toxicity to treatment as defined by the Common Toxicity Criteria of Adverse Events (CTCAE) ver. 3.0[9].

Ethics
The study was carried out in accordance with the Helsinki II declaration and was approved by the ethics board of the Capital Region of Denmark (protocol: H-2-2011-092). Participation required written informed consent.
ClinicalTrials.gov identifier: NCT02025231.

Patients
Inclusion criteria were: localized recurrent high-grade glioma (glioblastoma WHO grade 4 or anaplastic astrocytoma WHO grade 3 or anaplastic oligodendroglioma WHO grade 3); histologically confirmed diagnosis was required but not necessarily at recurrence; no other treatment options available; performance status 0-2; life expectancy > 3 months; PTV < 100 cm3 (for treatment groups 1-3) or PTV 100-300 cm3 (for treatment group 4); primary radiotherapy completed> 6 months previously.
Exclusion criteria were: disseminated disease as evaluated by the investigator; early progression following primary course of radiotherapy (< 3 months); contraindications to radiotherapy (e.g., local wound dehiscence); contraindications to MRI or ^{18}F-FET-PET (e.g., paramagnetic implants).

Test administration

The cognitive test battery was administered the study principal investigator (*SM*, M.D.) or by one of two study nurses who had received training in test administration. A registered neuropsychologist at our institution had trained *SM*. The test was performed at baseline and at every scheduled clinical follow-up evaluation. Evaluations were carried out 4, 10,

16, 22, 28, 34, 46 and 58 weeks after treatment. The memory test, however, was only carried out at baseline and at the 10.week evaluation (or at 4 weeks in case of early progression). Patients were briefly assessed prior to testing to ensure an adequate mental, physical and emotional condition.

Test battery

Trail Making Test A+B (TMT)

In part A of the test, subjects are required to connect numbered circles in correct sequence from 1 to 25 by drawing a line as quickly as possible. Part B is similar but also contains letters and the subject must now alternate between numbers and letters. The score is the time used for each test in seconds. The maximum allowed time was 3- (part A) and 5 minutes (part B). Patients who could not complete the test within this time received a score of 180 seconds and 300 seconds, respectively. The test is sensitive to changes in visual search, information processing speed and cognitive flexibility[10].

Symbol Digit Modalities Test (SDMT)

This test requires subjects to pair numbers (1-9) with symbols according to a digit-symbol 'key' on the top of the page. The score is the amount of correct numbers written within 90 seconds. Like the TMT, it is a complex test sensitive to a range of processes, notably information processing speed [11].

Memory test

This was developed at our institution and is in clinical use as part of a mental status examination. The subject is asked to name five easily recognizable drawings of common objects in each of six categories (e.g., vegetables and items of clothing) and name the category. Immediately after naming of the last category, free recall cued by category is attempted in the order presented, followed by a recognition trial where the subject is to point out 12 of the previously displayed objects mixed with 18 'distracter' images. In scoring, false positives are subtracted from correctly recognized items.

Verbal fluency test

The subjects are asked to produce as many words as possible within the category of 'animals' and 'words beginning with the letter 'S''. One minute was given for each of these two trials.

Grooved pegboard

The subjects are requested to place grooved pegs in the 25 holes of a board as fast as possible. Each hand was tested separately. The time in seconds to complete the test is the score and the maximum allowed time is five minutes. This test evaluates visuomotor coordination and –speed [12].

Statistics

Standard scores, or Z-scores, were calculated by the following general equation:

$$Z = \frac{X - \mu}{\sigma}$$

where X is the raw score, μ is the mean score in a reference population and σ the standard deviation in a reference population. For timed tests where a lower numerical score was better (TMT A+B and grooved pegboard), the negative Z-value was used so that positive Z-scores indicate better performance than the mean and negative Z-scores indicate a score worse than the mean for all tests. The calculations of Z-scores for each cognitive domain are described in table 1. Thresholds of Z= -1.5 and Z= -3.0 were used for categorizing patients' scores in each of the four cognitive domains as either normal, impaired or severely impaired.

A univariate Cox regression analysis was carried out to test the prognostic value of baseline scores and of changes in scores approximately one month after treatment compared to baseline. Survival times were calculated from the date of inclusion but the landmark method was used when comparing survival times according to change in scores at one month. The Kaplan-Meier method was used to estimate median survival times All statistical analyses were carried out using SPSS 19.

For evaluation of test failure on TMT-A, the reliable change index (RCI) was calculated using the method first described by Jacobson and Truax[13], and previously used in studies of recurrent glioma patients[5]. A confidence interval of 95 % was chosen for detection of reliable change. The test-retest reliability coefficients used for TMT-A was 0.43 which was based on tests of 20 normal individuals in a three-month interval (Gade, personal correspondence). Changes were compared to baseline and only deterioration was examined.

Results

Twenty-eight patients were enrolled in the study between December 2011 and December 2013 All patients underwent cognitive testing at baseline and 82% were retested one month after treatment. A total of 85 tests were administered, 81% of which were by *SM*.. The progression-free survival (PFS) was 3.0 months (95% CI: 2.1–3.7) and overall survival (OS) was 8.7 months (95% CI: 5.4-12). Baseline patient characteristics are listed in table 2.

Figure 1 shows the distribution of scores in each of the four cognitive domains at baseline and 4 weeks after radiotherapy. Median baseline Z-scores (and range) for TMT-A, TMT-B and SDMT were -2.2 (-21.9 – 1.2), -7.7 (-25.8 - 0.1) and -2.4 (-5.3 – 0.8) respectively. The proportion of patients with Z-scores < -1.5 was 56% for TMT-A, 79% for TMT-B and 71% for SDMT.

The assumptions of the proportional hazards model were found to be fulfilled. Table 3 shows the results of the univariate Cox regression analysis of PFS and OS. Four known prognostic characteristics are included along with results of the cognitive testing.

Seven patients remained progression free for at least four months and were considered evaluable for late toxicity. Figure 2 shows the TMT-A scores for these individuals at all time points. Changes in performance was evaluated by the RCI using only the individual test scores in order to keep the statistical calculations as simple as possible and to increase the clinical utility of the results. A similar analysis of TMT-B scores was performed. Although no patients failed by the RCI of the TMT-B, the results were somewhat similar (not shown). SDMT scores were far more stable (not shown) as would be expected with a larger reliability coefficient (r=0.74) (Gade, personal correspondence) and only one reliable deterioration occurred in this subset (at disease progression).

Discussion

There is evidence that re-irradiation for recurrent high-grade glioma can be carried out safely[14, 15] but the impact on cognitive function has not been evaluated. In this study, we subjected heavily pretreated HGG patients undergoing re-irradiation in a trial to repeated formal cognitive testing. The purpose was to evaluate the feasibility and clinical utility of the applied test battery as well as to determine the potential impact of re-irradiation on cognitive function in a prospective manner.

The test battery

Choosing the a test battery represents a compromise between practicality (time consumption and ability to be administered by a non-neuropsychologist) and sensitivity to detect change. The Mini Mental State Examination [16] exists in a Danish version and was considered for use in this study. But while being brief and easy to administer, it has been shown to lack sensitivity for both detecting impairment in brain tumor patients and for registering changes [17]. We assembled a test battery that would evaluate four major cognitive domains, with special emphasis on processing speed as this is known to be particularly sensitive to diffuse white matter change, which is a known late effect of radiotherapy [18]. TMT-A and TMT-B are widely used and easy to administer. The SDMT was included in this category. Z-scores across these three tests were highly correlated (Spearman's rho > 0.73 and $p<0.001$ for all three bivariate comparisons) and we found it meaningful to calculate a total Z-score for the cognitive domain.

A test battery for brain tumor patients described by Herman et al.[19] and used in several clinical trials of both recurrent HGG [6] and brain metastases [20]have used the Hopkins Verbal Learning Test (HVLT-R)[21] of memory. However, when this study was planned no validated Danish translation of the test existed. We therefore chose to use a validated and easily applicable visually based test of memory that evaluated both recall and recognition. Normative data for this test exists and they are derived from results of 88 healthy individuals between the ages of 36 and 84 years. Only one form of the test was available and no test-retest reliability coefficient is available. This precluded calculation of reliable change and it is a weakness of our study but it illustrates a challenge that researchers in smaller language areas often face. Likewise, only normative scores of verbal fluency using the letters 'S', 'N' and 'F' in Danish have been published [22]. As the letter 'S' is the easiest, this was used for every test along with the naming of animals.

The Cox regression analysis revealed several significant prognostic covariates among the baseline cognitive scores (please refer to table 3). This is not a novel finding. Meyers et al. in 2000 reported that cognitive function was an independent predictor of survival in patients with recurrent HGG participating in phase I or phase II trials[6]. In comparison, our patients had generally lived longer since diagnosis (24 mo. vs. 7 mo.) and had experienced more previous recurrences before entry (93% vs. 21% were treated for 2nd or later recurrence). Although the number of patients and events (progression or death) was not sufficient in our study to perform a multivariate analysis, we would

cautiously interpret our findings as a confirmation of Meyer et al.'s results but in a group of patients that were more heavily pretreated.
Interestingly, we found that the change in Z-score from baseline to 4 weeks in the domains of processing speed and memory also was prognostic for OS. This indicates that cognitive deterioration as assessed by simple tests may yield important information to clinicians in a setting where standard imaging tools (CT and MRI) are much less reliable than earlier in the course of disease[23]. A limitation of this analysis is that only 82% of the study population was tested at the 4-week evaluation. Although the reasons for missing the test have been accounted for (clinical deterioration (n=2), short follow-up (n=2) and staff shortage (n=1)) there may be a minor bias in the sample at this time-point.

We aimed to evaluate possible adverse effects of re-irradiation on cognition. This was challenging particularly because of the short progression-free survival times (3.0 months from time inclusion). As a consequence, only 53% of the patients were tested beyond the first evaluation performed 1 month after treatment. This time period is shorter than the time it takes to develop late cognitive effects of radiotherapy [24] and therefore only a subset of patients was actually at risk of late toxicity. At disease progression, patients were offered continued follow-up if no other treatment options were available, but only a few patients consented to this. Disease progression and radionecrosis are exceedingly difficult to distinguish from each other clinically and therefore evaluation of change in cognitive function due to radiotherapy *after* progression has been established is practically impossible. Other possible confounders that may have affected cognitive function included anti-epileptic drugs and corticosteroid treatment (75% and 39% of patients at baseline, respectively) [25] [26].

The question can be raised whether preservation of cognitive function is even an important issue for patients with such a dismal prognosis. On the basis of the results presented, we would argue that it is. The seven patients with 'long' progression-free survival times were young (median age=36 y), more likely to suffer from grade 3 tumors (43%) and had long overall survival times (>22 months, median not reached). One of these individuals (patient A in figure 2) suffered temporary cognitive decline (evaluated by TMT-A) that was likely attributable to radiotherapy. We therefore believe that cognitive function should be monitored in future studies of re-irradiation, even if disease control is only achieved in a minority of cases.

In conclusion, we found repeated cognitive testing feasible in a study of patients undergoing re-irradiation for recurrent HGG. Z-scores at baseline and changes in Z-scores in two domains one month after radiotherapy were prognostic for clinical outcomes in univariate analysis, which suggests that cognitive tests may provide early information regarding tumor progression. This may be useful as standard imaging techniques lack specificity in pretreated disease. The method we used was able to diagnose deterioration in processing speed likely caused by radiotherapy in one case. Quality of life is important with any non-curative treatment and we propose that future studies of radiotherapy in glioma patients should include cognitive testing.

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Conflicts of interest

None.

Ethical considerations

This study was carried out in compliance with Danish law.

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Cognitive domain	**Tests**	**Scoring**	**Z-score calculation**	**Reference values**
Processing speed	Trailmaking test A Trailmaking test B Symbol Digit Modalities Test	seconds (max. 180 s.) seconds (max 300 s.) correct number of substitutions in 90 seconds	$\mathbf{Z_{processing}}$ **=** mean of $Z_{TMT\text{-}A}$ $Z_{TMA\text{-}B}$ Z_{SDMT}	Tombaugh 2004 [10] (age- and education level adjusted) Gade, Nielsen [21]
Memory	Visual memory test of 30 objects from daily life	number of items recalled correctly	$\mathbf{Z_{memory}}$ **=** mean of $Z_{early\ recall}$ $Z_{delayed\ recall}$	Gade *(personal correspondence)*
Verbal fluency	Producing words beginning with the letter 'S' Producing animals	number of words for each category	$\mathbf{Z_{fluency}}$ **=** mean of $Z_{S\text{-}words}$ $Z_{animals}$	Nielsen [21], Gade *(personal correspondence)*
Motor speed and coordination	Grooved Pegboard test	seconds for dominant and non-dominant hand	$\mathbf{Z_{motor\ speed}}$ **=** mean of $Z_{dominant}$ $Z_{non\text{-}dominant}$	Dikmen 1999 [12]

Table 1 Tests, scoring, Z-score calculations and references.

Number of patients		**n = 28**	
Age, median		54 y	(30-74 y)
Performance status	0	36 %	
	1	43 %	
	2	21 %	
GBM		79 %	
Glioma gr. III		21 %	
Steroid T_x		39%	
Anti-epileptic drug T_x		75%	
Tumor location, side			
Right		61 %	
Left		32 %	
Bilateral		7 %	
Previous RT dose, Gy	60	82 %	
	44 - 45	14 %	
	34	4 %	
Previous temozolomide		100 %	
Previous bevacizumab		61 %	
Surgery prior to reirradiation		11 %	
Survival from diagnosis to study, median		24 mo.	(6-129 mo.)
- for GBM only		24 mo.	(6-129 mo.)
Recurrence number being treated			
1		2	
2		14	
3		10	
4-5		2	
Treatment in study			
3.5 Gy x 10		n=12	
3.5 Gy x 10 + 7 Gy boost to PET+ volume		n= 9	
5.9 Gy x 5		n= 3	
3.5 Gy x 10 (to larger tumors)		n= 4	

Table 2. Baseline patient characteristics (clinical characteristics, previous treatment, current treatment).

Covariate	Level	Hazard Ratio (95 % CI)		p-value	Hazard Ratio (95 % CI)		p-value
		PFS			OS		
Diagnosis	grade IV vs. grade III	2.6	(.7 – 9.1)	.15	4.7	(1.1 – 21.0)	.04
Steroid T_x baseline	yes vs. no	3.3	(1.2 – 9.2)	.02	3.8	(1.4 – 10.2)	.008
Age	per 10 years	1.5	(1.0 – 2.2)	.06	1.3	(.9 – 2.0)	.14
Performance status	1-2 vs. 0	2.6	(.9 – 7.5)	.07	2.4	(.8 – 6.7)	.10
Processing speed baseline	Z-score	0.9	(0.8 – 1.0)	.03	0.9	(0.8 – 1.0)	.08
Processing speed change	per decrease in Z-score of 1 SD	1.3	(1.0 – 1.8)	.03	1.5	(1.1 – 2.0)	.009
Memory function at baseline	Z-score	0.8	(0.7 – 1.0)	.03	0.8	(0.7 – 1.0)	.08
Memory function change	per decrease in Z-score of 1 SD	1.0	(.8 – 1.3)	.76	1.3	(1.0 – 1.6)	.04
Verbal fluency at baseline	Z-score	0.8	(.9 – 1.9)	.23	0.8	(.6 – 1.2)	.33
Verbal fluency change	per decrease in Z-score of 1 SD	1.1	(.4 – 2.8)	.84	1.7	(.5 – 5.8)	.42
Grooved pegboard at baseline	Z-score	0.9	(0.8 – 1.0)	.01	0.8	(0.8 – 1.0)	.006
Grooved pegboard change	per decrease in Z-score of 1 SD	1.2	(.9 – 1.5)	.18	1.2	(.9 – 1.6)	.17

Table 3 Univariate analysis of prognostic factors for PFS and OS.
Abbreviations: CI, confidence interval; PFS, progression-free survial; OS, overall survival following treatment; BTV, tumor volume defined by FET-PET; T_{max}/B, maximal activity;

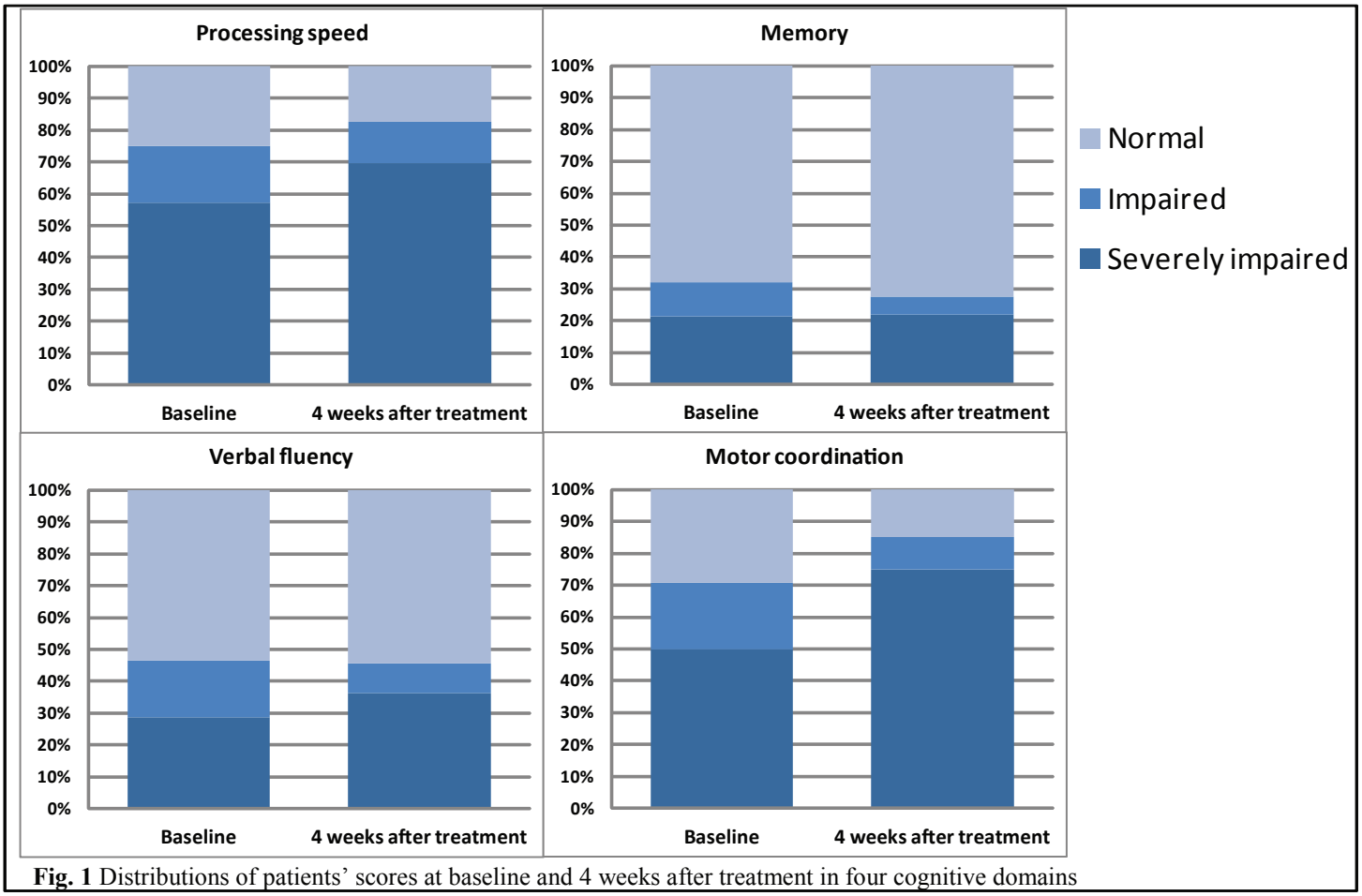

Fig. 1 Distributions of patients' scores at baseline and 4 weeks after treatment in four cognitive domains

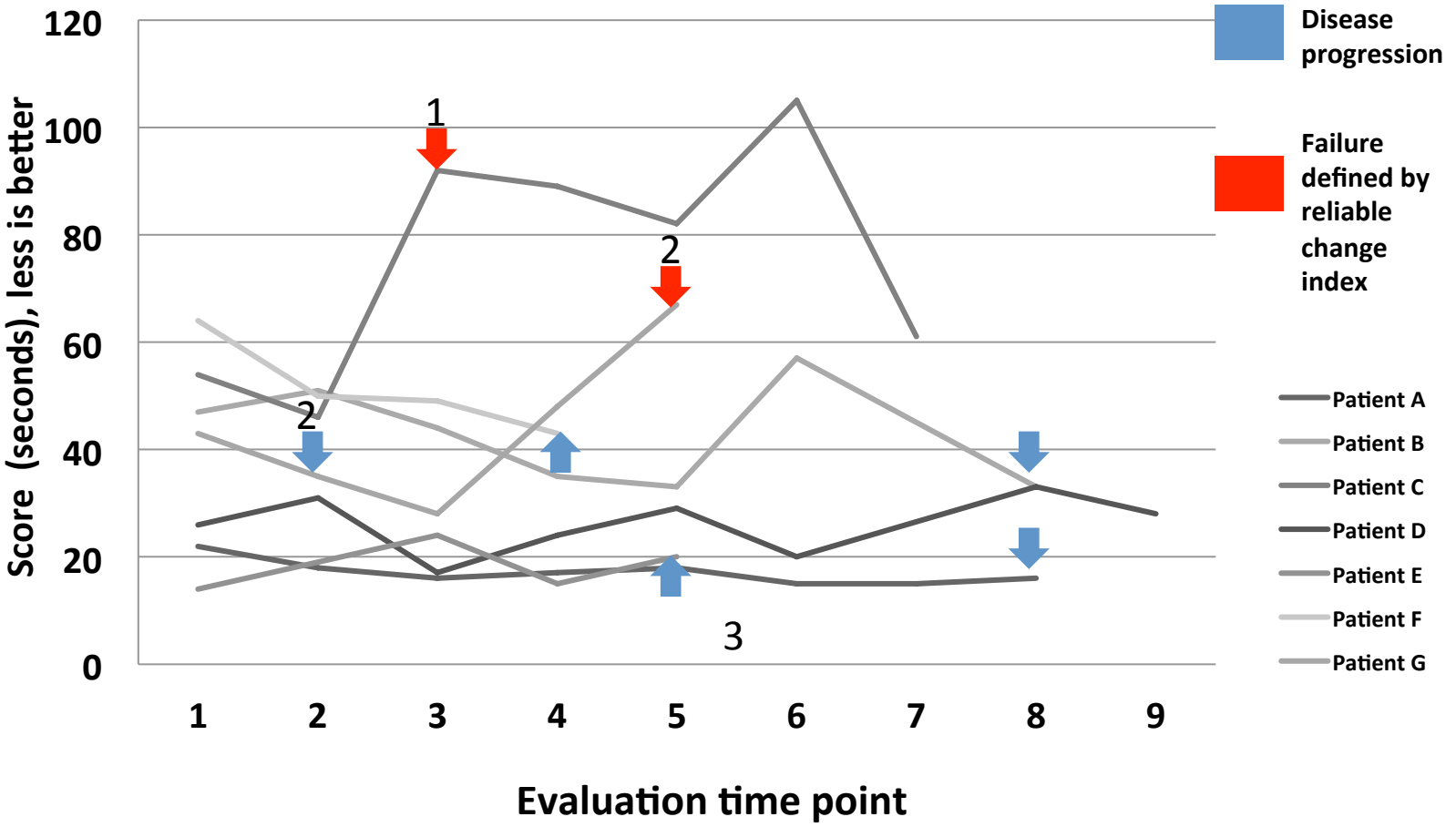

Fig. 2 Individual Trail Making Test-A scores for seven patients with long progression free survival
1) Progressing non-contrast- and contrast enhancing lesion. No change in 18F-FET-PET scan. Interpreted as radiation induced changes. The patient responded well to steroid treatment and was able to start tapering 6 months later. Lost to follow-up. 2) Progressing non-contrast- and contrast enhancing lesion at second time point. 18F-FET-PET scan showed regression. Lesions progressed on subsequent scans, including 18F-FET PET scans. Fails TMT-A five months after treatment and dies 2 months later. 3) Progressing non-contrast- and contrast enhancing lesion. 18F-FET-PET scan showed progression. No symptoms. Pathology report after sugery: radiation necrosis, few tumor cells.

Manuscript 3

Early changes in tumor perfusion during radiotherapy evaluated by DCE-MRI

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Abstract

Introduction: The survival times of patients with glioblastoma differ widely and biomarkers that would enable individualized treatment are needed. The purpose of this study was to measure tumor perfusion using T1*-dynamic contrast enhanced MRI (DCE-MRI) in patients with glioblastoma during early stages of radio- and chemotherapy (Tx) and identify possible biomarkers of treatment outcomes.

Material and methods: A prospective observational study was planned. Patients underwent DCE-MRI at baseline, after approximately 1- and 6 weeks of Tx and 3- and 6 months post Tx. In addition to MRI, positron emission tomography using 18F-fluoroethyl tyrosine (18F-FET PET) was carried out at baseline. DCE-MRI at 3 T generated maps of cerebral blood flow (CBF) cerebral blood volume (CBV), permeability (CBKi) and volume of distribution (CBVd) using a combination of model-free deconvolution and Patlak plots. Regions of interest in contrast enhancing tumor, PET-defined tumor and normal appearing white matter were contoured. Patients were categorized as responders or non-responders and the groups were compared at all time points. The prognostic value of changes in regional perfusion was assessed using univariate Cox regression analysis.

Results: Eleven eligible patients were included and 46 DCE-MRI examinations were carried out. Regional CBF (rCBF) in tumor increased for all patients early during Tx ($p=0.005$) and then fell to a level below baseline at post-Tx examinations ($p=0.016$). A similar but non-significant trend was seen for rCBV. There was no detectable difference between responders and non-responders with regards to baseline values or changes during- and after Tx. Perfusion in tumor ROIs defined using MRI and PET did not differ significantly.

Conclusion: Regional CBF in tumor increased significantly during early stages of treatment and decreased after treatment. Although no correlations to outcomes could be found, the results may be hypothesis generating and should be examined in a larger patient group.

Introduction

Glioblastoma is the most common form of primary brain cancer and among the most devastating of all cancers. The standard treatment is aggressive and consists of surgery followed by combined chemo-radiotherapy, which is supported by class I evidence [1]. However, while nearly all patients succumb to disease eventually, survival times can vary greatly.
It is clear that the field of neuro-oncology must move beyond the ‘one-size fits all’ frame of thought towards an individual approach guided by characteristics of each patient. It was recently demonstrated that elderly patients may benefit from a different approach[2] and two phase III studies examining the effect of addition of bevacizumab to the standard treatment did not yield a

survival advantage in an unselected population [3, 4]. Thus, biomarkers are needed that may aid in selecting the right treatment for the right patient.

Imaging biomarkers may include metabolic information such as tumor uptake of radiolabeled molecules using positron emission tomography (PET) or parameters of perfusion, (CBF) and cerebral blood volume (CBV) using computer tomography (CT) or magnetic resonance imaging (MRI). The most commonly used method for evaluating tumor perfusion is the dynamic susceptibility T2*-weighted (DSC) MRI method. It has proven useful in predicting glioma grade before surgery[5] and is FDA-approved and widely commercially available. Disadvantages include susceptibility to artifacts and the need for pre-bolus injection af contrast agent to correct for leakage [6]. Most previous studies evaluating brain tumor perfusion have focused on regional CBV (rCBV)[7],[8]. We have previously described a method of generating CBF- and CBV maps and permeability maps (CBKi) by dynamic contrast enhanced (DCE) MRI at 3.0 T using a single bolus injection of paramagnetic contrast agent [9, 10]. Leaky blood-brain barrier (BBB) is recognized as an important hallmark of malignancy in glioma but only a few studies have attempted to analyze BBB permeability in gliomas undergoing treatment in relation to outcomes [11][12].

A prospective observational study of patients receiving standard concomitant chemo-radiotherapy for glioblastoma was carried out. Patients were subjected to DCE-MRI perfusion scans at baseline, after approximately 1- and 6 weeks of radiotherapy and 3- and 6 months post radiotherapy (MRI_1-MRI_5). The aim of this exploratory study was to describe possible changes in tumor perfusion (rCBF, rCBV, rCBKi and rCBVd) using this technique and to evaluate correlations to clinical outcomes.

Patients and methods

Patients with glioblastoma WHO grade IV referred to concomitant chemo-radiotherapy ('Stupp'-regimen[1]) were eligible to be included. The study was approved by the local Ethics Board (case number: H-D-2008-002) and carried out in accordance with the Helsinki Declaration. Written informed consent was required. The study was observational and results achieved with DCE-MRI were not known to the treating physicians at the time of treatment due to the experimental nature of the technique. Patients went off-study at either disease progression, completion of the last DCE-MRI scan or withdrawal of consent. The time of progression was determined using the patient charts and decisions made in clinical practice in each individual case. These are based on the Macdonald criteria.

Patients

Inclusion criteria were: age 18-70, performance status 0-2, measurable residual tumor on post-operative MRI, signed informed consent. Exclusion criteria were: contraindications to MRI or contrast injection (pacemaker, non-compatible metallic implants, reduced kidney function [glomerular filtration rate<60 ml/min], previous allergic reaction to MRI contrast agent, pregnancy) and claustrophobia.

Radiotherapy

Target definition for radiotherapy (RT) was based on MRI (mandatory) and ^{18}F-fluoro-ethyl tyrosine PET (^{18}F-FET PET) (if available). The gross tumor volume (GTV) consisted of a tumor volume defined by MRI (GTV MRI) and a tumor volume defined by ^{18}F-FET PET. The GTV MRI was defined by a radiologist using the contrast enhanced MRI sequence of a planning scan

performed at radiotherapy planning (Siemens Magnetom Espree 1.5 T). Surgical cavities were contoured as part of the GTV MRI.
The PET images were acquired on an integrated hybrid PET/CT system (Siemens Biograph mCT scanner). An ^{18}F-FET PET frame of the entire brain was acquired at 20 to 40 minutes after i.v. injection of 200 MBq ^{18}F. The tumor was auto-contoured in 3D with Syngo-TrueD software (Siemens), defining tumor tissue using a threshold of 1.6x the mean SUV (standardized uptake value) in a background region of interest (ROI) placed in gray matter of the healthy hemisphere The total target volume consisted of the united volume of GTV MRI and GTV PET. A 20-mm margin was added to this volume to form the clinical target volume (CTV), to which 2 mm was added to form the planning target volume (PTV). A total dose of 60 Gray (Gy) was delivered in daily 2-Gy fractions over 6 weeks to the PTV. RT was delivered using volumetric modulated arc therapy (VMAT) on a Novalis Tx accelerator (Varian/BrainLab). Oral temozolomide was administered at 75 mg/m^2/day during RT and for six cycles (5d/28d) at 200 mg/m^2/day following RT[1].

DCE-MRI
DCE-MRI was performed on a 3.0 T MRI unit (Achieva; Philips Healthcare) at the Functional Imaging Unit (Diagnostic Department, Glostrup Hospital). Scans were acquired at baseline (0-7 days before start of radiotherapy [MRI_1]), following 10 Gy of RT (MRI_2), following 50 Gy of RT (MRI_3), 3 months post RT (MRI_4) and 6 months RT (MRI_5).

DCE imaging used a saturation-recovery gradient–echo sequence with 120 ms delay (TD) between prepulse and the first readout pulse. Readout pulses had a flip angle of 30°, TR=3.9 ms, TE=1.9 ms, centric phase ordering, parallel imaging factor 2, acquired matrix 96×61 interpolated to 256×256, field-of-view 230×182 mm^2, five slices, slice thickness/gap 8/1.5 mm [9].
The bolus of contrast (Dotarem 0.1 ml/kg body) was injected using an automatic injector and was followed by 20 ml saline. The bolus tracking had a time resolution of 1.25 s for 250 time points in approximately 6 minutes. In case of tumors that were too large to be covered by 4 slices, the procedure was repeated in a second run using new T1 measurements. Before each perfusion scan, an anatomic T2W sequence was obtained corresponding to the position of the four perfusion slices for the purpose of anatomical reference and for ROI registration .

A T1 map was generated before bolus injection by varying TD from 0.12 s – 10.0 s. The arterial input function (AIF) was generated by placing the most caudal slice at the level of the vertical segment of the internal carotid artery. The larger of the two internal carotid arteries was contoured and the pixel within this area with the largest signal change at bolus passage was used for subsequent calculations. To minimize the partial volume effect due to the small diameter of the vessel, the AIF was scaled to the venous output function derived from the sagittal sinus [13]. CBF was estimated by model-free deconvolution (Tikhonovs method) as previously described by Larsson et al. [9]. CBV and CBKi were estimated using the Patlak method [14]. In-house software for MATLAB R2012a (MathWorks®, Inc.) was used for these calculations.

In addition to the perfusion sequence acquistion described above, the following sequences were also generated: T1W-3D, fluid attenuation inversion recovery (FLAIR), diffusion tensor imaging (DTI), blood oxygen level BOLD (in resting state) and arterial spin labeling (ASL).

Regions of interest and registration of images
ROIs were contoured on MRI that was registered to a planning CT scan. Three 3-D ROIs were defined for measurements of perfusion: a) tumor volume defined by contrast enhancing tumor on a T1+Gd sequence used for radiotherapy planning. This volume was modified to exclude resection cavities; b) the ^{18}F-FET PET positive volume; c) normal appearing white matter (NAWM) drawn manually in healthy appearing white matter in the contralateral hemisphere within the section of brain encompassed by the perfusion slices. The ROIs remained unchanged throughout the study. For each of the five MRI examinations (MRI_1-MRI_5), the CT scan with the ROIs was rigidly registered to the anatomical T1W-3D sequence (isotropic, $1mm^3$) and each T1 sequence was subsequently registered to the axial T2W slices. Registrations were performed with the Functional Magnetic Resonance Imaging of the Brain Analysis (FMRIB) Software Library using either mutual information or normalized mutual information[15]. Each registration step was visually verified. The two registrations were combined and used to transform the CT-based ROIs to the DCE-slices.

Statistics
Baseline perfusion values in ROIs were calculated as mean values for NAWM and median values for tumor ROIs. The patients were categorized as either responders or non-responders to treatment according to their progression-free survival (dichotomized as longer or shorter than the median, respectively) from the date of diagnosis. Statistical comparison between these groups was carried out using the Mann-Whitney test (non-parametric) whereas paired data (two measurements from the same patient) were compared using the Wilcoxon test (non-parametric). A univariate Cox regression analysis was carried out to test the prognostic value of changes in perfusion values compared to baseline. P-values <0.05 were considered significant. All statistical analyses were carried out using SPSS 19 (IBM Corp.).

Results
Twelve patients were included in the study. One patient experienced a decline in blood oxygen saturation to 77% due to sleep apnea during the first scan whereupon he was excluded from the study. Eleven patients thus underwent a total of 46 scans. Seven patients underwent ^{18}F-FET PET scans at baseline as part of routine radiotherapy planning. The number of patients scanned at each time point were: MRI_1 n=11; MRI_2 n=10; MRI_3 n=11; MRI_4 n=10; MRI_5 n=4. At the time of MRI_5, 6 patients had experienced progressive disease and one patient had elected to discontinue participation. The median progression-free survival was 7.8 months.

		NAWM (n=11)	$Tumor_{MRI}$ (=11)	p-value ($Tumor_{MRI}$ vs. NAWM)	$Tumor_{PET}$ (n=7)	p-value ($Tumor_{PET}$ vs. $tumor_{MRI}$)
rCBF	ml/(100g/min)	11.6 ±3.4	18.9 ±7.7	0.006	20.7 ± 6.2	0.500
rCBV	ml/100g	1.0 ± 0.4	4.2 ± 3.2	0.003	4.3 ± 2.7	0.612
rCBKi	ml/(100g/min)	0.2 ± 0.1	1.2 ±0.9	0.003	1.0 ± 0.8	0.237
rCBVd	ml/100g	1.7 ± 0.8	7.5 ±5.2	0.003	7.0 ± 4.4	0.310

Table 1. Perfusion values at baseline for NAWM, $tumor_{MRI}$ and $tumor_{PET}$. Abbreviations: NAWM=normal appearing white matter, rCBF=cerebral blood flow, rCBV= cerebral blood volume, rCBKi=blood-brain-barrier permeability, rCBVd = volume of distribution.

There were no differences between $tumor_{MRI}$ and $tumor_{PET}$. NAWM at baseline was compared with all subsequent examinations. Generally, perfusion parameters did not change significantly within this ROI. However, a significant difference was found between rCBKi at MRI_1 and MRI_4 (mean:0.2±1 vs. 0.1±0.1 p=0.02).This was not significant at scan 5. At baseline, all perfusion values (rCBF, rCBV, rCBKi and rCBVd) were higher in tumor than in NAWM in all cases except one (where $rCBF_{NAWM}$>$rCBF_{tumor}$).
Figure 1 depicts the normalized values of rCBF, rCBV, rCBKi and rCBVd in tumor for responders and non-responders at baseline. No significant differences were found.

Figure 2 shows changes in rCBF, rCBV and rCBKi over time for all patients and divided into responders and non-responders. There was a significant increase in rCBF at MRI_2 compared to MRI_1 which applied to all patients. rCBF decreased again at MRI_3 and MRI_4. At MRI_4, values of rCBF and rCBV were significantly lower than at baseline for the whole group, but there was no significant difference between responders and non-responders. Comparing the absolute values (not shown) of rCBF and rCBV showed a similar significant decrease from MRI_1 to MRI_4, however, the absolute increase from MRI_1 to MRI_2 did not prove statistically significant (p=0.29).
Using univariate regression analysis, the hazard ratios for progression- and death for changes from scan 1 to 2 and from scan 2 to 3 were analyzed. No statistically significant correlations with outcomes were seen.

Figure 3 shows parameter maps at four timepoints for a non-responding patient with a short PFS of approximately 4 months. The patient had clinical and radiological disease progression at MRI_4 which was also confirmed histologically as the patient underwent re-resection. Only the CBKi map reflects this whereas both CBF and CBV are lower than at baseline.

Discussion

In this exploratory prospective study of glioblastoma patients receiving standard radio-chemotherapy, we found that rCBF was significantly increased one week into radiotherapy compared with baseline. This has not been described previously in the literature, but Cao et al. have described a similar increase in rCBV during early stages of RT [7]as well as an increased permeability [12]. The simplest explanation is that tumor growth could have occurred between the baseline scan and the early scan during RT. This time period was between 14 and 20 days. Perfusion might have increased due to both physiological factors and partial volume effects. However, in human tumor xenografts, an early stress response to ionizing radiation has been demonstrated within 24 hours of exposure consisting of a dose-dependent increase in vascular endothelial growth factor (VEGF) expression [16]. VEGF promotes formation of new blood vessels [17] and blockade of the VEGF pathway has early and measurable effects (~24 hours) on perfusion parameters (rCBV and rCBKi) [18]. It is therefore also possible that the early increase in perfusion was due to a transient rise in VEGF that later fell due to treatment response. Cao et al. proposed that corticosteroid treatment during RT could influence perfusion but we do not find this likely to be important in our study, as there was no systematic change in treatment or dosage of steroids. The early increase in rCBF was analyzed in relation to outcomes but we found no significant differences between responders and non-responders. At MRI_3 and MRI_4, rCBF was markedly decreased but still no correlations to outcomes were seen, which was also evident from the parameter maps shown for a patient in fig. 3. Taken together, it appears that rCBF is alterable by chemo-radiotherapy but not an important marker of tumor malignancy. Similarly, a leaky blood-brain barrier is effectively

sealed by the anti-VEGF antibody bevacizumab, but treatment with this drug has not been shown to increase overall survival for glioblastoma patients [3, 4].

The significance of tumor perfusion during treatment has been assessed previously using DSC-MRI. Cao, Galban and co-workers at the University of Michigan have shown in several papers [8, 19] that mean values for the whole tumor may not contain predictive information regarding treatment response. This group used an elegant but somewhat elaborate technique where voxel-by-voxel comparisons allowed for the generation of so-called parametric response maps (PRMs) that evaluated regional changes. It was shown that regional changes of a certain magnitude correlated to outcomes. Other groups have successfully employed similar functional maps in the context of diffusion-weighted MRI [20]. In this study, we did not attempt to carry out voxel-to-voxel analysis for two main reasons. Firstly, it requires a highly precise image registration that we believe may be difficult to achieve for all patients. Additionally, change in tumor morphology within the contoured contrast enhancing area further complicates a reliable voxel-to-voxel relationship, even when using a deformable registration approach. Furthermore, we believe that the subtraction of tumor/surgical cavities that we carried out in some respects minimizes the influence of 'dead' perfusion space on the median values used. Finally, the method we used generated voxels with a size of 3.1 x 2.5 x 8 mm. Differences in responses dependent on the microenvironments may even exist within a voxel, which then itself only represents a weighted average of values.

The methods used our study have some limitations. We chose to measure parameters of perfusion within a static ROI defined at the baseline scan. This has the practical advantage of not requiring contouring of tumor for every examination. However, with this method there is a risk of the tumor moving out of the ROI due to either increased or decreased edema during radiotherapy, treatment response, or tumor growth with central cavitation. The tumor mask (ROI for measurement) was therefore visually inspected for each scan and found to be generally acceptable although in some cases minor mismatches were visible. In addition, in four cases (two responders and two non-responders) we drew ROIs of contrast enhancing tumor directly on the CBKi-parameter map to evaluate the perfusion values. These corresponded closely with the original measurements and patterns of change (increases or decreases) were nearly identical for all four parameters. Nonetheless, contouring the tumor using contrast-enhanced sequences for each examination would likely have yielded more precise estimations of tumor-specific values but we find it unlikely that the results and conclusions would be altered. Another limitation of our method was the small vertical field of view (36.5 mm). As covering the whole tumor was prioritized in this study, in most cases (10/11) it was necessary to perform two bolus trackings ('runs'). For this reason, we chose to normalize the data to NAWM. But in the case of very caudally placed tumors, contouring adequately large white matter regions within the perfusion slices could be challenging, even when two runs were used. We unexpectedly found a significant decrease in the rCBKi of NAWM at MRI_4. It is unknown whether this was a random occurrence or an effect of having completed radiotherapy (which is known to induce a mild but temporary edema of the brain for some patients). The radiation doses to these areas have not been assessed but as they were required to be located within the perfusion slices, a non-zero dosage is to be expected even though the treatment was very conformal. But as mentioned, the decrease in tumor rCBV and -rCBF that we observed at this time point was significant even when using absolute (non-normalized) values.

In a number of cases, we found imaging artifacts due to movement, predominantly in the second runs. This was probably due to patient discomfort during long acquisition times. We find it likely that carrying out only one run with the slice placed centrally in the tumor would be physiologically

representative and adequate for measuring perfusion. This would also reduce the time expenditure of post-processing and registration, which was substantial. It may also be possible to achieve a greater spatial resolution by sacrificing the time-resolution, but this needs to be validated. We have previously shown that the signal-to-noise ratio in a 1.5 T MRI scanner is too low to generate perfusion maps using DCE-MRI [9]. Thus, a scanner with a field strength of at least 3.0 T is necessary and this is also a disadvantage of this technique

Seven patients underwent ^{18}F-FET PET scans at the approximate time of the baseline scan for radiotherapy planning. The PET-positive tumor ROIs were included in this analysis solely for exploratory purposes. We had no a priori theory about how this volume would compare to a contrast enhancing tumor (defined by MRI) although we speculated that rCBKi values in contrast enhancing tumor would be larger at baseline. Indeed, the mean rCBKi was slightly higher but this was not statistically significant (please refer to table 1.). No differences could be detected at the other time points (not shown). The changes in ^{18}F-FET PET images during the course of RT have been evaluated in a cohort of patients at our institution and the results are expected soon.

This study had the advantage of being comprised of a well-defined group of patients receiving the same standard treatment. Adherence to both treatment and the scanning protocol was very good. But the small number of patients as well as the relatively poor survival of the group as a whole makes it challenging to draw any statistically valid conclusions. Responders were defined as having longer PFS than the median but in two of these five cases, the PFS was less than a year. Thus, the biological differences between these two groups may not have been as significant as one could wish, but this is a common issue in neuro-oncology.

In conclusion, using DCE-MRI at early intervals during radio-chemotherapy of glioblastoma, we have found significant early changes in rCBF that have not been described previously. We have described a distinct time-curve for perfusion parameters during RT, but we did not find these to be correlated to outcomes. This included rCBKi, which has not received much attention previously, as it cannot be measured by the most widely used method of generating perfusion weighted MRI sequences, DSC-MRI. We have shown that perfusion assessment is feasible with DCE-MRI, especially if validation studies will support the use of a larger field of view at the expense of a lower temporal resolution. Possible correlations to outcome should be investigated in a larger cohort of patients.

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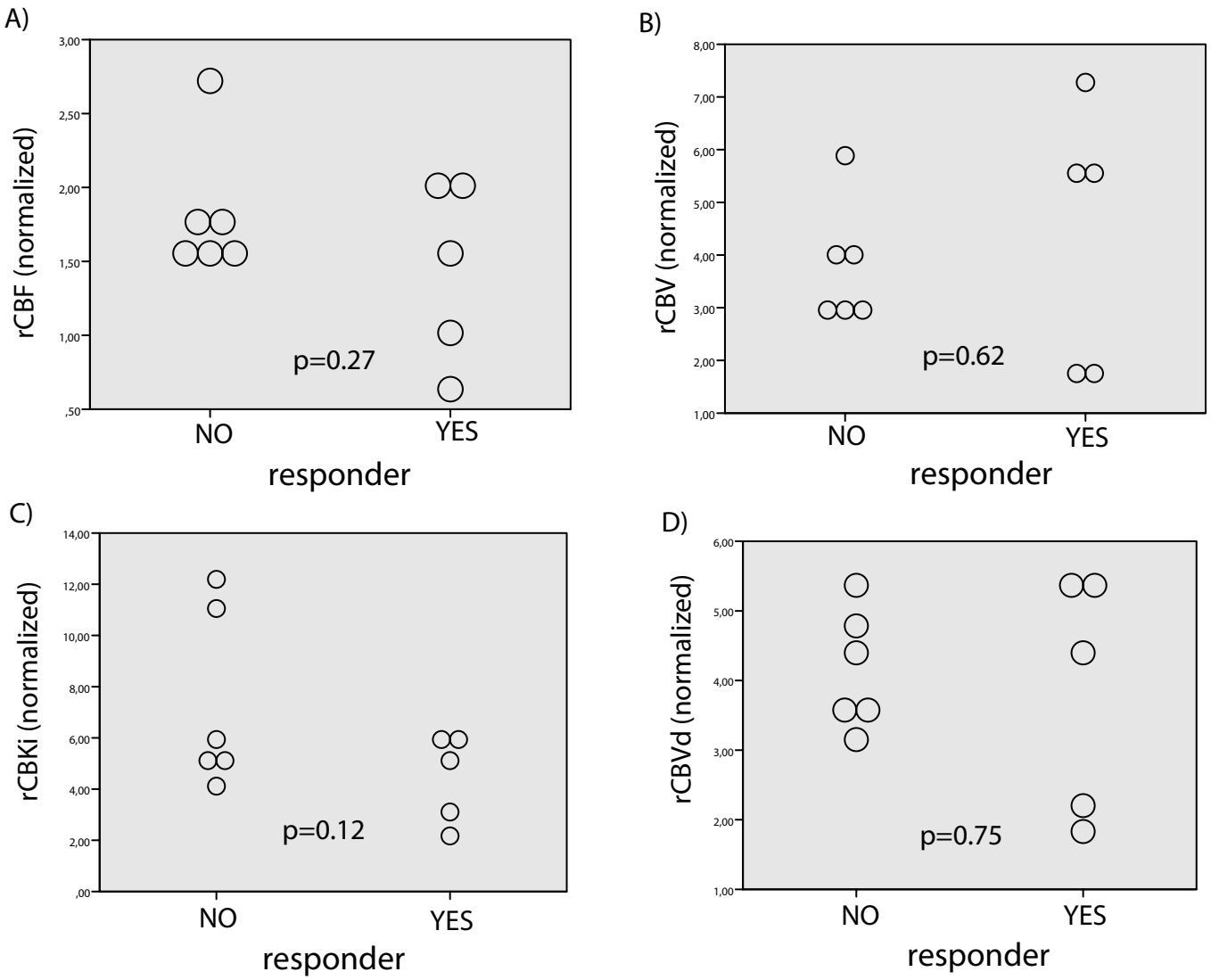

Figure 1. Values of perfusion parameters in tumor normalized to normal appearing white matter at baseline for non-responders vs. responders. Each circle represents one patient.
A) rCBF B) rCBV C) rCBKi D) rCBVd

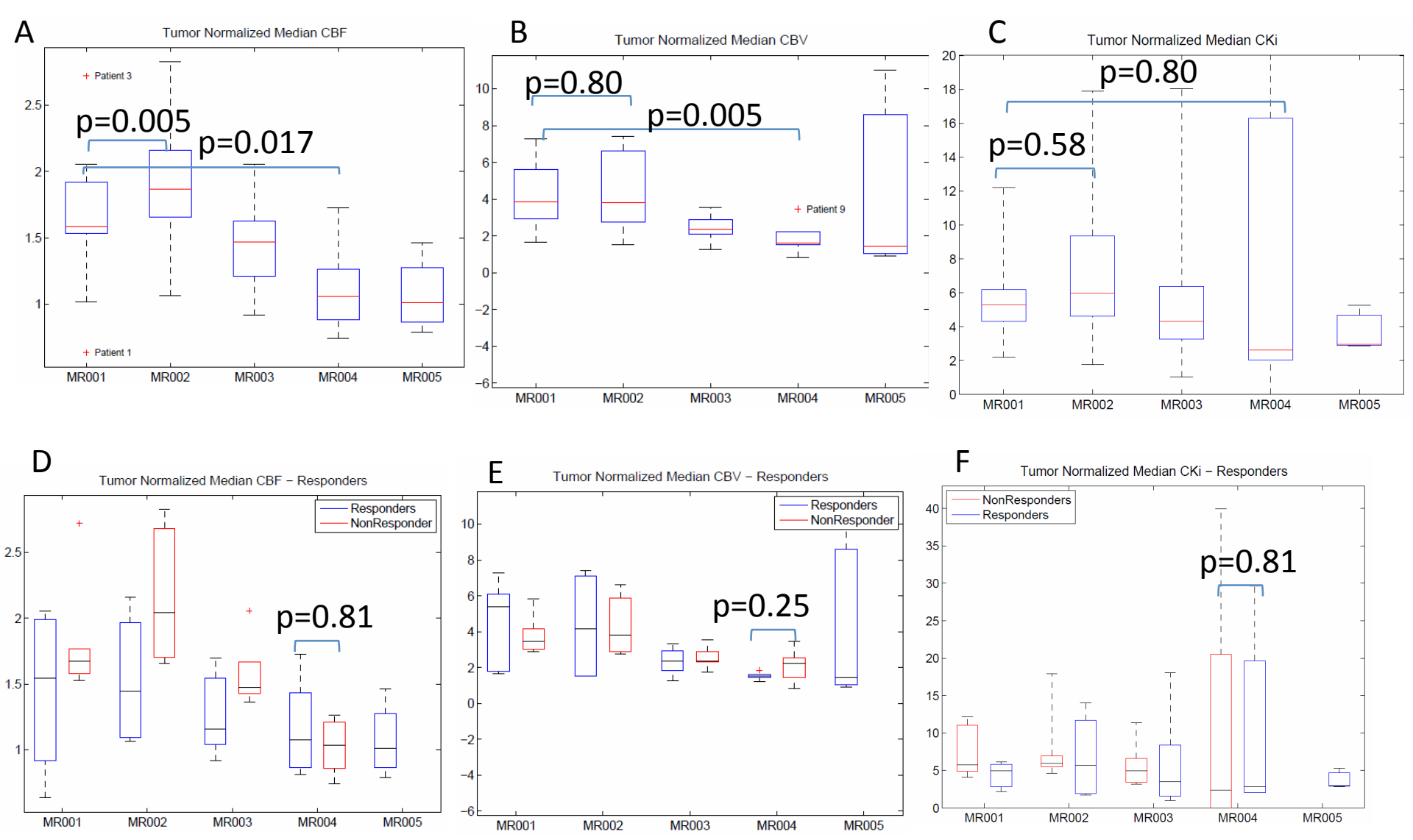

Fig. 2 Values of perfusion parameters normalized to normal appearing white matter at all timepoints. A) – C) all patients. D) – F) divided into non-responders (red bars) and responders (blue bars). Red line denotes the median. Boxes include 25%-75% of values. Whiskers include all datapoints.

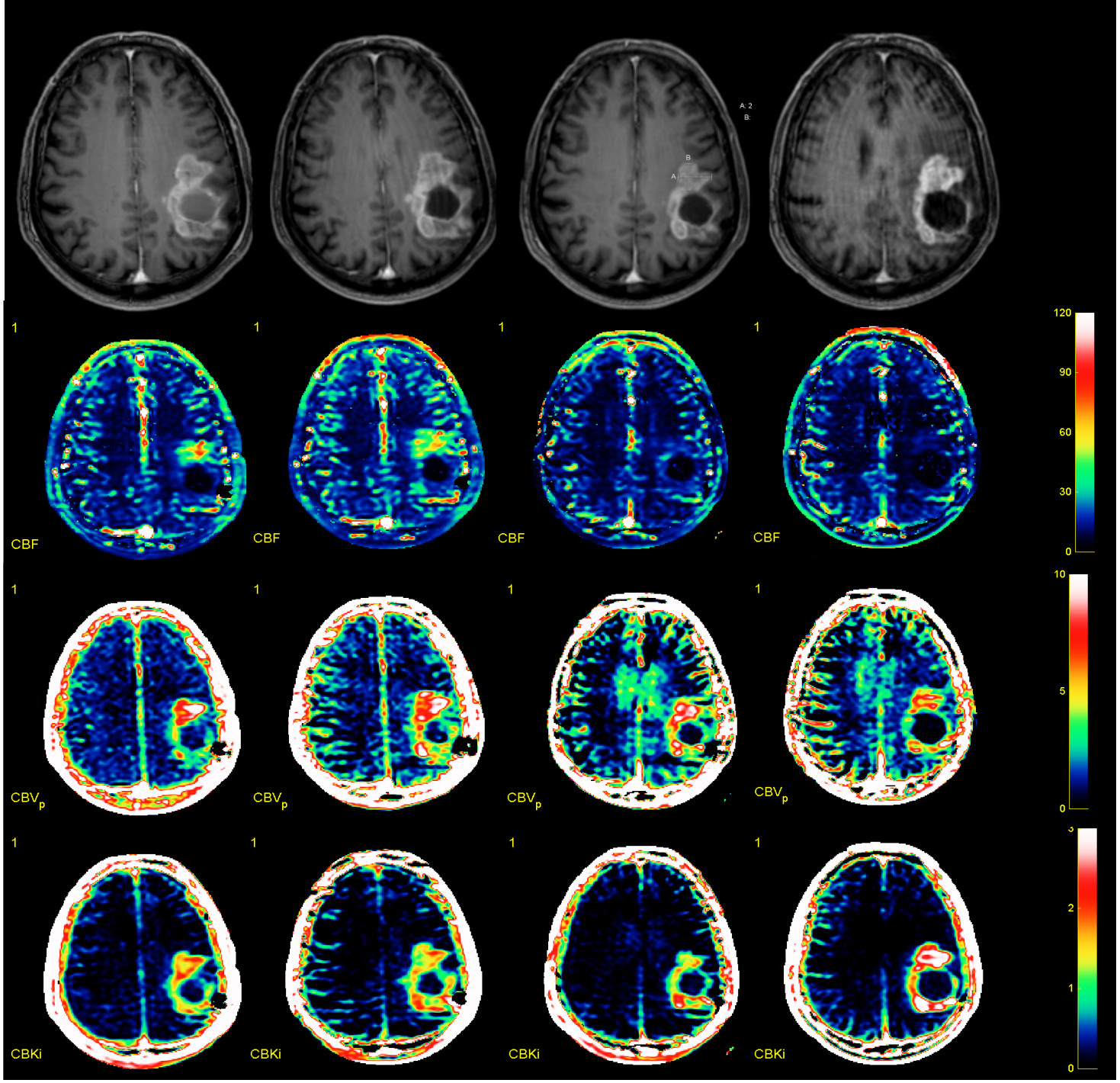

Fig. 3
Parameter maps for one patient at time points MRI_1 to MRI_4.

Row 1: T1W+contrast
Row 2: CBF.
Row 3: CBV.
Row 4: CBKi.
The patient had disease progression at the last time point (rightmost column).

The color keys on the right show corresponding absolute values of CBF [ml/(100g/min)], CBV [ml/100g] and CBKi [ml/(100g/min)].