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FLUORESCEIN-GUIDED SURGERY FOR RESECTION OF MALIGNANT GLIOMAS

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Anno Accademico 2012-2013

A Chiara e il gattino
A Mara e Gianni

"If you want something to be done, just do it yourself."
Charlie Teo

"All truth passes through three stages: first, it is ridiculed;
second, it is violently opposed;
third, it is accepted as being self-evident."
Arthur Schopenhauer

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1. LIST OF ABBREVIATIONS

AA: Anaplastic astrocytoma

BBB: Blood-brain barrier

CHT: Chemotherapy

EOR: Extent of reception

eV: ElettronVolt

FDA: Food and Drug Administration

FLIM: Fluorescence lifetime imaging microscopy

GA: General anesthesia

GBM: Glioblastoma multiforme

GFAP: Glial fibrillary acidic protein

GTR: Gross total resection

HGG: High grade gliomas

ICG: Indocyanin green

iMRI: Intraoperative magnetic resonance imaging

i.v.: Intravenous

KPS: Karnofsky performance scale

L: Left (*tables only*)

LGG: Low-grade gliomas

MG: Malignant gliomas

MGMT: O-6-methylguanine-DNA methyltransferase

MMSE: Mini mental state evaluation

MRI: Magnetic resonance imaging

NADH: Reduced nicotinamide adenine dinucleotide

NIHSS: US National Institute of Health Stroke Scale

OS: Overall survival

PFS: Progression-free survival

PpIX: Protoporphyrin IX

Pt(s).: Patient(s) (*tables only*)

R: Right (*tables only*)

RANO: Response assessment in neuro-oncology

RT: Radiotherapy

S0: Ground state

S1: Higher energy state

TMZ: Temozolomide

TR-LIFS: Time-resolved fluorescence spectroscopy

UV: Ultraviolet

VP: Venticulo-peritoneal

vs: Versus

WHO: World Health Organization

5-ALA: 5-aminolevulenic acid

2. INTRODUCTION

Malignant Gliomas (MG), or High Grade Gliomas (HGG), are aggressive primary brain tumors with poor prognosis despite maximal therapy available consisting in a combination of surgery, chemotherapy (CHT) and radiotherapy (RT) [47]. Gliomas account for nearly 80% of primary malignant brain tumors, contributing to approximately 13.000 deaths and 18.000 new cases annually in the United States [70]. This devastating disease accounts for more years of life lost than any other type of tumor [70]. According to the current World Health Organization (WHO) grading system classification, MG include grade III and IV gliomas [47, 84]. Glioblastoma multiforme (GBM), grade IV WHO glioma, is the most common type of glioma and carries a very poor prognosis. The current standard of care results in an average life expectancy of 14 months after diagnosis [70].

Though the role of surgery in MG remains controversial, there are some retrospective studies and one prospective study that suggested a significant impact of the extent of resection (EOR) on the outcome of patients affected by MG [37, 43, 48, 77]. However, the surgical removal of the whole enhancing mass on the preoperative Magnetic Resonance Imaging (MRI), the so called gross total resection (GTR), was reported to be feasible only in a low percentage of cases [43, 48], due to intraoperative difficulties in tumor tissue recognition at the margin of the resection [89].

A number of new technical tools, such as neuronavigation [98], intraoperative magnetic resonance imaging (iMRI) [36, 46, 55] and cranial ultrasound [23, 91], were therefore developed to obtain better surgical results and they are part of the common armamentarium used for this king of surgery. Nevertheless, only a randomized trial on iMRI demonstrated a class I evidence of its application on EOR [71]; iMRI guided surgery, though, is significantly more time consuming, it can interfere with the surgical workflow (compatibility of instruments or equipment) [36] and, finally, may not be available in every center due to high cost of installation and maintenance.

Photodynamic detection, which takes advantages of a photosensitive drug (fluorophore) specific for the tumor tissue and with a well-defined range of fluorescence, has been used in neurosurgery for removal of HGG [45, 53].

This study aims to evaluate the safety and the efficacy of fluorescein guided surgery for removal of MG.

3. FLUORESCENCE OVERVIEW

3.1 Basic mechanisms of fluorescence

Electromagnetic radiation can excite electrons in atoms or molecules from their ground state (S0) to a higher energy state (S1). Excitation energy between 1.5 elettronVolt (eV) and 3.5 eV is required for aromatic organic molecules; this corresponds to wavelengths between 800 nm and 300 nm. Fluorescence occurs when electrons relax from their S1 to S0 state by emitting photons of light. Excitation light is of lower wavelength (higher frequency and higher energy) and the corresponding fluorescent radiation is of longer wavelengths (lower frequency and lower energy); this phenomenon is known as the Stokes shift [44, 45, 94]. This transfer typically happens in pico- to nano-seconds.

3.2 Fluorescence lifetime

Fluorescence lifetime is the average amount of time a fluorophore remains in the excited state following excitation. Different molecular constituents of cells display intrinsic auto-fluorescence. As a result of the variability of cellular (cytosol, mitochondria, et cetera) or tissue (normal, necrotic, neoplastic, et cetera) environments in which these molecules are found, variations in fluorescence lifetimes can be used as a possible mechanism of contrast to distinguish tissue [6, 7, 32, 49, 94, 100]. Several groups have reported on the utility of fluorescence lifetime for delineating normal from pathologic tissue

[10, 12, 74]. A major advantage of using time-resolved fluorescence assessments using either spectroscopic or imaging devices is that lifetime assessments are independent of blood, irregular brain surfaces, or tissue illumination, unlike measurements of fluorescence intensity alone [3, 4, 10].

Time-resolved fluorescence spectroscopy (TR-LIFS) measures fluorescence lifetime, but rather than imaging mode, it does so using a point spectroscopy device [6-8, 49, 61, 62, 94, 100]. Butte et al. [6, 7] previously demonstrated the implementation of TR-LIFS to detect fluorescence lifetime differences between normal brain tissue and glioma *ex vivo* and more recently reported the potential of applying TRLIFS for intraoperative diagnosis of gliomas. Normal cortex and white matter showed two fluorescence peaks at 390 nm and 460 nm, whereas the 390 nm emission peak was absent in low-grade gliomas (LGG) (N = 5) and reduced HGG (N = 9) [6]. These differences found *in vivo* suggest that TR-LIFS may function as potential adjuncts for facilitating delineation of the brain-tumor interface; however, additional studies are needed to validate its accuracy as an intraoperative tool for delineation of brain tumors.

Fluorescence lifetime imaging microscopy (FLIM) is an imaging technique used by Sun et al. [85] to determine the practicality of using an endoscopic fluorescence lifetime imaging for intraoperative identification of GBM. These authors evaluated the technique by validation between FLIM and MRI, intraoperative gross judgment, and gross pathological assessment. In this study, the FLIM instrument was adapted onto an endoscopic probe which

was positioned perpendicular to tissue surface. The fluorescence intensity and lifetime centered at 460 nm (major emission band for reduced nicotinamide adenine dinucleotide (NADH)) were significantly weaker and longer, respectively, in GBM compared to normal parenchyma following ultraviolet (UV) excitation. This pilot study presented initial results on the potential application of FLIM for intraoperative tumor identification.

3.3 Fluorophores

3.3.1 5-aminolevulenic acid (5-ALA)

Formation of ALA is the initial rate-limiting step in the porphyrin synthesis pathway that ultimately leads to heme-synthesis. Exogenous administration of 5-ALA functions as a pro-drug, which is then metabolized and promotes accumulation of fluorescent protoporphyrin IX (PpIX) in tissues through the hemi-biosynthesis pathway [95]. Although malignant gliomas show an increased accumulation of PpIX, the exact mechanism responsible for excess PpIX production in neoplastic cells after exogenous 5-ALA administration is not yet fully understood. The main mechanism of accumulation of PpIX in malignant tissue was initially believed to be the result of 5-ALA crossing a disrupted blood-brain barrier (BBB).

However, several studies have demonstrated that this is not the only mechanism, and that and specificity for the malignant cells might exist. Stummer et al. [81] studied 144 biopsies obtained from 66 patients. The

intensity of PpIX fluorescence was spectrographically assessed. Histopathologic analysis was conducted in terms of cellular density, MIB-l labeling index (as an indicator of proliferative activity) and the area of CD-31 staining was measured to determine neovascularity. A multi regression model demonstrated that CD-31 staining was barely significant, meaning that neovascularity and BBB abnormality was not the only factor related to fluorescence accumulation. Evidence suggests that PpIX accumulates more in HGG compared to LGG due to the increased proliferation, disruption of the BBB, neovascularization, differential expression of membrane transporters, and intracellular enzymes [34, 60, 79, 81]. Selective accumulation of ALA-induced PpIX between normal brain and tumor tissue provides necessary contrast and gradient for tissue delineation [28, 81-83, 95]. Intra-operative imaging of PpIX fluorescence allows the neurosurgeon to visualize tumor and thus optimize the potential for maximal resection (Fig. 1-2) [13, 45, 64, 78, 81, 83, 90, 92, 93].

Multiple studies have aimed to assess the effects of ALA fluorescence-guided glioma resection on the extent of gross total resection and progression-free survival (PFS) [13, 79].

A randomized controlled multicenter phase III trial was conducted by the ALA-Glioma study group and results published in 2006 [79]. In this trial, 322 patients with suspected malignant gliomas were enrolled and randomized to conventional white-light microsurgery (N=161) compared to 5-ALA fluorescence-guided resection (N=161). The patients were administered ALA

orally at a dosage of 20 mg/kg body in 50 mL of water 3 hours before induction of anesthesia. Surgeons conducted resection as completely as was thought safe and feasible based on imaging and their experience. Volumetric analysis of the preoperative and postoperative MRI studies were conducted by a centralized group of radiologists who were blinded for the allocation of the patients in the study. Postoperatively, all patients underwent standard protocol fractionated RT with the same recommended dose within 14 days of surgery. CHT protocols were given only to those with tumors of known susceptibility [79]. MRI assessed the extent of tumor resection within 72 hours and at 6 months. These patients were evaluated for PFS, residual tumor, overall survival (OS), neurologic deficit, and side effects. Full analysis was conducted on 270 patients with a mean follow-up of 35.4 months. This study demonstrated a 29% overall reduction in the number of patients with residual tumor on early MRI in the ALA group compared to the group given standard surgery under white light. PFS at 6 months was higher among the patients assigned to ALA compared to white light. Each of these findings was statistically significant. The OS, however, did not significantly differ between groups.

Despite the reported benefits in terms of improved rates of GTR with application of ALA, this technology has limitations, mainly based on subjective fluorescence interpretation, especially at tumor margins. Most studies have revealed high sensitivities and specificities for areas of intense

fluorescence. However, specificity tends to be lower in areas of vague fluorescence at the tumor margins.

Furthermore, the distorting optical properties of spatially heterogeneous tissue lead to subjective interpretation of PpIX fluorescence. This phenomenon is a limitation of this technique as currently practiced. Both excitation light and fluorescence emissions, as attenuated in tissue, make the detected fluorescence highly prone to subjectivity. This subjective fluorescence significantly decreases the correlation between the true levels of fluorophore and the visualized fluorescence. This phenomenon, which is dependent on the intrinsic metabolic and structural tissue changes that cause selective accumulation of PpIX, is partly responsible for reduced sensitivity and specificity reported in the above studies. Thus in the series published by Hefti et al. [24], tissues with a solid visualized fluorescent signals showed a sensitivity of 98% and a specificity of 100%; these values decreased to 76% and 85%, respectively, for vague fluorescence. Specificity limitations due to biological reasons such as nonspecific accumulation of PpIX in abscesses, metastasis, lymphoma, and necrotic tissue have also been reported [89]. This phenomenon may be related to inflammation and infiltration by reactive astrocytes and macrophages [50, 64, 79].

Occasionally, HGG may also not demonstrate PpIX fluorescence after 5-ALA administration [24]. In these cases, a tight BBB is thought to limit 5-ALA reaching tumor cells, preventing consistently successful fluorescence [21, 26]. Novel developments using quantitative methods may allow improved

detection and quantitative determination of PpIX levels which would minimize this limitation [24, 45, 93, 95]. Valdes et al. [95] reported the use of an intraoperative probe to estimate the absolute concentrations of PpIX, correcting for the distorting effects of tissue optical properties. This study across a range of tumor histologies showed a statistically significant improvement ($p<0.0001$) in detection accuracy for tumor of 87% (specificity = 92%; sensitivity = 84%) compared with 66% (specificity = 100%; sensitivity = 47%) using conventional visual fluorescence imaging. An additional study by this group showed a statistically significant correlation between increasing quantitative levels of PpIX and cellular proliferation and histopathological grade in gliomas (WHO grade I-IV), further demonstrating the need for quantitative methods to detect the more aggressive regions of tumor [93].

Another phenomenon that should be remarked is photobleaching. This is the chemical degradation of a fluorophore that results from exposure to light [44]. The stronger the intensity, the longer the exposure, and the lower the concentrations and photochemical stability of the fluorophore, the more likely is the risk of photobleaching. The operator's interpretation of fluorescent signal is also influenced by photobleaching of PpIX. This bleaching effect is mainly relevant at the tumor margins where PpIX concentrations are low [89].

Other factors may also limit the reliability of PpIX fluorescence. Such fluorescence covered by non-fluorescent tissue (normal thin brain tissue or blood) may be overlooked during resection [24, 89] as blue-violet light has a penetration power of only about 0.5 mm from the tissue surface [24].

Moreover postoperative management of patients undergoing ALA fluorescence can be demanding too. To avoid erythema induced by the drug's phototoxicity, patients are currently limited to low-light conditions for 24 to 48 hours after surgery [60, 78, 89]. Usually 5-ALA is administered orally 2 to 3 hours before tumor resection as the fluorescent labeling of the tumor cells is based on an enzyme-kinetic conversion which limits the use of 5-ALA in emergency cases [24] and can inconvenience surgical schedules.

Finally, but not less important, at this stage 5-ALA is not approved yet by the Food and Drug Administration (FDA) for surgical resection of brain tumors and it is not available in every center due to the mandatory certification needed and to its cost (around 900 Euros for each vial).

3.3.2 Hypericin

Hypericin is a photosensitizer and fluorescent dye that has demonstrated preferential accumulation in malignant tissue [56, 63, 65]. Ritz et al. [63] recently demonstrated the potential advantages of hypericin in fluorescence-guided recurrent glioma resection. They performed a 5-patient phase I study and injected a water soluble formulation of hypericin (0.1 mg/kg body weight) 6 hours before surgery.

Using a modified operating room microscope, they were able to distinguish tumor tissue from normal brain using the red fluorescence induced by hypericin. Histopathological review of 110 tissue samples demonstrated a specificity of 90% to 100% and sensitivity of 91% to 94%. No side effect with

intravenous (i.v.) administration of hypericin was evident. Future studies with larger cohorts of patients will further elucidate the utility of hypericin for fluorescence-guided surgery of gliomas.

3.3.3 Indocyanin Green

Indocyanin green (ICG) is a cyanine dye used largely for ophthalmologic purposes and has been established as the standard for visualization of choroidal and retinal pathologies [59, 75]. ICG binds strongly to plasma proteins, accounting for its slower vascular leakage within tissues. It resides within the vasculature for a longer period of time in the tissues with higher blood flow, allowing for intraoperative angiographic visualization [22]. ICG is metabolized in the liver and excreted via the hepatobiliary system with a half-life of approximately 3 to 4 minutes [75]. Although it has a low toxicity profile, i.v. administration can cause side effects, including sore throat, hot flashes, and occasional anaphylaxis. ICG has an excitation peak around 800 nm and a major emission peak of 830 nm in tissue, and this lower frequency (longer wavelength) light allows deeper penetration of excitation light and permits ICG angiography to detect fluorescence emitted from deeper tissues to image patterns of circulation [22, 75]. ICG fluorescence is not visualized through the surgeon’s viewing binoculars since its fluorescence (830 nm) is not in the visible range of the electromagnetic spectrum, but rather requires special near infrared cameras for detection and analysis software for image display.

The ability of ICG to stain and demarcate brain tumor margins has been investigated in rat glioma models [22, 31]. These studies showed that i.v. ICG allowed demarcation of implanted glioma from normal adjacent brain parenchyma. Additionally, tissue staining coincided with the histologic margins within 1 mm at the brain tumor interface, and minimal residual tumor tissue was identified after resection of stained tissue. These preclinical studies suggested that ICG could function as a potential adjunct for intraoperative fluorescent delineation of brain tumor margins [31]. Based on these animal models, Haglund et al. [22] reported on 9 patients with LGG and HGG who underwent tumor resection using ICG fluorescence. In this study, a maximum dose of 2 mg/kg of ICG was i.v. administered. They used enhanced optical imaging and offline interpretation of the pictures. Optical imaging of the resection margins in malignant tumors showed faster ICG uptake and slower clearance within the tumor as compared with normal brain. This phenomenon was more pronounced among higher-grade tumors. After resection, optical imaging of the tumor margins was obtained. The sites with delayed clearance were biopsied. Histopathological analysis of these samples was positive for tumor. Despite of some promising initial findings, this technology is cumbersome and images need to be analyzed off-line. No other investigations using enhanced optical imaging with ICG for glioma surgery have been reported. However, ICG videoangiography has become an invaluable tool in vascular neurosurgery [19] and it has been used as an

intraoperative tool to define the vascularity associated with some tumors [18, 31].

3.3.4 Fluorescein Sodium

Fluorescein is a green fluorescent synthetic organic compound which has a myriad of medical applications. Fluorescein sodium ($NaC_{20}H_{10}Na_2O_5$), in particular, is a water-soluble salt form of fluorescein with a major blue excitation peak in the region of 465-490 nm and a major green emission peak in the region of 510 to 530 nm [33, 45, 72]. Pathological changes in tissues are recognized by detection of the green-yellow fluorescence, both macroscopically and microscopically. Causes of increased fluorescence include malignancy, vascular leaking defects, pooling defects, and abnormal vasculature or neovascularization. Causes of low fluorescence include blocking defects, filling defects, and a normal BBB. The fluorescence of fluorescein sodium occurs naturally and may be observed by the human eye (at high doses) or via a modified surgical microscope [5, 72].

Steinwall and Klatzo [76] demonstrated that fluorescein may cross through an altered, but not an intact, BBB [54]. The passive diffusion of fluorescein beyond edematous portions and the ability for neuroglial cells to pinocytose fluorescein is considered very small [61]. Ginsbourg and LeBeau [20] also concluded that fluorescein-stained tissue was confined to neoplastic areas whereas cortical tissue stain was not seen at peritumoral margins.

It is usually available as a water-soluble dye that has been extensively used in the field of ophthalmology since 1961, particularly to perform retinal angiography, with very few side effects [41, 42, 57, 99]. Fluorescein sodium, however, may have side effects, especially if injected quickly or in high doses [51].

Although much of its use has been limited to ophthalmology, fluorescein fluorescence was used as an adjunct for navigation and resection of intracranial tumors as early as 1947-48 [52, 53].

The way of action of fluorescein is different from that of 5-ALA. Fluorescein usually accumulates in specific cerebral areas as a result of leakage from a damaged BBB. In the case of the MG, the invasiveness of these lesions changes the morphology of the BBB and disrupts its continuity along brain vessels modifying their permeability. This allows fluorescein to become concentrated specifically at the tumor site, making the tumor tissue more clearly visible, particularly if a dedicated filter on the surgical microscope is available, with a possible increase in GTR rate [1] (Fig. 3-4-5-6).

In 1982, Murray [54] reported a study in which 23 patients underwent craniotomies for malignant brain tumors. During this study, 111 fluorescein-stained and 75 non-stained tissue biopsies were reviewed for histopathological examination using a special arc-lighting system connected to a conventional surgical headlight. Of the 75 non-stained specimens, 71 (94.7%) were negative for tumor and 4 were positive. Ninety-four (84.7%) fluorescent sections demonstrated neoplasia and 17 showed features

consistent with necrotic debris. Their results emphasized the need for further improvement in reliability of this fluorescent technique, especially along the tumor margins.

Koc et al. [33] reported a prospective nonrandomized study to evaluate the influence of fluorescein sodium fluorescence-guided glioma resection on the extent of GTR, overall prognosis, and side effects. Of the 80 patients enrolled, 47 received high-dose (20 mg/kg body weight) i.v. fluorescein sodium after craniotomy prior to incision of the dura, whereas in the second group (n = 33), standard resection was conducted. Using this high dose of fluorescein, these authors used a standard operating room microscope to visualize the fluorescent signal without a filter or special camera. Postoperatively, all patients underwent fractionated RT with the same recommended dose to the lesion within 20 days of surgery. The results showed a significant increase in the number of patients receiving GTR (83% versus (vs) 55%) with administration of fluorescein; however, there was no statistically significant difference in OS between the two groups.

Shinoda et al. [73] reported GTR in 84.4% of patients receiving fluorescein sodium compared with 30.1% in those with conventional white light imaging. Among the patients presenting with removable lesions, GTR was achieved in 100% of patients pretreated with fluorescein sodium compared to 44.8% without. Despite these encouraging results, there was no observed difference in the overall prognosis. There were no significant side effects noted in these studies.

The results of these studies in terms of non-significant impact on OS appear to be similar to those reported by the multicenter phase III randomized trial conducted by the ALA-Glioma Study Group.

Kuroiwa et al. [40] assembled an intraoperative microscope with the ability to enhance fluorescence intensity and normal-to-tumor tissue contrast, but the equipment was cumbersome and impractical at the time it was designed.

Due to its small molecule size and reversible binding to albumin and red blood cells, fluorescein sodium may leak extravascularly, leading to staining of peritumoral regions. More recently, fluorescein conjugated to albumin has been used to increase the concentration gradient in the tumor/brain interface and therefore facilitate resection. A clinical trial of a small group of patients (n=13) receiving 5-aminofluorescein-human serum albumin conjugate has demonstrated promise in improving the extent of resection [35].

Fluorescein is much less expensive compared to 5-ALA (5 Euros for each vial) and can be easily administered i.v. at the moment of anesthesia induction. Few side effects have been reported in the ophthalmologic literature [41, 42, 57, 99].

A brief comparison between 5-ALA and fluorescein with their main characteristics is reported in Table 1.

4. METHODS

4.1 Study design

Based on the aforementioned characteristics, sodium fluorescein was chosen to carry out a prospective phase-II trial (FLUOGLIO) with a two-stage Simon design in order to evaluate the safety and to obtain initial information about the efficacy of fluorescein-guided resection of MG using a dedicated fluorescence kit integrated in the surgical microscope.

The study was registered on the European Regulatory Authorities website (EudraCT No. 2011-002527-18).

4.2 Patient population

Inclusion criteria of the FLUOGLIO trial were: 1) Patients age ranging from 18 to 75-year old; 2) with suspected, newly diagnosed, untreated HGG, based on a recent (less than 14 days) brain MRI with and without i.v. paramagnetic contrast agent administration with volumetric sequences; 3) located as to enable complete resection of the whole contrast-enhancing area as decided by individual study surgeon were eligible to participate in the FLUOGLIO trial.

Exclusion criteria were: 1) histological diagnosis different from HGG (grade III glioma, Anaplastic astrocytoma (AA) or grade IV glioma, GBM); 2) tumors starting from the midline, basal ganglia, cerebellum, or brain stem; 3) multifocal tumors; 4) the presence of a large, non-contrast enhancing area,

suggesting LGG with malignant transformation; 5) medical reasons precluding MRI (pacemaker, prostheses not suitable for MRI, at cetera); 6) inability to give consent because of dysphasia or language barrier; 7) Karnofsky performance scale (KPS) 60 or less; 8) renal insufficiency; 9) hepatic insufficiency; 10) history of active malignant tumors at any other body site.

The sample size and stopping rules were determined according to a minimax Simon two-stage design, with 80% power and α=5%, in which a response rate (proportion of tumor complete resections) of ≥50% was considered promising and a response rate of ≤30% was unacceptable.

In the first stage, it was foreseen that 15 patients should be enrolled, and, in case five or fewer responses were observed, further study should be terminated because of lack of treatment efficacy. Otherwise, an additional 31 patients should be enrolled for a total of 46.

At the end of the second stage, if 18 or fewer responses were observed the treatment would be rejected, whereas if 19 or more responses were recorded the treatment could be considered sufficiently active to warrant further study.

The first patient was enrolled in September 2011. All patients gave written informed consent, and the FLUOGLIO study was approved by the Ethics Committee of the Foundation IRCCS Neurological Institute Carlo Besta of Milan, Italy, and registered on the European Regulatory Authorities website (see previous paragraph).

Twenty-eight patients were screened for participation in this prospective trial. There were 18 males and 10 females, ranging from 45 to 74-year old (median 63). Four patients were excluded from the study: two of them due to histological diagnosis different from a HGG (one lymphoma and one PNET). In the other two cases some residual fluorescent tissue was left during the operation: the first case was a left parietal GBM and a small amount of fluorescent tissue was left because of contralateral motor activation during neurophysiological monitoring, while the second one was a left mesial temporal HGG; in this case some fluorescent tissue was visible in the cerebral peduncle and in the mesencephalon and the surgeon decided not to resect it. 24 patients were finally enrolled (23 GBM and 1 AA).

Clinical and neurological characteristics of the patients are described in Table 2.

4.3 Pre- and postoperative clinical and radiological assessment

General physical performance was recorded by the use of the KPS. Blood laboratory tests were included. Neurological function was assessed by Mini Mental State Evaluation (MMSE) and by the US National Institute of Health Stroke Scale (NIHSS).

Pre-operative clinical evaluation was performed up to 14 days before surgery, while immediate post-operative evaluation was completed within 72 hours after surgery.

All patients were submitted to pre-operative and post-operative pre- and post-contrast MRI (1.5-3 Tesla, Siemens), with volumetric sequences, within 14 days before surgery, and within 72 hours after surgery. Pre-operative tumor volume and residual tumor after surgery were calculated by an independent neuroradiologist, using an open-source software (Osirix for Macintosh, www.osirix-viewer.com). Residual tumor was defined as contrast enhancement with a calculated volume more than 0,175 cm^3 [79].

Surgery was followed by RT with concomitant and adjuvant Temozolomide (TMZ), according to Stupp protocol [84]. No restrictions were imposed on treatment after disease progression. Type and frequency of treatments after Stupp Protocol (II^{nd} line CHT, further surgeries and radiosurgery) were recorded.

Clinical and radiological follow-up were performed within 4 weeks after the end of radiochemotherapy and then every 2 months for GBM patients and every 3 months for AA patients.

Clinical evaluation included the same tests performed at pre- and early post-operative evaluation. Radiological controls included a pre and post-contrast MRI performed with volumetric sequences (1.5-3 Tesla, Siemens).

Progression was defined as the occurrence of a new tumor lesion with a volume greater than 0,175 cm^3, or an increase in residual tumor volume, according to the response assessment in neuro-oncology (RANO) Criteria [96].

Due to the fact that all the patients were operated on under general anesthesia (GA), immediate and post-operative reaction to the administration of fluorescein could be evaluated only for objective data such as: hypotension, cardiac arrest, shock, seizures, bronchospasm and anaphylaxis.

4.4 Surgical protocol

At the intubation and before the skin incision, all patients included in the study received i.v. 5-10 mg/kg of a 20% solution of sodium fluorescein (Monico Spa, Italy).

The study protocol stipulated complete resections of the fluorescent area, which was considered safely feasible by the surgeons. For this reason, two patients were excluded due to not complete fluorescent tissue removed by the surgeon (see above, paragraph 4.2).

Surgery was done by using a dedicated surgical microscope (Pentero with fluorescence kit, BLU 400 or YELLOW 560, Carl Zeiss, Germany) that allows visualization of light emitted in the wavelength of fluorescein (maximum excitation of 494 nm and maximum emission of 521 nm). The operating room light should be dimmed for optimum reproduction of the fluorescent light. In the first 6 cases, the same fluorescence kit for 5-ALA-guided resection of gliomas (BLU 400) was used; in these cases a dose of 10 mg/kg of fluorescein was used. Since January 2012, the new fluorescence kit YELLOW 560, specifically designed by the Zeiss Company for excitation in

the wavelength range from 460 to 500 nm and for observation in the wavelength range from 540 to 690 nm, has been available. For this reason, the dose of fluorescein was reduced to 5 mg/kg.

Neuronavigation (Treon, Tria and S7 Stealth Station, Medtronic, USA) was permitted only for surgical planning, initial tumor localization and orientation during tumor removal, but not for EOR evaluation.

In cases located near or in the proximity of cortical or subcortical eloquent areas, neurophysiological monitoring was performed as already described in a previous article [11].

The mechanism of action of fluorescein is related to the passage through a damaged BBB and accumulation in extracellular space. Thus, it helps in visualizing the pathological tissue in the same way as for contrast enhancement during MRI. In this study, fluorescein was injected i.v. after patient intubation and before skin incision. In this way, it was possible to avoid accumulation of fluorescein in cerebral area inadvertently damaged during craniotomy or dural opening. Microscope is brought in the operative field after craniotomy. During resection, the microscope could be switched alternatively from fluorescent to white-light illumination, as desired by the surgeon, by pressing the specific button on the microscope handgrip. However, with the YELLOW 560 filter it was possible to visualize the fluorescent signal and at the same time the non-fluorescent tissue in more natural color [1]. For this reason, most of the surgery was completed with the filter activated. Tumor resection was completed in an inside-out fashion

usually by ultrasonic aspiration, until all the fluorescent tissue exposed was removed, as considered feasible by the surgeon (Fig. 3-4-5-6).

For patients harboring tumors located in non-eloquent areas, and giving their specific written informed consent, an ancillary protocol was designed in order to evaluate the accuracy of fluorescein in identification of tumor tissue. This included specific biopsies at the tumor margins (see next paragraph).

4.5 Histological analysis and MGMT methylation status

Histological analysis of the tumor was performed with standard procedures and classified on the basis of the current 2007 WHO classification by an independent neuropathologist [47].

For tumors located in non-eloquent areas, biopsies at the tumor margin were performed to evaluate the accuracy of fluorescein in tumor tissue identification (see previous paragraph). Specifically, for each patient, 4 biopsy samples were performed at the tumor margin in areas located distant one from the other, 2 in the fluorescent and 2 in the non-fluorescent tissue in order to calculate sensitivity and specificity (see next paragraph). In this case, histological analysis was performed by a pathologist that was blind regarding the fluorescence characteristics of the biopsies, in order to discriminate between clear tumor tissue and peritumoral area (gliosis or tumor cell infiltration); immunohistochemistry for GFAP (glial fibrillary acidic protein) was also performed and proliferating index with Ki67 (MIB 1) was calculated as well. Methylation patterns in the CpG islands of O-6-

methylguanine-DNA methyltransferase (MGMT) were determined as previously described [16].

4.5 Statistical analysis

The sample was described by means of the usual descriptive statistics: mean, median and standard deviation for continuous variables and proportions for categorical ones. T-student test was used to compare pre- and postoperative clinical conditions.

In order to evaluate fluorescein accuracy in tumor identification, sensitivity and specificity were calculated as follow. Sensitivity: number of fluorescent samples true positive (with histologically confirmed HGG)/ all samples with confirmed HGG. Specificity: number of non-fluorescent samples true negative (histologically confirmed not neoplastic)/ all samples histologically confirmed not neoplastic.

PFS was calculated from surgery until disease progression and death/last follow-up, if censored. 6 months PFS was defined as the proportion of patients without radiological progression at the 6^{th} month after surgery.

OS was calculated from treatment onset until death/last follow-up, if censored. PFS and OS were estimated by the Kaplan-Meier method.

5. RESULTS

5.1 Intraoperative fluorescence characteristics

Even though both fluorescence kits in the Zeiss microscope (BLU 400 and YELLOW 560) allowed good visualization of the tumor area in all the patients included (Fig. 3-4-5-6-7), slight differences between the two filters do exist.

BLU 400 is the fluorescence mode developed for 5-ALA-guided removal of HGG [79]. However, due to its wide range of emission and excitation, this filter also allowed good visualization of the light emitted by fluorescein in the area with BBB disruption. In particular, there was good discrimination between the fluorescent area, which appeared yellowish green, and the non-fluorescent tissue, which was dark blue (Fig. 7). Nevertheless, this contrast was more evident through the oculars for the first surgeon. Furthermore, the motion capture system of the microscope allowed the video reproduction only at a low frame rate, consequently creating a low-quality video. In addition, the fact that the area surrounding the tumor appeared dark blue did not allow performing all the procedures with the fluorescent mode activated, as the surgeon would not have had perfect control of vascular structures.

The YELLOW 560 module, on the other hand, improved visualization of the pathological and normal anatomy in the fluorescent mode. In fact, once the YELLOW 560 mode had been activated and the filters optimized for fluorescein (see above) moved into the microscope's light path, specific defined amounts of blue and red light were mixed for generating a white light

impression of the non-fluorescent tissue. This allowed delineating the fluorescent signal (appearing greener and brighter than the one visualized with the BLU 400 mode) and at the same time visualizing the non-fluorescent tissue in more natural color, without significant differences between the ocular views of the first surgeon and those of the assistant. Furthermore, in this way, most of the procedure could be performed under filter activated, reducing the discomfort of switching continuously between white-light to fluorescence illumination to control bleeding or manipulate vessels during tumor resection. This reduced eye fatigue during surgery too.

In addition, the high definition of the motion capture system allowed for good quality of images and video reproduction.

In summary, the main difference between the two filters was related to the fact that, due to the higher specificity of YELLOW560 module, the fluorescent tissue appeared greener and brighter, with a dose of fluorescein that was reduced by the half.

As mentioned above, the microscope is brought in the operative field after craniotomy. At the activation of the filter, the dura appeared fluorescent due to the characteristic features of its vascularization (Fig. 3). Tumor tissue showed an intense fluorescence, even if some necrotic parts of it could appear darker.

Sometimes, in case of partly cystic lesions, the liquid inside the cyst was intensively fluorescent.

5.2 Percentage of resection

Median pre-operative tumor volume was 33.1 cm^3 (range 1.3-87.8 cm^3). 20 patients were submitted to a complete removal of all enhancing tumor as verified by an early postoperative MRI (83% of the cases) (Figure 8 and Table 2). The remaining patients had a mean tumor resection of 92.6% (range 82.6-99.9%) (Figure 9 and Table 2).

5.3 Accuracy of fluorescein in tumor identification

A total of 42 biopsies (22 in fluorescent tissue and 20 in non-fluorescent tissue) were performed at the tumor margin in 11 patients (4 biopsy in each patient, except one patient in which only fluorescent tissue biopsies were performed). Nineteen out of the 22 biopsies in fluorescent area showed clear HGG tissue. Nineteen out of the 20 biopsies in non-fluorescent area showed the absence of HGG tissue. These data were confirmed by the immunohistochemistry analysis: the 19 samples from fluorescent areas with HGG tissue showed GFAP-positive gliomatous cells, with a high Ki67 (MIB 1) index (range 5-25 %). Conversely, in the 19 non-fluorescent biopsies negative for MG tissue, immunostaining showed the presence of only non-proliferating reactive astrocytes (Fig. 10-11).

These data gave a preliminary estimation of sensitivity of fluorescein in identifying tumor tissue of 95% (19/20), and a preliminary estimation of specificity of fluorescein in identifying tumor tissue of 86% (19/22).

5.4 Reactions to Fluorescein, post-operative neurological status and complications

According to the method of analysis described above, no adverse reaction to fluorescein administration could be registered in this cohort of patients, including those with different histological diagnosis that were excluded from the analysis. Other adverse events during the follow-up period are listed in table 3.

No statistically significant difference was found between pre- and immediate postoperative NIHSS (1.46±0.38 vs 2.42±0.71, p=0.148) and KPS (median 90 vs 80, p=0.145).

In the early post-operative period (since the first post-operative month), three patients presented a complication. One patient with left occipital GBM (n.1) developed an entrapped temporal horn 16 days after surgery that required ventriculo-peritoneal (VP) shunt. One patient with left frontoinsular GBM (n.8) developed post-operative hemiplegia and aphasia due to a small infarction in the internal capsule and was sent to rehabilitation with improvement in aphasia and movement in the right limb. One patient with right frontal GBM (n.18) presented a post-operative infection of the bone flap, requiring wound revision and antibiotic therapy.

5.5 PFS and OS

Nineteen out of 24 patients completed post-operatively the Stupp protocol. After a median follow-up of 12 months (range 2-27 months), estimated 6

months PFS was 71.4% (60.6-82.2%) (Figure 12). Eleven patients died during the follow-up period for tumor progression (10 cases) or for unrelated cause (myocardial infarction in one case, n. 15). Median survival was estimated in 11 months (Figure 13).

6. DISCUSSION

6.1 Impact of EOR on the outcome of MG patients

There is evidence in the literature that EOR has an impact on PFS and OS for patients with MG, but no Class I data are available. Lacroix et al [43] retrospectively analyzed a database of 416 patients with GBM. They concluded that an EOR of 98% or more was associated with a survival advantage (median survival 13 months vs 8.8 for less than 98%, $p<0.0001$). Sanai et al [67] retrospectively reviewed a series of 500 patients with GBM. They found a stepwise correlation between EOR and OS starting at an EOR of 78% with 95% and greater EOR being the strongest predictor of OS.

Nevertheless, complete resection, intended as the surgical removal of the whole enhancing mass on the preoperative MRI, the so called GTR, was reported to be feasible only in a low percentage of cases [43, 48], due to intraoperative difficulties in tumor tissue recognition at the margins of resection [89]. In particular, in the retrospective series published by McGirt et al in 2009 [48], considering patients operated on in the period 1996-2007 and excluding locations precluding a complete removal, a GTR was feasible only in 39% of the cases at the first diagnosis. These results were similar to the ones from the control group of patients of the 5-ALA study, operated on without the aid of fluorescent visualization, in which a complete resection was reported to be feasible only in 36% of the cases [79].

The 5-ALA study represented a unique opportunity to study the impact of EOR in OS.

Stummer et al. [80] published a follow-up study adjusted for bias using the data from the 5-ALA randomized controlled trial. For this purpose, the authors re-stratified the patients from both the 5-ALA and white light groups based on complete versus incomplete resection, resulting into two homogeneous cohorts with well-matched characteristics. The analysis revealed treatment bias in terms of younger patients and those with tumors in non-eloquent areas receiving more complete resections. Patients without residual tumor survived longer (16.7 vs 11.8 months, $p<0.0001$). This study provided level 2b evidence that complete resection had a significant impact in OS. Survival within the incomplete resection group based on EOR was not analyzed.

The use of fluorophores has been designed as a surgical adjuvant to enhance tumor visualization increasing the rates of GTR and thus attempting to improve OS [45].

The only randomized study that has attempted to investigate the impact of fluorescence-guided resection in EOR and PFS is the phase III randomized controlled trial conducted by Stummer et al [79]. This trial was designed to compare rates of GTR with 5-ALA fluorescence vs standard white light microsurgical resection. In this study, GTR was achieved in 65% of patients in the 5-ALA group as opposed to 36% in the white light group. Results showed a statistically significant increase in PFS for the 5-ALA group (5.1

months) vs the white light group (3.6 months). However, there was not a significant difference in OS (15.2 months for 5-ALA group vs 13.5 months for the white light group). The study was underpowered for the analysis of this variable. It must be noted that both arms in the study had an OS equal or superior than other large series published that included adjuvant chemoradiation [67, 84]. Overestimation of GTR is unlikely, since the analysis of postoperative MRI studies were conducted by a blinded independent group of radiologists. However, only tumors with a distinct ring-like pattern of contrast-enhancement and a core area of reduced signal were included in the study. Tumors of the midline, basal ganglia, cerebellum, brain stem, multifocal contrast-enhancing and significant non-contrast-enhancing areas were excluded. This pre-operative assessment, made by the study surgeons, might have led to inclusion in both groups of tumors more amenable to GTR, with or without fluorescence. Also, tumors located in eloquent areas were 53% for the 5-ALA group and 59% for the white light group. This proportion is lower than that reported in other large series, such as Sanai et al. [67], who reported 69%. All these factors might have contributed to very aggressive resections with very small residuals in the control arm (median residual volume 0.7 cm^3), thus making it difficult to prove a significant difference with the fluorescence-guided arm.

Another fluorophore that has been extensively used to increase tumor visualization in HGG surgery is sodium fluorescein.

6.2 Literature review on fluorescein applications for MG surgery

By analyzing the data available in the literature, three major applications of sodium fluorescein during surgery for MG could be documented: Fluorescein-Guided resection of MG with white-light illumination; Fluorescein-Guided Resection of MG with a surgical microscope equipped with a dedicated filter for fluorescein; Confocal Microscopy for intraoperative histopathological analysis on MG.

6.2.1 Fluorescein-Guided resection of MG with white-light illumination

The first reported application on the use of fluorescein during surgery of MG was described by Moore in 1947 [52, 53]. In this study [52], 46 patients received 1 gr of fluorescein intravenously immediately before or during ventriculography, in order to confirm the location of subcortical tumors. The majority of tumors detected by needle biopsy were malignant astrocytomas. The material sampled suggested the presence of tumors sometimes by direct white light visualization, but, usually, by UV light directly in the operating room. The biopsy was then verified by histological examination, with a correct diagnosis assured by fluorescein visualization in 96% of cases. Similar applications were reported in subsequent series [2, 86].

The accuracy of fluorescein to identify tumor tissue was systematically studied by Murray KJ, by performing a total of 186 biopsies in 23 patients with brain malignancies. In that study, he obtained a sensitivity of 96% and specificity of 81% [54].

The use of fluorescein to improve the percentage of resection of MGs has only been explored in recent years. Some studies were performed without the aid of a specific illumination light and filter to allow for a better discrimination of fluorescein fluorescence [9, 33, 73].

Shinoda et al in 2003 reported the first retrospective analysis on a series of 32 patients operated on with the aid of fluorescein, but without a special filter adapted to the surgical microscope [73]. The operation was therefore completed under white light illumination and with standard surgical microscope. In this case, a high-dose of fluorescein was intravenously administered (20 mg/Kg) in order to detect fluorescence in the surgical field, at the time of dural opening. For this reason, immediately after intravenous injection, the normal cortex, vessels, dura mater and tumor were stained yellowish, but after approximately 5 minutes only the tumor retained the yellow dye. The yellow appearance was different inside the tumor, with the center more deeply stained and the periphery faintly stained. However, even the periphery appeared clearly discernable from the negative peritumoral area. This difference in fluorescence seemed to be correlated to the histological characteristics of the tumors. In fact, in 4 of the cases analyzed by histology, the authors found GBM tissue in the center deeply stained area, while less dense tumor cells and scattered endothelial proliferation in the faintly stained area. Based on post-operative MRI, interestingly enough, the percentage of GTR was almost 85%, and it was significantly better than the percentage of 30% obtained by the same authors in a historical control group

operated without fluorescein. However, no statistically significant difference in survival could be found between these groups.

Similar results were reported by Koc et al [33], who described the result of a prospective analysis on two non-randomized groups, one of which operated by the aid of fluorescein (20 ml/Kg at the time of dural opening) without a specific filter on the microscope (group 1), and one without fluorescein administration (group 2). The percentage of GTR based on post-operative MRI was 83% in group 1 and 55% in group 2. Both groups were submitted only to RT, without concomitant or adjuvant CHT. OS was therefore poor, with 44 weeks in group 1 and 42 weeks in group 2, without a significant difference. A similar trial was performed by Chen at al [9], reporting a significant increase in GTR in patients undergone fluorescein-guided surgery for MG. A total of 22 patients were enrolled in this study, divided into the study group (n=10) which included patients that received i.v. fluorescein injection (15-20 mg/Kg at the time of dural opening) and the control group (n=12) without fluorescein administration. GTR rate was 80% in the study group and 33.3% in control group. No complications were detected by Chen et al [9] in a study where pre-operative allergy test were performed in order to avoid any possible allergic reactions. Persistent yellow staining of skin, mucosa and urine were the only adverse events observed by Shinoda et al [73] in the next 24h after operation. Even though low complication rate has been described in the ophthalmologic field [41, 42, 57, 99], some side effects can occur after high-dose fluorescein administration. Few anaphylactic

reactions have been reported with severe bradycardia and hypotension [14, 88].

These data are summarized in table 4.

6.2.2 Fluorescein-Guided Resection of MG with a surgical microscope equipped with a dedicated filter for fluorescein

Due to the specific characteristics of fluorescein, with a maximum absorption at 494 nm and maximum emission of 521 nm, some filters have been developed to help in the discrimination of fluorescence during surgical approaches for MG removal.

The first experiences were made with filters not directly integrated in the surgical microscope, but adapted to guarantee a visualization of fluorescein that was coherent with its absorption and emission wavelength peaks. Kabuto et al in 1997 used two filters for the excitation and emission of fluorescein that could be manually fitted to and removed from a surgical microscope during operation for MG in 5 cases [29]. These authors injecting high dose of fluorescein (1 gr i.v.), reported a clear visualization of the tumor under the filter, but they also found a faint yellow color under white light illumination. Observation during tumor removal could be changed to ordinary white light illumination by removing the filters, in order to perform surgical maneuvers requiring a better visualization of the structures in and around the tumors, such as vessels.

A similar combination of high-dose fluorescein (20 mg/Kg upon dural opening) and excitation and barrier filters inserted in the operating microscope (OME-9000, Olympus) was proposed more recently for GBM removal, by Okuda et al [58]. Ten consecutive patients were included in this trial, and no side effects were described. Depending on the resection requirements, the high fluorescein dose allowed surgical maneuvers to be performed both in fluorescence mode and under normal white xenon-light illumination. According to these authors this seemed to improve the reliability of surgical removal of GBM. In all the 5 cases studied with an intended GTR, this was confirmed post-operatively.

Kuroiwa et al described for the first time a technique in which the fluorescence filter was directly integrated in the microscope (Zeiss OPMI) [40]. In this study as well, visualization could be switched during operation from filter to white light illumination. In addition, due to specificity of the filter for absorption and emission wavelength of fluorescein, these authors were the first to use a reduced dose of i.v. fluorescein, specifically 8 mg/kg, which was injected upon dural opening. As reported by Shinoda, without filter visualization [73], they documented immediately after injection a fluorescence staining in the arteries, veins of the surface, brain parenchyma and tumor. But 5 minutes after injection the brain surface fluorescence lessened, and tumor removal started 20 minutes later. Ten patients were included in this study. In 8 cases, a complete tumor removal could be obtained. The same group [39] studied a larger series of 30 patients using

the same technique, and in 5 cases they compared the intra-operative finding with standard histological analysis. Fluorescein-positive areas revealed abnormal tumor vessels and dense tumor cells, while fluorescein-negative areas showed only scant tumor cells infiltration and no abnormal vessel. In a different paper, the same group demonstrated the application of fluorescein injection for frameless stereotactic biopsies of deep contrast-enhancing brain tumors [38].

Since 2012, a dedicated module for fluorescein filter (YELLOW 560), has been specifically designed by the Carl Zeiss Company (Germany) for excitation in the wavelength range from 460 to 500 nm and for observation in the wavelength range from 540 to 690 nm, and directly integrated in the Pentero surgical microscope. The YELLOW 560 module improved visualization of the pathological and normal anatomy in the fluorescent mode [1]. In fact, once the YELLOW 560 mode had been activated and the filters moved into the microscope light path, specifically defined amounts of blue and red light were mixed for generating a white light impression of the non-fluorescent tissue. This allowed for a delineation of both the fluorescent signal and the non-fluorescent tissue in more natural color. In this way, it was possible to protect the normal structures during surgical maneuvers under fluorescence visualization [1]. Furthermore, due to the high specificity for fluorescein of this filter, the fluorescein dose could be further reduced in clinical application [1, 69].

In 2013, two papers concerning the use of Yellow 560 filters for surgery of MGs have been published [1, 69]. Schebesch et al [69] conducted a retrospective analysis on 35 patients, including 22 with MG (12 initial glioblastomas, 5 recurrent glioblastomas and 5 grade III gliomas), operated on from May to August 2012. A total of 2 ml (200 mg corresponding to approximately 3-4 mg/kg) of fluorescein were intravenously administered after bone flap removal prior to durotomy. Most of their results came only from a qualitative analysis and there was no definitive data presented regarding EOR in MG. In addition, no histological studies on fluorescent and non-fluorescent tissue were performed. In this study, there were no adverse reactions registered. With this low-dose of fluorescein, only the urine became colored yellow for 6 hours post-operatively with no sclera or skin discoloration experienced by the patients.

Our group was the first to propose a phase II prospective trial on fluorescein-guided resection of MG with a dedicated filter on the surgical microscope (FLUOGLIO study) [1].

These data are summarized in table 5.

6.2.3 Confocal Microscopy for intraoperative histopathological analysis on MG

Confocal microscopy is an innovative technology that has been developed in the last decade. Its introduction represents a major breakthrough in the conventional histopathological analysis for selected tissue biopsy. Confocal

microscopy has been integrated in different medical specialties, since its initial application in Gastroenterology, where it was used to evaluating endoscopically inapparent barrett's esophagus [15, 30], or colorectal cancer [27]. It has also been used for endomicroscopic diagnosis of cervical intraepithelial neoplasia [87] or uroepithelial cancer [97].

Its application in neurosurgical field followed in turn. But, in order to do this, a specific tool needed to be developed [17, 66, 68]. The essential elements of this neurosurgical tool was a rigid probe containing a miniaturized scanner that allowed an operator-dependent specific depth-visualization that was usually in the range of 0-250 micron. This technical tool was handheld and sterilizable, and perfectly designed for neurosurgery. The scanner was connected to an external monitor in order to visualize the tissue against which the probe was directed, which was then sent to a personal computer unit.

This technique allowed an in vivo visualization of the histopathological features and cytoarchitecture of the analyzed samples, giving a significant aid in the intraoperative tumor identification [17]. Furthermore this approach seemed to overcome the limitations presented by the traditional intraoperative frozen-section evaluation that can be subject of mechanical or chemical alteration or can present artifacts [17]. In the studies performed by Eschbacher J et al and Sanai N et al from the Barrow Neurological Institute [17, 66], the clinical protocol included 25 mg of i.v. fluorescein sodium in order to increase the qualitative difference of the contrast-enhanced tumor

margin. According to this study the imaging process was initiated 5 minutes after fluorescent tracer administration. Once the different surgical sites were studied and the images acquired, biopsy samples were taken in order to perform the traditional histological analysis. In the blinded study that was performed [17], a neuropathologist interpreted the confocal images and the biopsy samples. In 28 images, an accuracy of 92.9% was reported. As these data suggest, this relevant device was less time-consuming and it had a comparable sensitivity to the traditional frozen-section analysis.

6.3 The FLUOGLIO study

From the analysis of the literature, fluorescein injection seemed to be a good method to obtain a high rate of GTR during surgery for MG. Percentage of resection in the analyzed series varied from 75% to 100%. In some papers, a significant increase of total resection was obtained if compared with cases that had been operated on without fluorescein use [9, 33, 73]. In addition, even if the mechanism of action is not related to a specific uptake of fluorescein by tumoral cells (as for 5-ALA), thus allowing for the possibility of false positive cases (metastases, surgical trauma, et cetera), different studies performed with and without filters on the surgical microscope showed a high level of accuracy in tumor identification [1, 39, 40, 54].

However, it needs to be noted that a proper phase III trial has not been published which is crucial to provide the kind of definitive data concerning any possible increase in PFS as well as the survival of patients operated with

fluorescein-guided technique. It is also important to recognize, additionally, that even with fluorescein-guided technique and under specific filter visualization, a complete removal of favorable MG is not always possible. As for other types of fluorescence tools currently been used in neurosurgical applications [18, 19], and as it has been suggested for 5-ALA [89], it is possible to enhance the discrimination of the pathological tissue with fluorescein only if it is properly exposed during the surgical approach. In two of the cases included in the FLUOGLIO study [1], for example, a small remnant was left due to its location around the corner of visualization during the approach. In addition, sometimes, in tumor located in close proximity to eloquent areas, a portion of fluorescent tissue could be left at the tumor margin if cortical or subcortical brain mapping shows positive responses.

The data presented on this cohort of patients confirmed that the use of i.v. fluorescein during resection of HGG is safe and it is associated with a high-rate of complete resection of contrast enhanced tumor at the early post-operative MRI.

Coherently with the experience of ophthalmologists for retinal angiography [99], and based on its known safety profile, no adverse reaction related to the use of fluorescein was recorded so far in the FLUOGLIO study.

Even though no adverse reactions were found in previous small series with doses of 20 mg/kg of fluorescein [73], the use of specific filters enabled reduction of the total dose of fluorescein injected [1, 40, 69] with a possible impact on reduction of side effects. In fact, even if adverse events including

anaphylactic reactions after fluorescein injection have been rarely reported in ophthalmological and neurosurgical literatures [14, 41, 42, 57, 88, 99], no side effects were registered with the use of low-dose fluorescein in more recent papers.

Moreover, it should be noticed that in neurosurgical application side effects could be even less frequent than ophthalmological cases due to the fact that fluorescein administration was performed with the patient intubated under GA.

The volumetric analysis performed in our cohort of 24 patients with HGG on early postoperative MRI showed a total resection of the contrast-enhancing tissue in 83% of cases. These data, although still limited by the small number of cases and, more importantly, the absence of a control group operated on without fluorescein, are really promising.

The inclusion and exclusion criteria were in fact similar to the prospective phase-III trial on 5-ALA by Stummer et al [79]. Specifically, patients with tumors located as to enable a complete resection of the contrast-enhanced area were enrolled. Nonetheless, the case series presented also included lesions in eloquent areas and with a median volume of 33.15 cm^3 (Table 2). Therefore, it seems reasonable to consider the rate of total removal not related to the inclusion of small tumors in non-eloquent areas. Furthermore, since patients were operated on by surgeons with different levels of experience and skill, these results could be generalized to other

neurosurgical departments in the world and not be related to a hypothetical better result of a highly skilled surgeon.

It should be underlined that the accumulation of fluorescein in the brain tumor area is related to the passage through a damaged BBB, and not to a specific uptake by the high grade glial cells as for 5-ALA. Therefore, as previously mentioned [89], this could lead to a reduced accuracy in terms of tumor identification. Nevertheless, different studies performed in the past with and without filters on the surgical microscope, showed a high level of accuracy in tumor identification [40]. These data were confirmed in the present series of biopsies of fluorescent and non-fluorescent tissue at the tumor margin, with a sensitivity and specificity in tumor identification that were respectively 95% and 86%. The use of the specific filter on the surgical microscope could have led to better accuracy in tumor identification. This could also explain why the patients did not experience a statistically significant difference in terms of neurological functions in the post-operative period. In addition, the new filter Yellow 560 allowed a dose reduction of fluorescein, due to the higher specificity of the filter characteristics. Theoretically, this could further reduce the number of side effects.

Since the MGMT methylation signature has a relevant effect on disease outcome [25], its status was analyzed in all patients, being unmethylated in the majority of them (71%).

Median long-term follow-up (12 months) is still not sufficient to draw a definitive conclusion about long-term outcome. However, preliminary data on

6 months PFS were promising with more than 70% of patients without progression.

7. CONCLUSIONS

The analysis of the data collected so far in 24 patients, suggested that the use of i.v. fluorescein during surgery of HGG with a dedicated filter integrated in the surgical microscope is safe and could be associated to a high-rate of complete resection. If confirmed at the end of the study, these results will represent the prerequisite to build up a proper prospective randomized controlled phase III trial to definitively prove a positive effect of i.v. fluorescein on EOR of HGG.

Finally, we could speculate that a combination of fluorescein-guided resection of MG by the use of a dedicated filter on the surgical microscope and confocal microscopy to confirm the absence of cancerous residual cells at the tumor margin could provide an exceptional method to extend the surgical removal to a cellular level.

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9. TABLES

Table 1

Comparison between main characteristics of fluorescein and 5-ALA for surgery of MG.

	5-aminolevulinic acid (5-ALA)	**FLUORESCEIN**
TYPE OF SUBSTANCE	DRUG: precursor of hemoglobin	DYE: sodium salt of fluorescein
MECHANISM OF ACTION	Selectively elicits the synthesis of fluorescent porphyrin IX in tumoral cells	Passes throughout a damaged BBB
DOSAGE	20 mg/Kg	3-10 mg/Kg
TIME AND MODE OF INJECTION	2-3 hours before surgery, orally administered	At the time of surgery, i.v. administered
FLUORESCENCE VISUALIZATION	Specific Filter for visualization: needed (ZEISS-LEICA) Filter characteristics: good discrimination of fluorescent tissue; poor visualization of peritumoral area	Specific Filter for visualization: needed (unless high dosage) (ZEISS) Filter characteristics: good discrimination of fluorescent tissue; visualization of peritumoral area in more natural colors
EFFECT ON GTR RATE	65% vs 36% ˚	75-100%*
EFFECT ON PFS	6-PFS 41% vs 21.1% ˚	No definitive data available
EFFECT ON OS	No definitive data available	No definitive data available
COSTS	Around 900 Euros for each vial	Around 5 Euros for each vial

˚ data against white light illumination derived from a randomized controlled multicenter study (see text).

* data derived from the present study and available literature, including only case series, retrospective studies and prospective non randomized controlled studies.

Table 2

Patients included in the FLUOGLIO study up to December 31st, 2013.

Patient	Tumor location	Tumor size (cm3)	Histology	MGMT status	% of resection	NIHSS pre-op	NIHSS post-op	KPS pre-op	KPS post-op
1	L occipital	28.0	GBM	Unmethylated	100%	3	4	70	60
2	L temporal	12.8	GBM	Methylated	100%	0	0	100	100
3	R parietal	49.8	GBM	Unmethylated	100%	3	4	70	80
4	R parietal	87.8	GBM	Methylated	99.9%	7	8	70	60
5	R frontal	12.2	GBM	Unmethylated	100%	0	1	90	80
6	L occipital	41.0	GBM	Unmethylated	100%	0	0	100	100
7	L temporo insular	31.8	GBM	Unmethylated	88.6%	1	2	90	80
8	L frontoinsular	34.5	GBM	Unmethylated	100%	4	13	90	50
9	R frontal	9.6	GBM	Unmethylated	82.8%	0	3	90	70
10	L temporal	2.4	AA	Methylated	100%	1	1	90	90
11	R frontal	20.0	GBM	Unmethylated	100%	1	1	90	90
12	R frontal	83.8	GBM	Unmethylated	100%	2	1	80	90
13	R temporal	86.1	GBM	Unmethylated	100%	1	0	70	80
14	R parietal	12.5	GBM	Unmethylated	100%	1	4	100	90
15	R frontal	69.7	GBM	Unmethylated	100%	2	5	70	50
16	L frontal	8.3	GBM	Unmethylated	100%	1	2	90	70
17	R frontal	31.8	GBM	Methylated	100%	0	0	100	100
18	R frontal	8.9	GBM	Unmethylated	100%	0	0	90	90
19	L temporal	28.8	GBM	Unmethylated	100%	1	2	80	70
20	R parietal	38.9	GBM	Unmethylated	99.3%	1	2	90	80
21	L parietal	1.3	GBM	Methylated	100%	0	0	100	100
22	R parietal	1.9	GBM	Methylated	100%	0	0	100	90
23	L frontal	18.7	GBM	Unmethylaed	100%	2	2	90	80
24	R parietal	73.5	GBM	Methylated	100%	4	3	70	80

Table 3

List of adverse events, treatment required and outcome.

Pt. No.	Tumor location	Adverse event	Treatment required	Outcome	Correlation to fluorescein
1	L occipital	Left temporal horn excluded	VP shunt	Complete recovery	NO
2	L temporal	leucopenia after I° cycle of adjuvant TMZ	Reduction of TMZ dose during II° and III° cycle	Complete recovery	NO
3	R parietal	1. lung infection 2. extradural collection and brain edema with hemiparesis	1. hospitalization for i.v. antibiotics administration 2. extradural fluid collection and bone removal	1. stop CHT 2. slight hemiparesis (improved)	NO
8	L frontoinsular	Right hemiplegia and aphasia	Rehabilitation	Improvement of aphasia and of right inferior limb paresis	NO
9	R frontal	1. Trombocytopenia after I° cycle of adjuvant TMZ 2. Partial motor seizure during RT	1. Platelet transfusion 2. Antiepileptic drug	Complete recovery	NO
10	R frontal	Brain edema after RT	Hospitalization for i.v. steroids and mannitol administration	Complete recovery	NO

Table 4

Fluorescein application during surgery for MG with white-light illumination.

Fluorescein's application	**Author**	**Year**	**No. of pts.**	**Fluorescein dose (mg)**	**Time of Fluorescein injection**	**GTR rate**
Localization of brain tumor	Moore	1947	12	1000	Not specified	Not examined
Localization of brain tumor	Moore	1948	46	1000	Before ventriculography (approximately 2 hours before surgery)	Not examined
Localization of brain tumor	Belcher	1951	-	-	-	-
Localization of brain tumor	Svien	1951	not specified	1000	not specified	not examined
Identification of brain tumor	Murray	1982	23 (14 MG) (for 186 biopsies)	1000	not specified	not examined
Resection of MG	Shinoda	2003	32	20 mg/Kg	before dural opening	84.4%
Resection of MG	Koc	2008	47	20 mg/kg	before dural opening	83%
Resection of MG	Chen	2012	22	15-20 mg/kg	before dural opening	80%

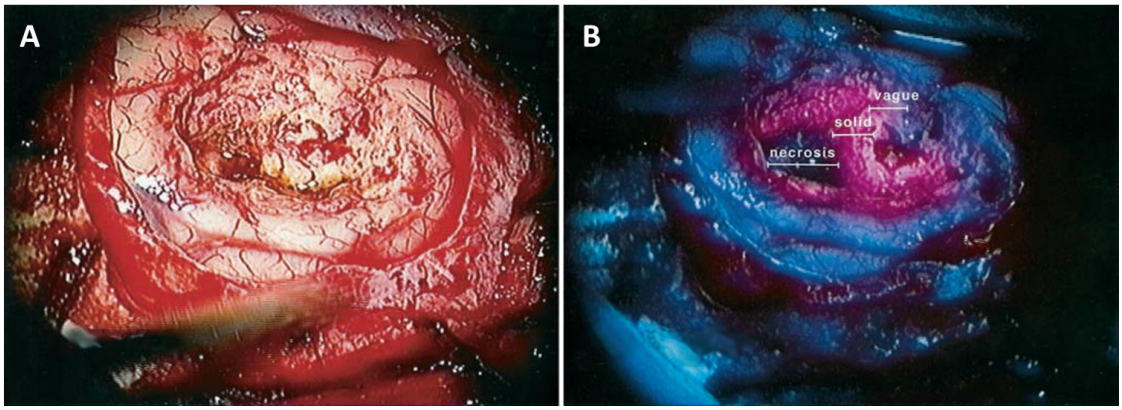
Table 5

Fluorescein application during surgery for MG with specific filters in the surgical microscope.

Dedicated Filter	Author	Year	No. of pts	Fluorescein dose (mg)	Time of Fluorescein injection	GTR rate
manually fitted and removed	Kabutol	1997	5 (3 MG)	1000	Before tumor resection (not further specified)	Not examined
integrated in the microscope (OPMI, Zeiss)	Kuroiwa	1998	10 (8 with tentative GTR)	8 mg/kg	upon dural opening	100%
integrated in the microscope (OPMI, Zeiss)	Kuroiwa	1999	30	8 mg/Kg	upon dural opening	83.3%
integrated in the microscope (OPMI, Zeiss)	Kuroiwa	1999	20 (3 with frameless stereotactic system)	8 mg/Kg	before incision of the dura mater	95%
inserted in the operating microscope (OME-9000, Olympus)	Okuda	2012	10 (5 with intended GTR)	20 mg/kg	upon dural opening	100%
YELLOW 560 filter integrated in the microscope (Pentero, Zeiss)	Schebesch	2013	35	3-4mg/kg	after bone flap removal, prior to durotomy	Not examined
YELLOW 560 filter integrated in the microscope (Pentero Zeiss)	Acerbi	2013	20 (ongoing trial)	5mg/kg	after anesthesia induction, before skin incision	75%

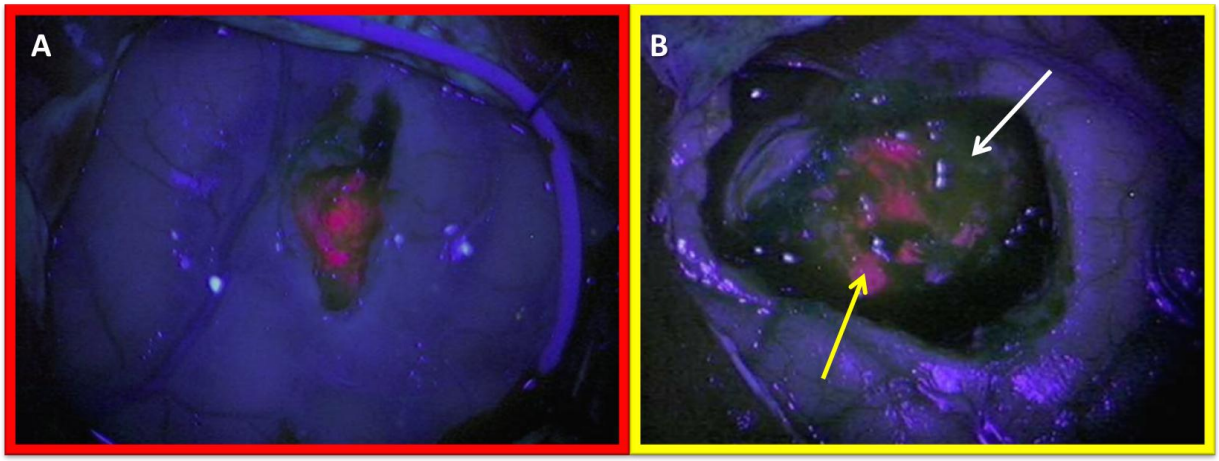
10. FIGURES

Figure 1



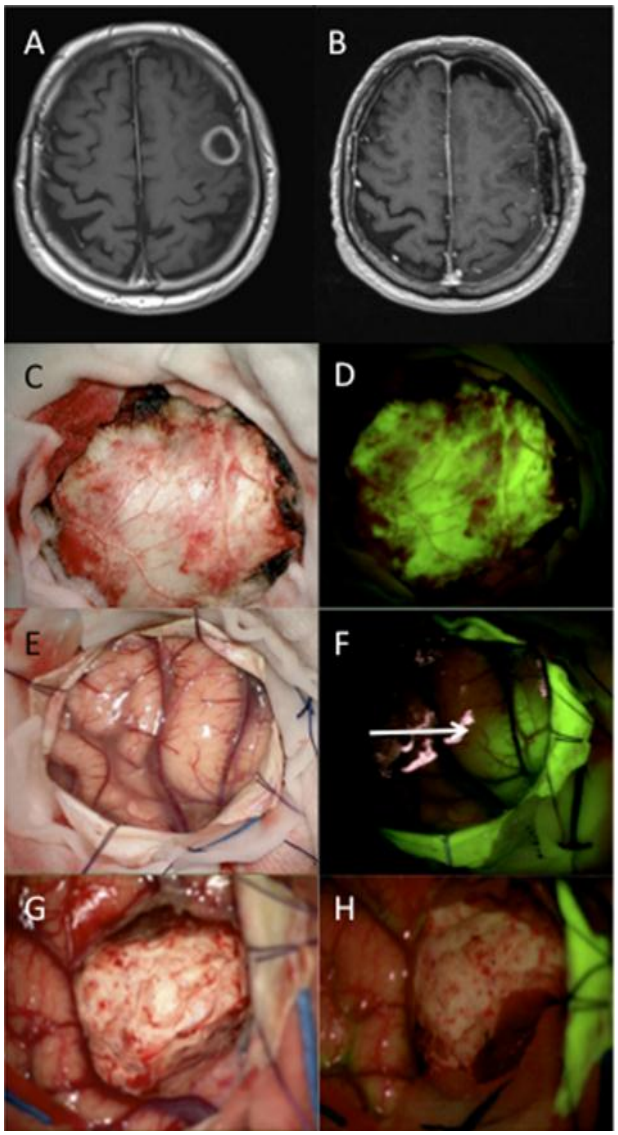
Intraoperative view showing tumor cavity viewed under conventional white light (A) and violet-blue illumination (B). Note the different qualities of tumor fluorescence in the necrotic part (*necrosis*), in the solid very cellular part (*solid*) and at the tumor margins (*vague*). *(From: Stummer W et al, J Neurosurg, 2000, ref no. 78).*

Figure 2



Intraoperative view showing tumor fluorescence with 5-ALA at the beginning of surgical resection (A) and almost at the end of resection (B). Note in B the darker area where resection is completed (*white arrow*) and the fluorescent area where the is still tumor tissue to be removed (yellow *arrow*). *(Adapted from: Li Y et al, Wolrd Neurosurg, 2013, ref no. 45).*

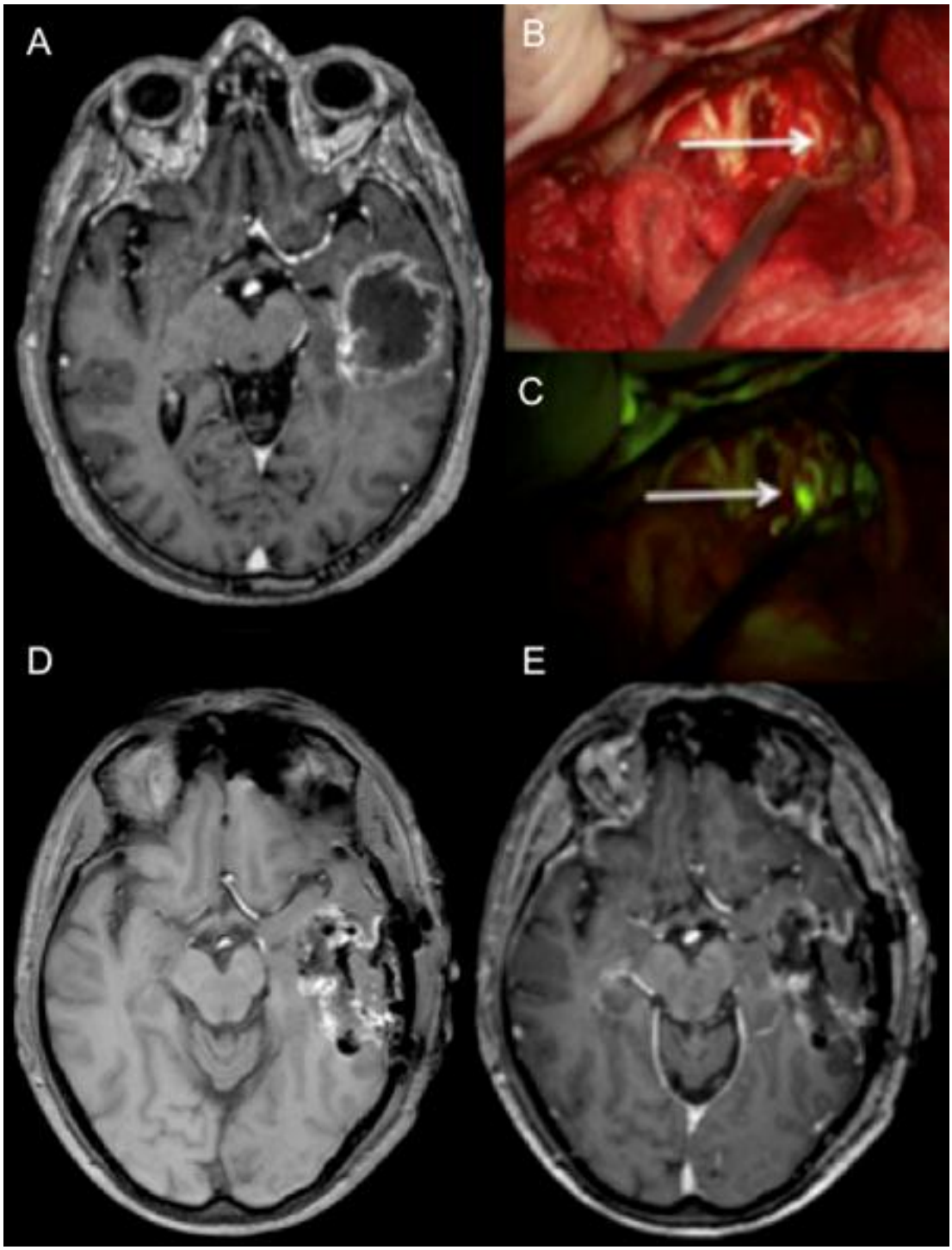
Figure 3



Preoperative (A) and post-operative (B) MRI and intraoperative images (C-H) of a case of left frontal GBM operated on with fluorescein-guided technique by the use of Yellow 560 filter. A) Preoperative axial MR (T1 with gadolinium), showing a left frontal partially cystic tumor, compatible with a diagnosis of MG. B) Post-operative axial MR (T1 with gadolinium), showing the complete resection of the tumor. C) Dural exposure after craniotomy with

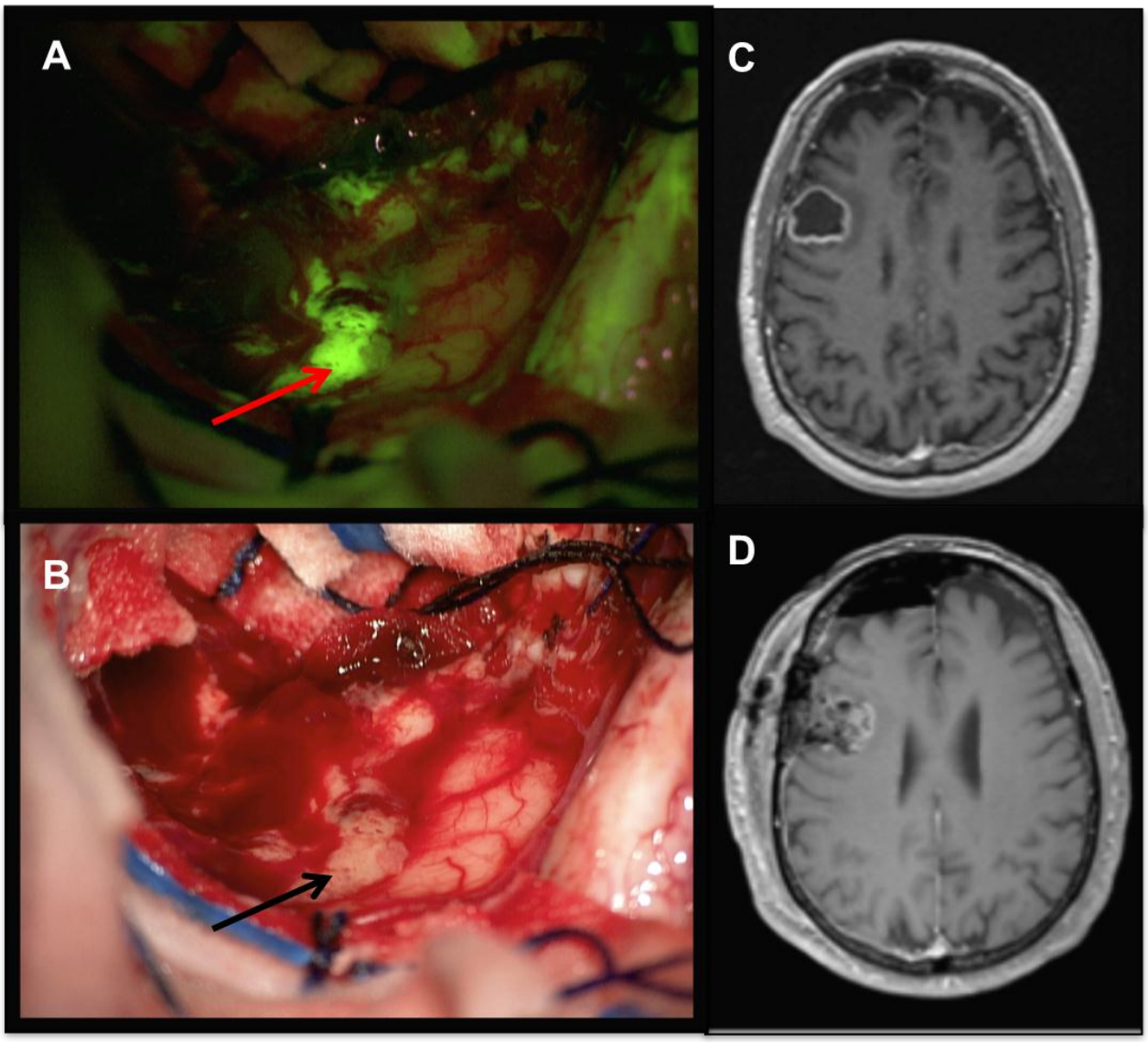
white light illumination. D) Dural exposure after Yellow 560 filter activation, showing the yellow-green discoloration due to the fact the in dural tissue no BBB is present. E) After dural opening, brain parenchyma is exposed, without clear demonstration of the location of the tumor. F) After Yellow 560-filter activation, there is a clear delineation of the tumor area (*white arrow*) with respect to the peritumoral brain. G) At the end of the removal, no residual tumor tissue is evident with white light illumination. H) The absence of residual tumor is confirmed by the activation of Yellow 560, showing no fluorescent areas.

Figure 4



Preoperative (A) and postoperative (D-E) MRI and intraoperative images (B-C) of a case of left temporal GBM operated on with fluorescein-guided technique by the use of Yellow 560 filter. A) Pre-operative axial MR (T1 with gadolinium) showing a huge mass of enhancing tumor, compatible with a diagnosis of MG. B) During removal with ultrasonic aspiration with white light illumination, it was difficult to clearly distinguish tumor tissue (*white arrow*) from peritumoral areas. However, the activation of Yellow 560 filter (C) allowed for the identification of the fluorescent tumor (*white arrow*). D-E) Post-operative axial MR T1 images without (D) gadolinium showed the surgical cavity with hyperintensity due to hemoglobin products, while T1 with gadolinium (E) confirmed the absence of contrast-enhancement, thus showing GTR of the tumor.

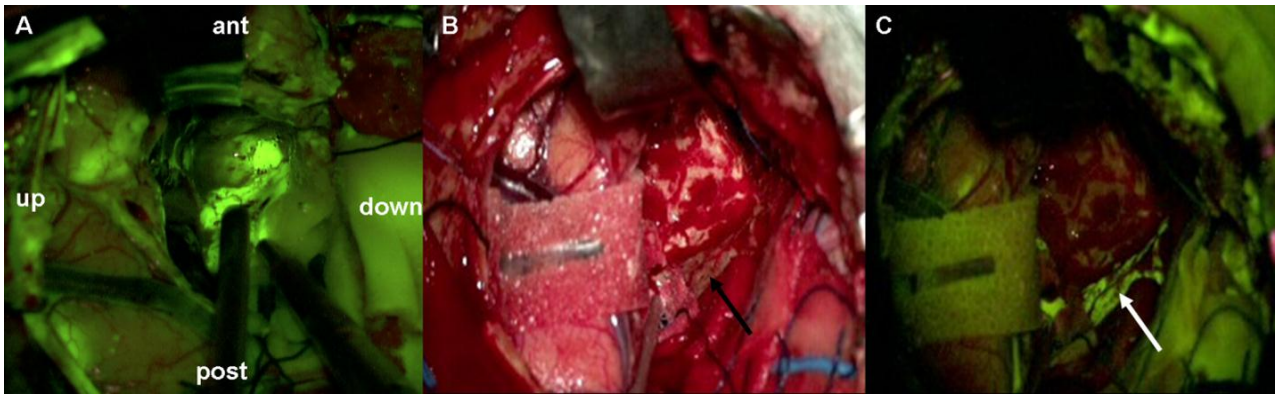
Figure 5



A-B) Intraoperative view during a right frontal GBM resection. With the YELLOW 560 filter activated (A) it is possible to visualize an area of tumor that still needs to be removed (r*ed arrow*); the same picture with white light illumination (B) and the tumoral tissue (*black arrow*). C) Preoperative axial T1-weighted MR with i.v. contrast administration showing a right frontal GBM

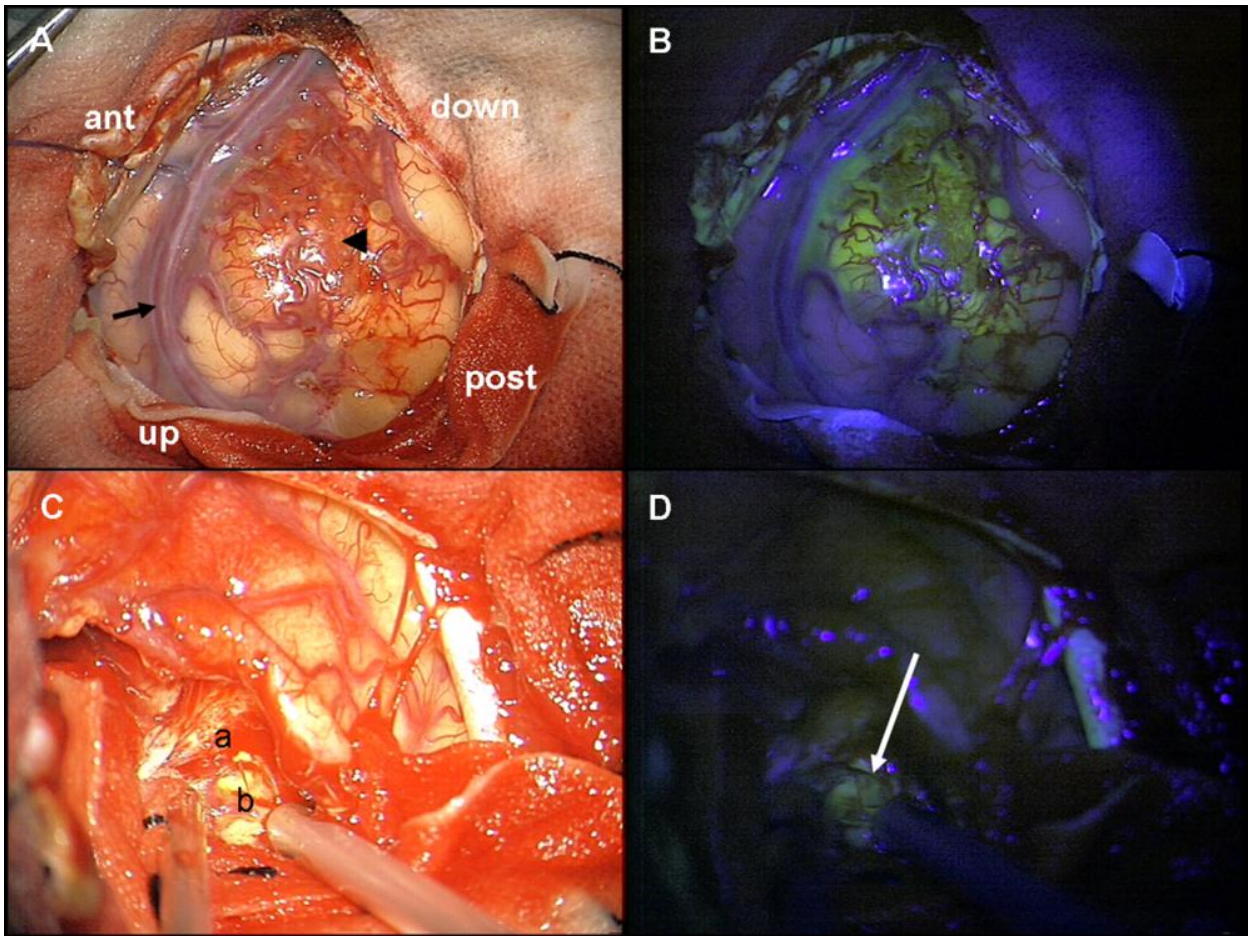
(tumor volume 8.93 cm^3). D) Early (<72 hours) postoperative axial T1-weighted MR with i.v. contrast administration showing a complete tumor resection without any area of contrast enhancement.

Figure 6



Intraoperative pictures of a right frontal GBM, operated on with the YELLOW 560 fluorescence kit. The patient is in supine position with the head turned toward the left; at the left side is the vertex (*up*), while on the right is the sylvian fissure and temporal lobe (*down*). A) The fluorescent area is bright yellowish green, the normal brain parenchyma surrounding the tumor is slightly dark, but with similar colors as with normal white light view. B-C) Intraoperative pictures during tumor removal: at the inferolateral margin of the tumor, the area depicted in B (*black arrow*) appears similar to the surrounding zone; after the YELLOW 560 filter has been activated (C), the same area appears fluorescent (*white arrow*), thus indicating tumor tissue that still needs to be removed.

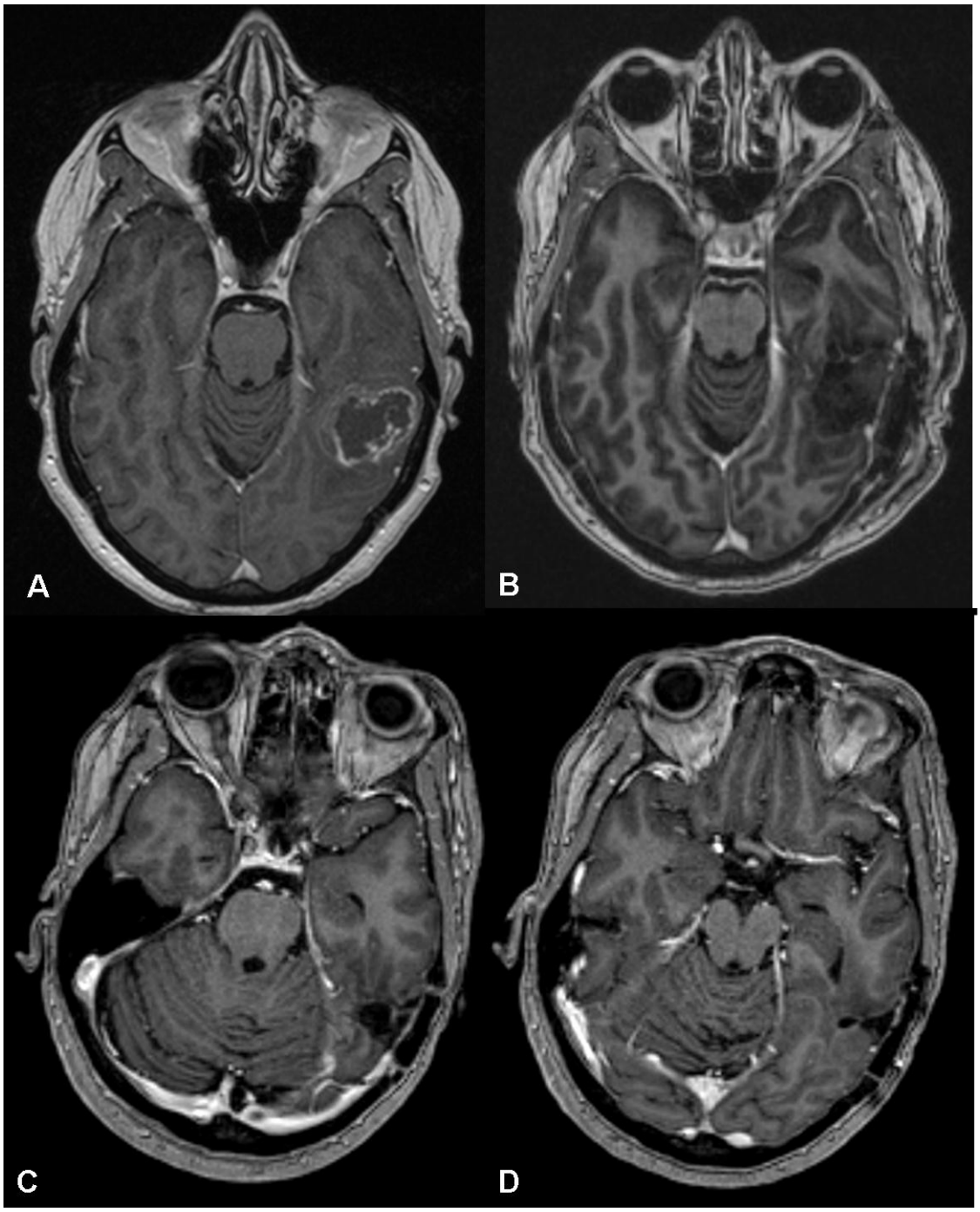
Figure 7



Intraoperative pictures of a right parietal GBM, operated on with the BLU 400 fluorescence kit. The patient is in the left lateral position; at the bottom is the vertex (*up*) and on the left the frontal lobe, anterior to the tumor (*ant*). A) After dural opening in normal white light view. Note the superficial vascularization of the tumor area (*arrowhead*) with arterialized vein on the post-central sulcus (*arrow*). B) The same superficial area as in A, after activation of BLU 400 filter. The tumor area is delineated as a yellowish zone, surrounded by normal blue parenchyma. C) During tumor resection with the two-sucker

aspiration technique, at the posterior-inferior margin of the tumor. All the exposed areas (*a* and *b*) appear similar. D) After activation of the BLU 400 filter, the *b* area is fluorescent (*arrow*), while the *a* area is not.

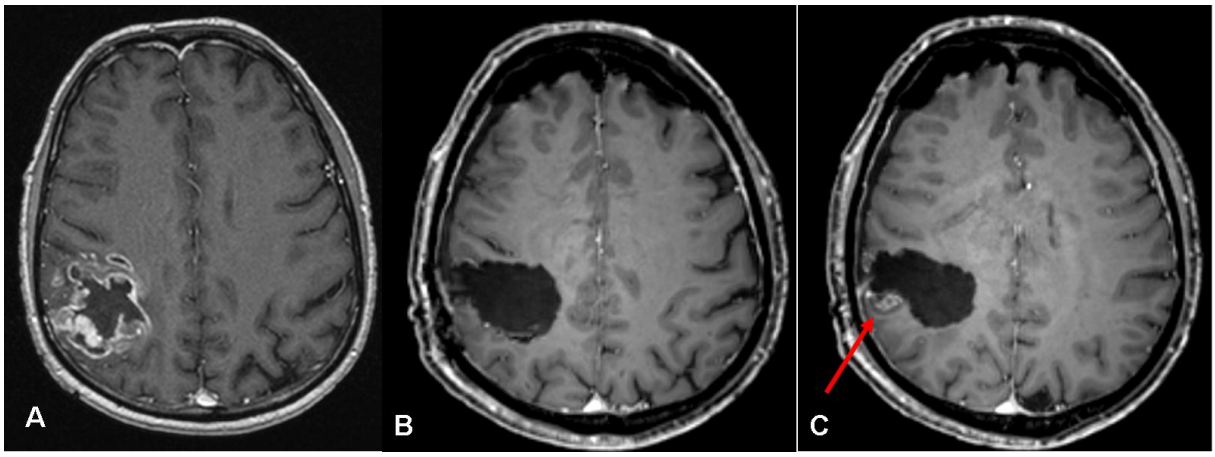
Figure 8



A) Preoperative axial T1-weighted MR with i.v. contrast administration showing a left posterior temporal GBM (tumor volume 12,8 cm^3). B) Early

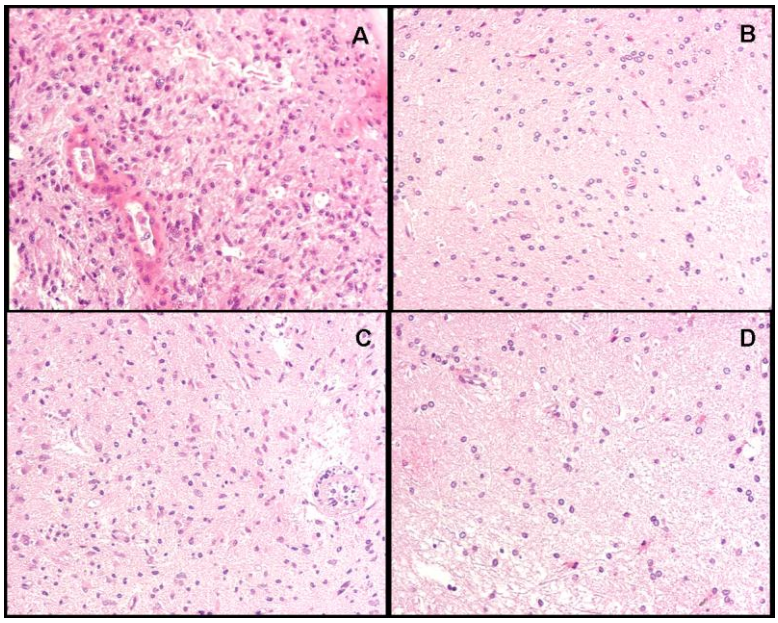
(<48 hours) postoperative axial T1-weighted MR with i.v. contrast administration showing a complete tumor resection without any area of contrast enhancement. C-D) 24 months follow-up axial T1-weighted MR with i.v. contrast administration without any tumor recurrence.

Figure 9



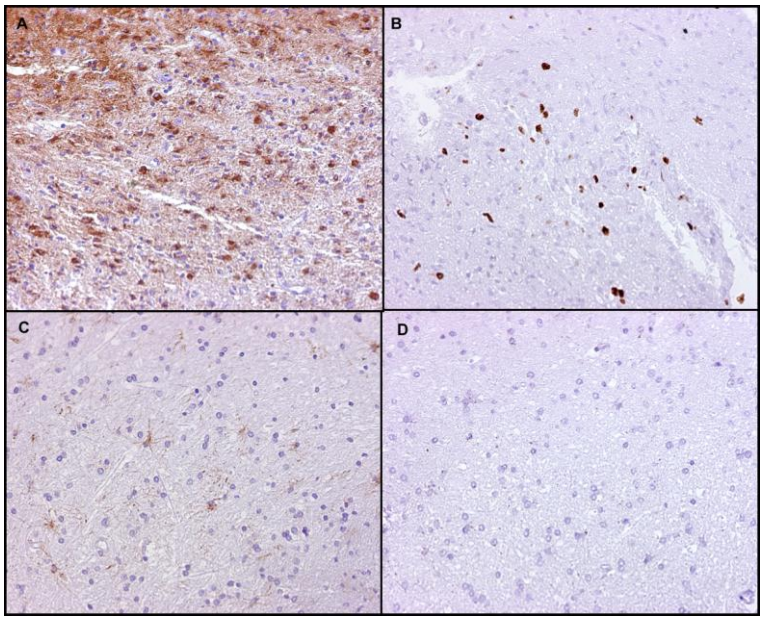
A) Preoperative axial T1-weighted MR with i.v. contrast administration showing a right frontal GBM (tumor volume 38.92 cm^3). B-C) Early (<48 hours) postoperative axial T1-weighted MR with i.v. contrast administration showing a very extensive, but not complete tumor removal (*red arrow* in C); residual tumor volume was 0.28 cm^3.

Figure 10



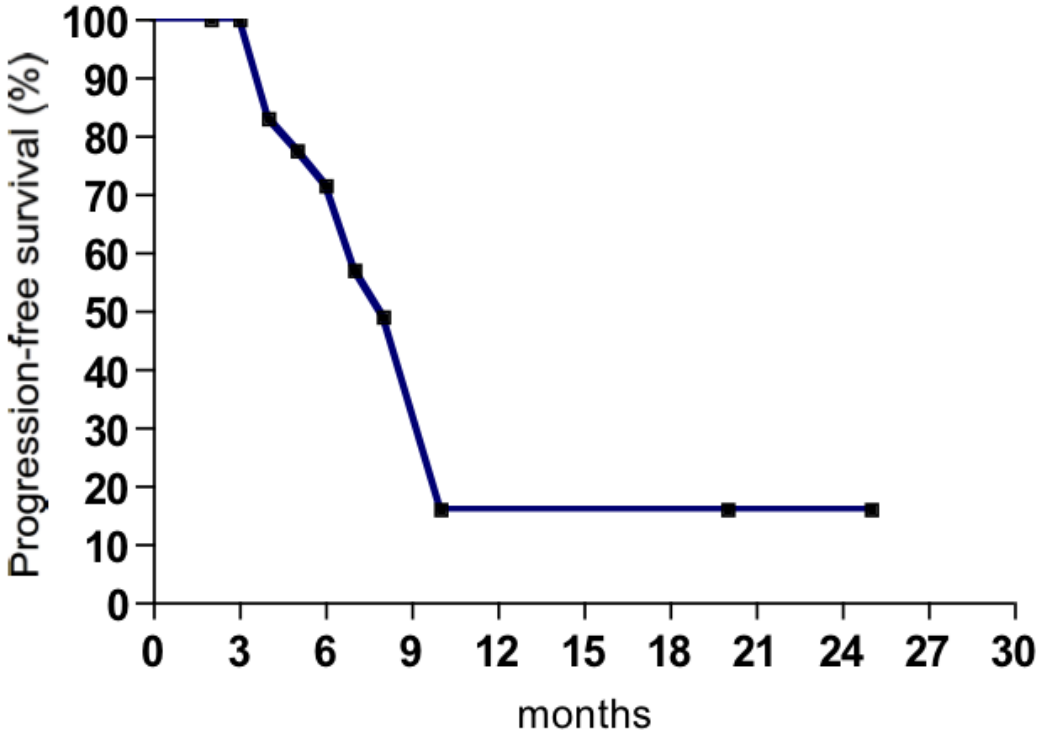
Histological pictures (hematoxylin-eosin) of biopsies at the tumor margin from patient no. 4 (A-B) and no. 11 (C-D). A and C were obtained from biopsies of fluorescent tissue at the tumor margins and showed a sample of true GBM (A) and nervous tissue widely infiltrated by HGG cells (C) (both considered as true positive for the estimation of sensitivity). B and D were obtained from biopsies of non-fluorescent tissue at the tumor margin and showed normal nervous tissue (B) or some gliosis (D) (both considered as true negative for the estimation of specificity).

Figure 11



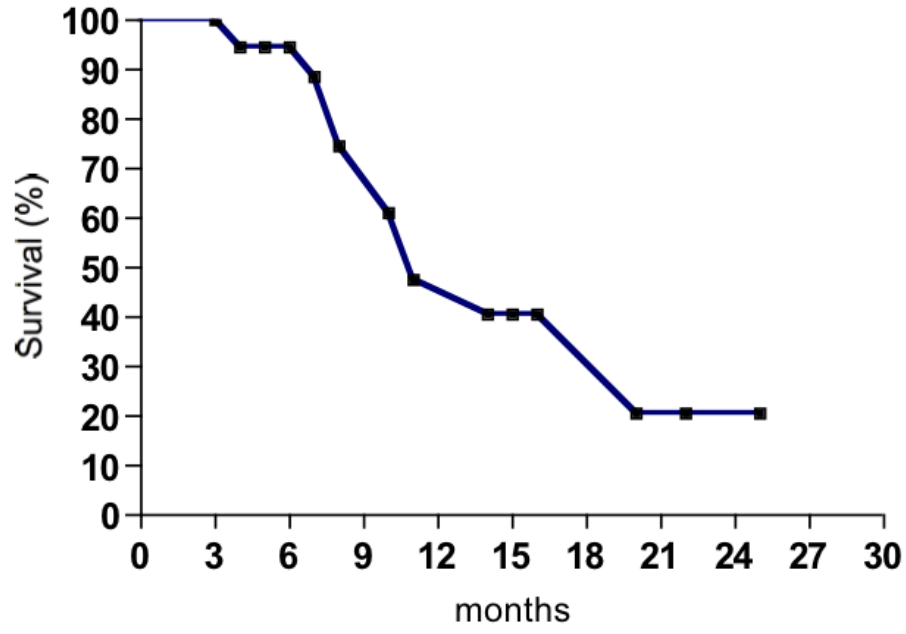
Immunostaining pictures of biopsies at the tumor margins in patient no. 11. A and B were obtained from biopsies of fluorescent tissue and showed GFAP-positive gliomatous cells (A) with high proliferation index by Ki67 (MIB1) immunostaining (B). C and D were obtained from biopsies of non-fluorescent tissue and showed only reactive astrocytes (C) without proliferating nuclei at Ki67 (MIB 1) immunostaining (D).

Figure 12



Kaplan-Meier curve of PFS.

Figure 13



Kaplan-Meier curve of OS.