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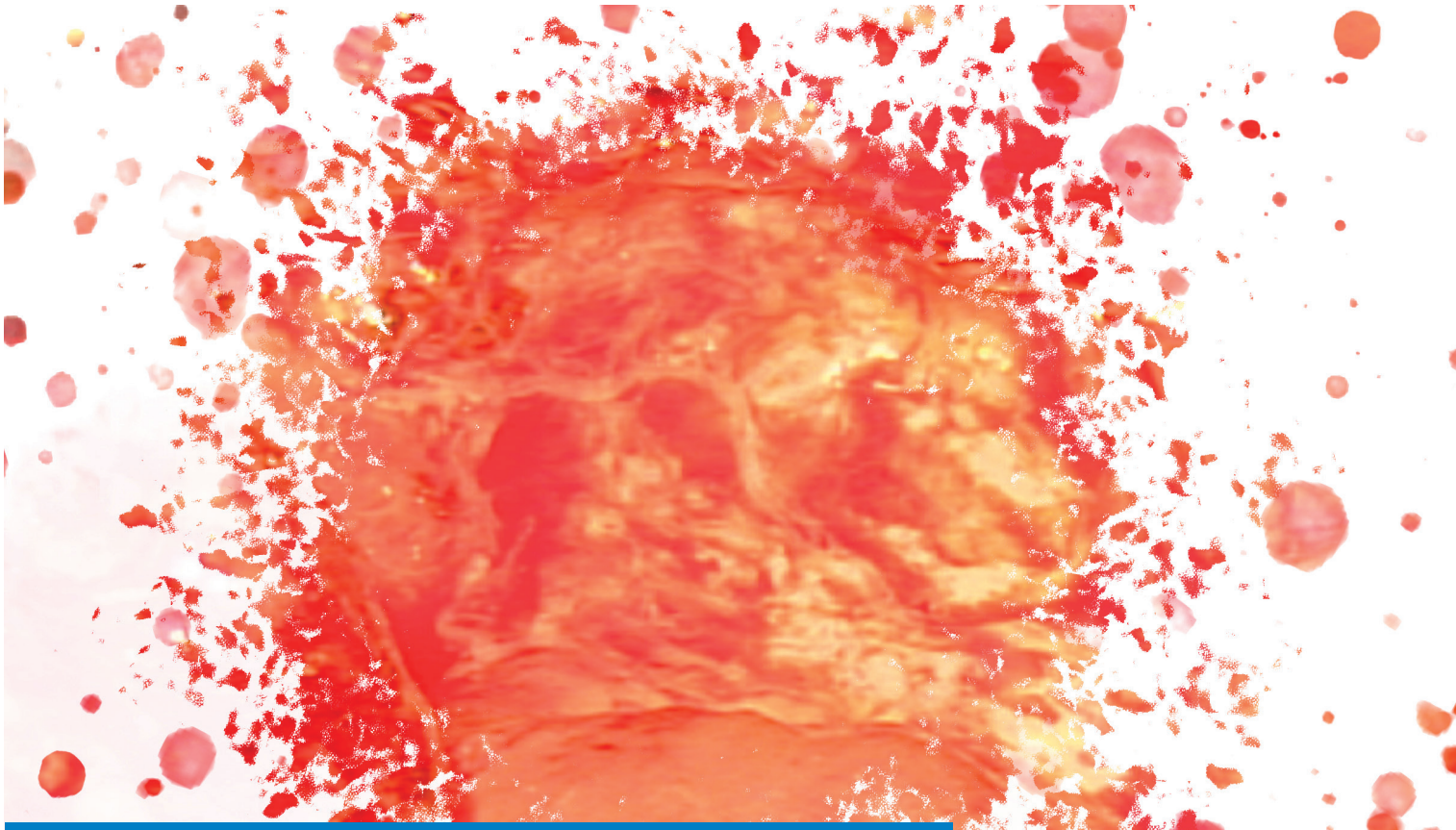

ON GLIOBLASTOMA PATIENT SURVIVAL

SURGICAL, RADIOLOGICAL, RADIOTHERAPEUTIC AND MOLECULAR BIOLOGICAL PROGNOSTIC FACTORS

Giorgio Hallaert
2021

Doctoral Supervisors
J.P. Kalala, MD, PhD
D. Van Roost, MD, PhD
T. Boterberg, MD, PhD

Thesis submitted to fulfill the requirements
for the degree of Doctor in Medical Sciences

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De overleving van patiënten met een glioblastoma

Chirurgische, radiologische, radiotherapeutische en moleculairbiologische prognostische factoren

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Promotoren
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Proefschrift neergelegd om te voldoen aan de vereisten tot het behalen van de titel van Doctor in de Medische Wetenschappen

γλοιός, -ου, ὁ	=	*resin, gum, something sticky*
βλαστάνω	=	*to grow, to sprout, to form*
προγιγνώσκω	=	*to know, perceive, learn or understand beforehand*
πρόγνωσις, -εως, ἡ	=	*foreknowledge*

τὸν ἰητρὸν δοκέει μοι ἄριστον εἶναι πρόνοιαν ἐπιτηδεύειν: **προγιγνώσκων** γὰρ καὶ προλέγων παρὰ τοῖσι νοσέουσι τά τε παρεόντα καὶ τὰ προγεγονότα καὶ τὰ μέλλοντα ἔσεσθαι, ὁκόσα τε παραλείπουσιν οἱ ἀσθενέοντες ἐκδιηγεύμενος, πιστεύοιτ᾽ ἂν μᾶλλον γιγνώσκειν τὰ τῶν νοσεόντων πρήγματα, ὥστε τολμᾶν ἐπιτρέπειν τοὺς ἀνθρώπους σφέας ἑωυτοὺς τῷ ἰητρῷ.

Ἱπποκράτης ὁ Κῷος;

esse quam videri

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ABBREVIATIONS

ACTB	β-actin gene
5-ALA	5-aminolevulinic acid
BTPC(s)	brain tumor propagating cell(s)
CNS	central nervous system
CpG	*5'-cytosine-phosphate-guanine-3'*
CTV	clinical target volume
Cq	quantification cycle
D2HG	D-2-hydroxyglutarate
DNA	desoxyribonucleic acid
EOR	extent of resection
FDG	fluoro-deoxy-glucose
FET	fluoro-ethyl-tyrosine
FFPE	formalin-fixed paraffine-embedded
FISH	fluorescence in-situ hybridization
G-CIMP	glioma CpG island methylation phenotype
GBM	glioblastoma
GRADE	Grading of Recommendations Assessment, Development, and Evaluation
GTR	gross total resection
Gy	Gray
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
KPS	Karnofsky Performance Scale
MGMT	O^6-methylguanine-DNA-methyltransferase
*MGMT*p	O^6-methylguanine-DNA-methyltransferase gene promoter
MPRAGE	magnetization prepared rapid gradient echo
MRI	magnetic resonance imaging
NSC(s)	neural stem cell(s)
PCR	polymerase chain reaction
PET	positron emission tomography
PR	partial resection
PTV	planning target volume
qMSP	quantitative methylation specific polymerase chain reaction
RTV	residual contrast-enhancing tumor volume
SuTR	supratotal resection
SVZ	subventricular zone
SVZ_{neg}	not contacting the subventricular zone
SVZ_{pos}	contacting the subventricular zone
WHO	world health organization
WT	wild type
YPLL	years of potential life lost

INTRODUCTION

Hippocrates of Kos emphasized already 2500 years ago the importance of prognosis in medicine. The term 'prognostic awareness' denotes the awareness of the patient (or the caregiver) of incurable disease and shortened life expectancy (1). Higher prognostic awareness in patients with advanced cancer may enhance quality of life, guide end-of-life discussions and reduce needless aggressive treatment near death (2,3). Patients are entitled to receive objective information based on scientifically supported data. However, evidence shows that patients suffering from high-grade glioma or glioblastoma often have low prognostic awareness and inaccurate perception of prognosis (3,4). High-grade glioma patients seem to talk infrequently about their prognosis with their physicians (4). At the same time, patients indicate that they want to receive prognostic information early on in their disease (1). These factors and data are also of paramount importance because they form the basis of the shared decision-making process which allows the glioblastoma patient to take part in vital decisions, e.g., whether to engage in neurosurgical resection or rather opt for biopsy only.

Glioblastoma patient survival has been and, unfortunately, remains dismal although prognosis has substantially improved over years, especially since the introduction of temozolomide (5,6). Age and patient performance at diagnosis have been recognized for decades as important prognostic factors. Surgery and radiotherapy were early pillars of treatment. Other prognostic factors remained elusive for a very long time. However, in recent years new insights emerged from progress in medical imaging, in cytoarchitectonic studies of the adult human brain and especially in glioblastoma genetics.

This thesis will elaborate on certain surgical, radiotherapeutic, radiological and molecular biological prognostic factors concerning glioblastoma patient survival.

Glioblastoma Classification and the Isocitrate Dehydrogenase Mutation

Virchow introduced the term 'glioma' in the 19th century, but the first scientific classification of gliomas was published in 1926 by Bailey and Cushing in their authoritative work *A Classification of the Tumors of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis* (7). Bailey and Cushing also coined the term *glioblastoma*[1] and elaborated on the high malignancy and dismal prognosis associated with this tumor. Fourteen years later, Scherer, working at the Bunge Institute in Antwerp, wrote about the distinction between primary and secondary glioblastomas (8). Since its first publication in 1979, the *World Health Organization (WHO) Classification of Tumours of the Central Nervous System* is the globally accepted brain tumor classification system. Until recently, this classification was based primarily on histological features. The current revised fourth edition of the WHO classification, published in 2016, introduces a combined histopathological-molecular classification with a central role for isocitrate dehydrogenase (*IDH*) mutation and the 1p/19q

[1] Before, this tumor was known as *medullary sarcoma* in the English-speaking medical community, sometimes also as *spongioblastoma*, and as *fungus medullare* in German medical literature.

co-deletion in the grouping of gliomas (9). Seventy-six years after the pioneering work of Scherer, it was established that secondary glioblastomas typically harbor an *IDH* mutation while primary glioblastomas belong to the *IDH*-wildtype (*IDHwt*) group. According to the redrawn family tree of gliomas in the 4th revised WHO edition (Fig. 1), glioblastoma is now part of the large group of diffuse astrocytic and oligodendroglial tumors and is the most malignant glioma (formerly WHO grade IV). Three types of glioblastoma are described: glioblastoma, *IDH*-mutated; glioblastoma, *IDH*-wildtype; and glioblastoma, not otherwise specified (NOS). The differences between *IDH*-mutated and *IDH*wt glioblastoma are summarized in table 1.

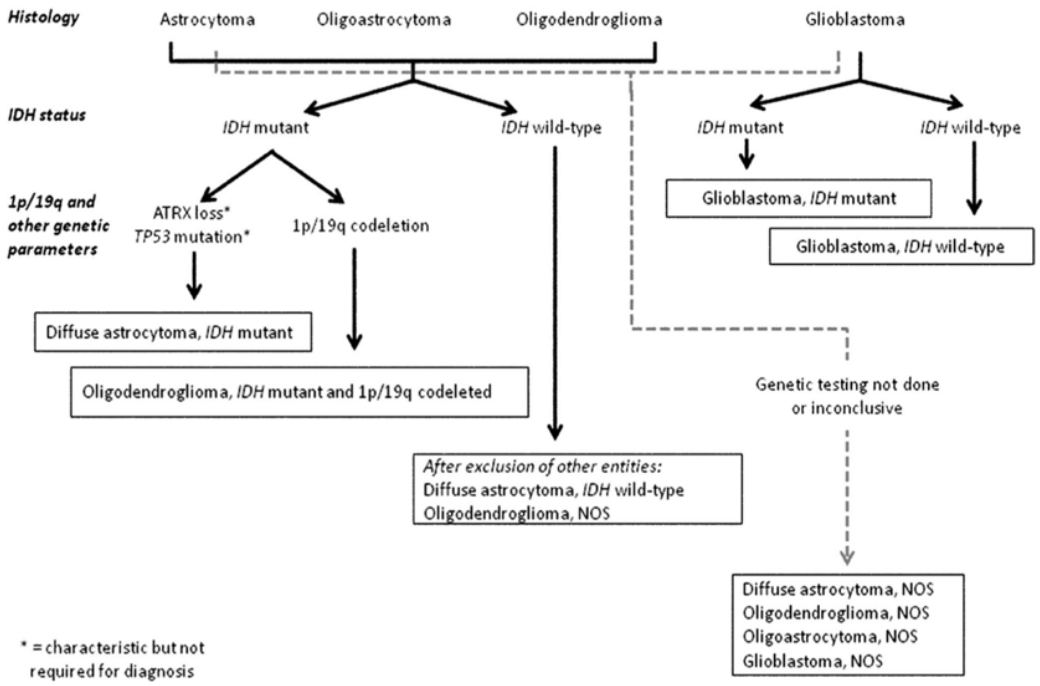


Figure 1. Simplified algorithm for the classification of the diffuse gliomas, based on both histological as well as genetic features; 4th revised WHO classification of tumors of the central nervous system (9).

Table 1. Key characteristics of *IDH*-mutated and *IDH*-wildtype glioblastoma (9).

	***IDH*-wildtype glioblastoma**	***IDH*-mutated glioblastoma**
Synonym	Primary glioblastoma	Secondary glioblastoma
Precursor lesion	Unknown	Diffuse astrocytoma; anaplastic astrocytoma
Proportion of glioblastomas	**~ 90%**	**~ 10%**
Median age at diagnosis	**~ 62 years**	**~ 44 years**
Male-to-female ratio	1.5 : 1	1.1 : 1
Median overall survival		
Surgery & radiotherapy	9.9 months	15 months
Surgery & chemoradiotherapy	**15 months**	**31 months**
Location	Supratentorial	**Preferentially frontal**
Necrosis	**Extensive**	**Limited**
Telomerase Reverse Transcriptase *(TERT)* gene mutations	72%	26%
Tumor Protein *(TP) 53* gene mutations	27%	81%
Alpha thalassemia/mental retardation syndrome X-linked *(ATRX)* gene mutations	Exceptional	71%
Epidermal Growth Factor Receptor *(EGFR)* amplifications	35%	Exceptional
Phosphatase and Tensin Homolog *(PTEN)* gene mutations	24%	Exceptional

Because of the pivotal importance of *IDH* mutations for gliomas and glioblastomas, both for classifying purposes but as well as a prognostic parameter, some basic aspects of this mutation will be highlighted. The role of *IDH* mutations in gliomas was first described in 2008 and 2009 (10,11). IDH is an essential metabolic enzyme of the citric acid cycle (Krebs cycle), involved in the oxidative decarboxylation of isocitrate to alpha-ketoglutarate. Three enzymes exist: IDH1, IDH2 and IDH3; IDH3 is a distinct enzyme from IDH1 and 2, despite the similar name giving, and will not be discussed further since it has no role in gliomas. IDH1 is found in the cell's cytoplasm, while IDH2 resides in the mitochondria. IDH1 and 2 are homodimers which generate nicotinamide adenine dinucleotide phosphate (NADPH) using $NADP^+$ as a cofactor. In fact, IDH1 is the largest source of NADPH in the human brain (12). IDH1 and IDH2 take part in several important cellular functions, such as glutamine metabolism, lipogenesis and the regulation of the cellular redox status (13). The *IDH*1^{R132H}-mutation (CGT → CAT) is present in about 90% of cases of *IDH*-mutated gliomas; in 2.5% the *IDH2*^{R172}-mutation is found (11). The enzymatic activity of mutated IDH is significantly reduced and results in accumulation of D-2-hydroxyglutarate (D2HG). The exact role of D2HG as an oncometabolite

remains unknown but evidence suggests (Fig. 2) that *IDH* mutations and high levels of D2HG lead to DNA alterations, histone methylation and blockage of normal differentiation processes (13). *IDH* mutations are considered early events in glioma genesis leading to a genetically more homogenous group of tumors as opposed to *IDH*wt gliomas (14). In glioma, the *IDH* mutation is strongly associated with a so-called *glioma CpG island methylation phenotype (G-CIMP)*, resulting in DNA hypermethylation (15). The methylation of the O^6-methylguanine-DNA-methyltransferase gene promoter (*MGMT*p) is a typical example of the G-CIMP hypermethylation profile (13). It has been shown experimentally that introducing the *IDH*1 mutation into human astrocytes induces vast DNA hypermethylation (16). However, the vast majority of newly diagnosed glioblastomas will not bear the *IDH* mutation (Table 1).

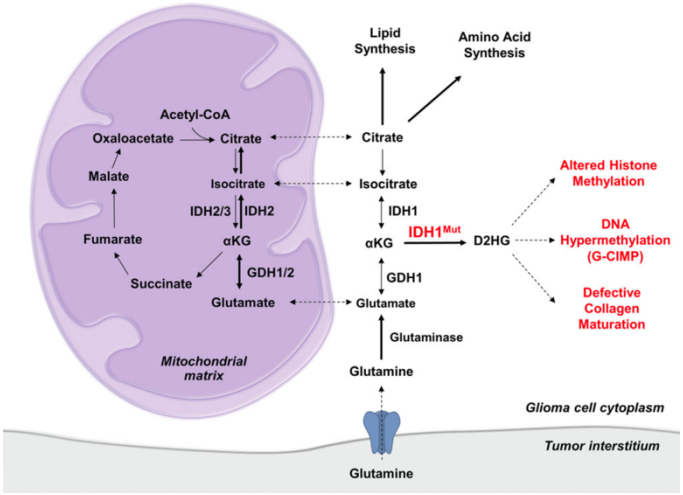


Figure 2. Schematic overview of altered metabolism in IDH1-mutated gliomas (13).

The *IDH*1^{R132H} mutation can be detected by immunohistochemical (IHC) staining with an *IDH*1^{R132H} antibody, which is a cheap and widely available technique. However, DNA-PCR based techniques have higher sensitivity and are also able to detect rarer *IDH* mutations. Unfortunately, these techniques come with higher financial costs.
In the patient cohort studied in this thesis, the *IDH* mutation was determined using DNA sequencing on stored tumor tissue. The focus of this thesis is on the IDHwt group because the overwhelming majority of glioblastoma patients studied here belongs to this subgroup.

Glioblastoma Incidence and Demographic Characteristics

Recent data from the United States of America show that glioblastoma accounts for 14.6% of a five-year total of 405,740 patients suffering from primary brain and other central nervous system (CNS) tumors and for 48.3% (Fig. 3) of primary malignant brain tumors (17). On average 90% of newly diagnosed glioblastoma patients suffer from *IDH*wt tumors. Reported age-adjusted incidence rates of glioblastoma are variable and range from 0.59 (Korea) to 3.69 (Greece) per 100,000 person-years (18). Of note, in the age group of 70 years or older, glioblastoma accounts for an incidence of 17.5 per 100,000 person-years (2).

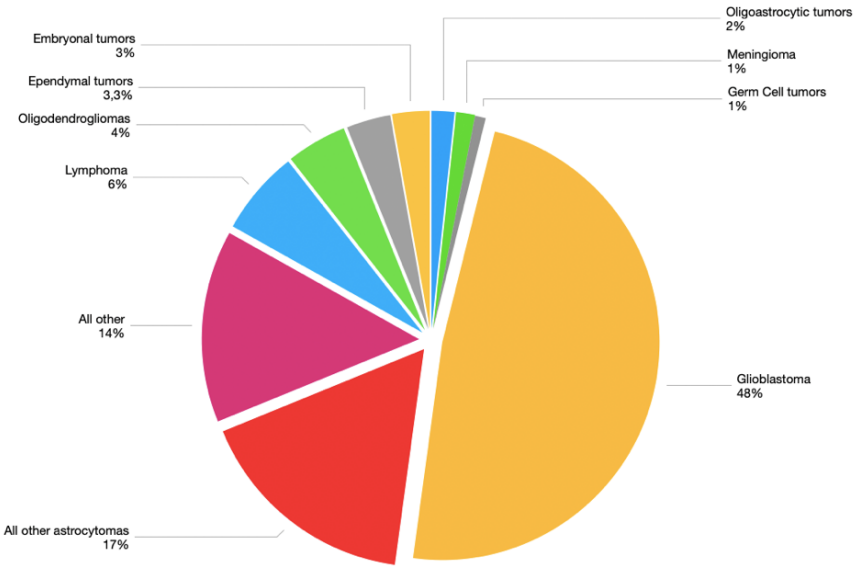


Figure 3. Distribution of primary malignant brain and other central nervous system tumors (2).

According to the Belgian Cancer Registry[2], 573 patients were diagnosed with glioblastoma in the year 2018 for a population of 11.4 million. In comparison to the incidence of prostate cancer (172/100,000/year) and female breast cancer (185/100,000/year) in Belgium[3], glioblastoma (5/100,000/year) is luckily a rare disease.

In the adult population, the incidence of glioblastoma raises steadily with age, impacting the economically active part of the population (2). The median age at diagnosis for glioblastoma patients is 65, but only 44 years for *IDH*-mutated glioblastoma patients. Years of Potential Life Lost (YPLL) is an important parameter, reflecting the economic and social repercussions of premature death. YPLL measures the average time a person would have lived if she or he would not have died prematurely due to the disease. Average YPLL for glioblastoma is 20 years, the highest YPLL across all cancers (19).

Men are more likely to suffer from glioblastoma than women (M:F = 1.5:1) (17). In Belgium, there were 351 newly diagnosed male glioblastoma patients in 2018, compared to 222 female patients (M:F ratio of 1.6:1)[2].

Demographic data from the USA show that the incidence of glioblastoma is significantly lower amongst African Americans as compared to non-Hispanic whites (20). A valid explanation for this observation is currently lacking.

[2] Personal communication from the Belgian Cancer Registry with the author on September 24, 2020; unpublished data. The website of the Registry shows only bulk data of malignant CNS tumors.
[3] https://www.gezondheidbelgie.be/nl/gezondheidstoestand/niet-overdraagbare-aandoeningen/kanker

Symptoms, Imaging and Diagnosis

There is no clinical presentation that is specific of glioblastoma. The presenting signs and symptoms depend on the location and size of the tumor but also on the extent of brain edema. Typically, the onset of neurologic symptoms follows a subacute course. The most common symptoms include headache, seizures, and focal neurologic symptoms (e.g. language deficit; motor weakness, memory loss, visual disturbances, personality changes) (21).

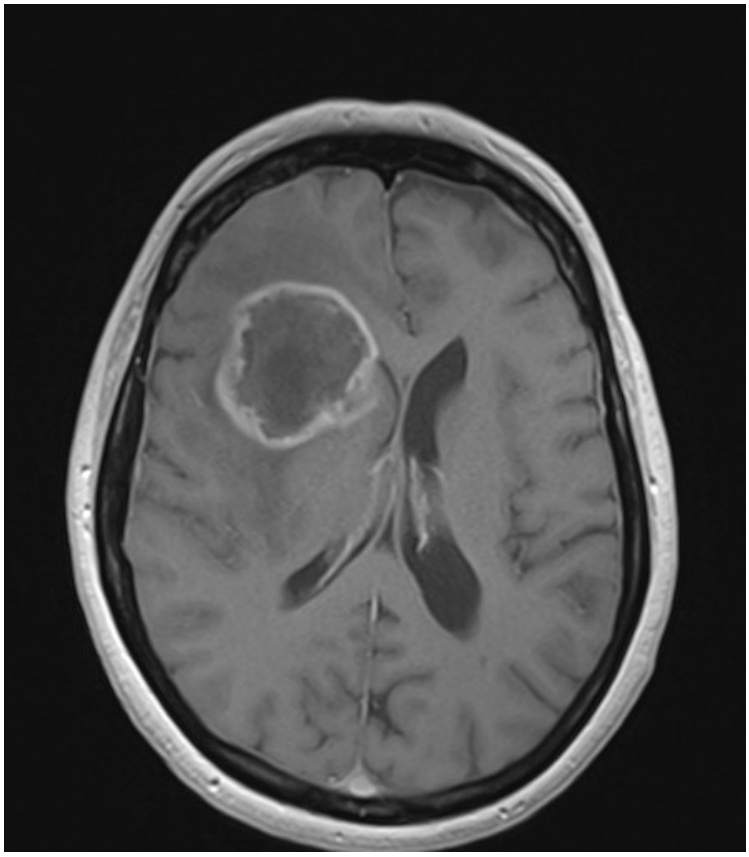


Figure 4. A representative post-gadolinium axial T1-weighted MR image of an IDH-wildtype glioblastoma with subventricular zone contact and a large necrotic core region.

Once the suspicion of a brain tumor, possibly a glioblastoma, has been raised, magnetic resonance (MR) imaging of the brain is the preferred imaging modality of the brain. On T1 weighted images, glioblastoma is a hypointense lesion. An *IDH*wt glioblastoma on T1 post-gadolinium imaging is typically an intense ring-enhanced lesion, with a central hypointense area (suggestive of necrosis) and surrounding oedema (Fig. 4). Non-enhancing glioblastoma is rare but possible. *IDH*-mutated glioblastoma shows little if any central necrosis and has a predilection for the frontal lobes (22). It was already shown in 1987 by Kelly et al. that tumor infiltration extends at least into the T2 and FLAIR hyperintense zone, beyond the boundaries of contrast enhancement (23). MR spectroscopy of glioblastoma shows a typical choline peak and a decreased level of N-acetyl aspartate. Glioblastoma has highly increased metabolic rates, like most malignancies. Most glioblastomas show increased uptake of fluoro-deoxy-glucose (FDG) and also of fluoro-ethyl-tyrosine (FET). Therefore, metabolic imaging in glioblastoma may be useful not only for diagnosis, but also for selecting the appropriate target for stereotactic biopsy and for treatment follow-up (24,25).

Definitive diagnosis of glioblastoma is based on histopathological diagnosis. A tumor tissue sample can be obtained using stereotactic biopsy, but in most cases a (partial) resection of the tumor will be performed. Either way, a sufficient amount of tissue should be obtained in order to allow both histological and molecular biological examination. As already mentioned, the 4th revised 2016 edition of the WHO classification necessitates both histopathological

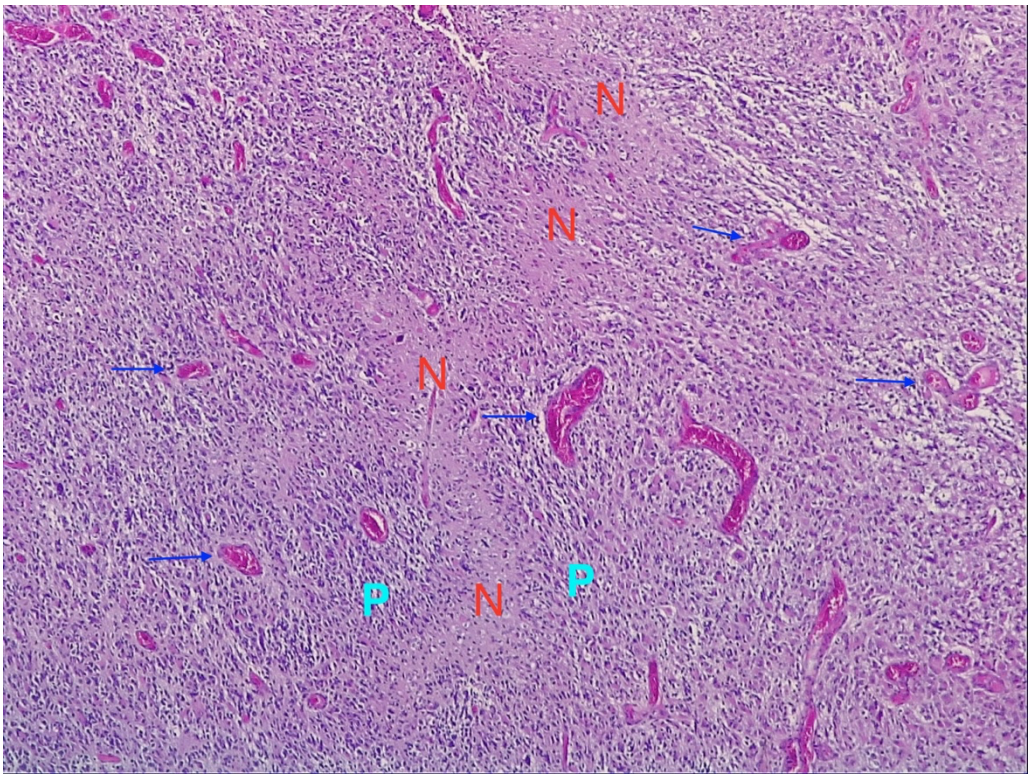


Figure 5. Microscopic images of *IDH*wt glioblastoma. A. A representative histological image of glioblastoma showing a highly cellular tumor, zones of necrosis (N) surrounded by dense accumulation of tumor cells, together forming palisading necrosis (P) and microvascular proliferation (blue arrows).

study and molecular testing (*IDH* mutation and in selected cases 1p/19q co-deletion) to establish a diagnosis of glioblastoma. Briefly, microscopy of typical glioblastoma shows a highly cellular glioma (Fig. 5). The tumor cells are poorly differentiated, sometimes pleomorphic. They have nuclear atypia and high mitotic activity. Diagnostic characteristics that separate glioblastoma from high-grade glioma are the presence of prominent microvascular proliferation and/or necrosis (9). Necrosis often occurs in the form of palisading necrosis, formerly known as 'pseudo-palisading' (26). Vascular proliferation is frequently associated with thrombosis, which may become apparent to the neurosurgeon during resection as the presence of so-called "black veins" (Fig. 6). If these typical histopathological characteristics are present, IHC for the IDH^{R132H} mutation should be performed. In patients younger than 54 years, sequencing to search for *IDH* mutation is preferred to IHC (9). Presently, other molecular tests (*TERT*; *TP53*; …) are not necessary for diagnosis of glioblastoma. The presence of pathological cells with rounded nuclei, frequently with perinuclear halos ("fried egg appearance") and a "chicken-wire aspect" of branching capillaries should rise suspicion of anaplastic oligodendroglioma and necessitates molecular testing (IHC for *IDH* mutation and FISH for 1p/19q co-deletion, or sequencing techniques).

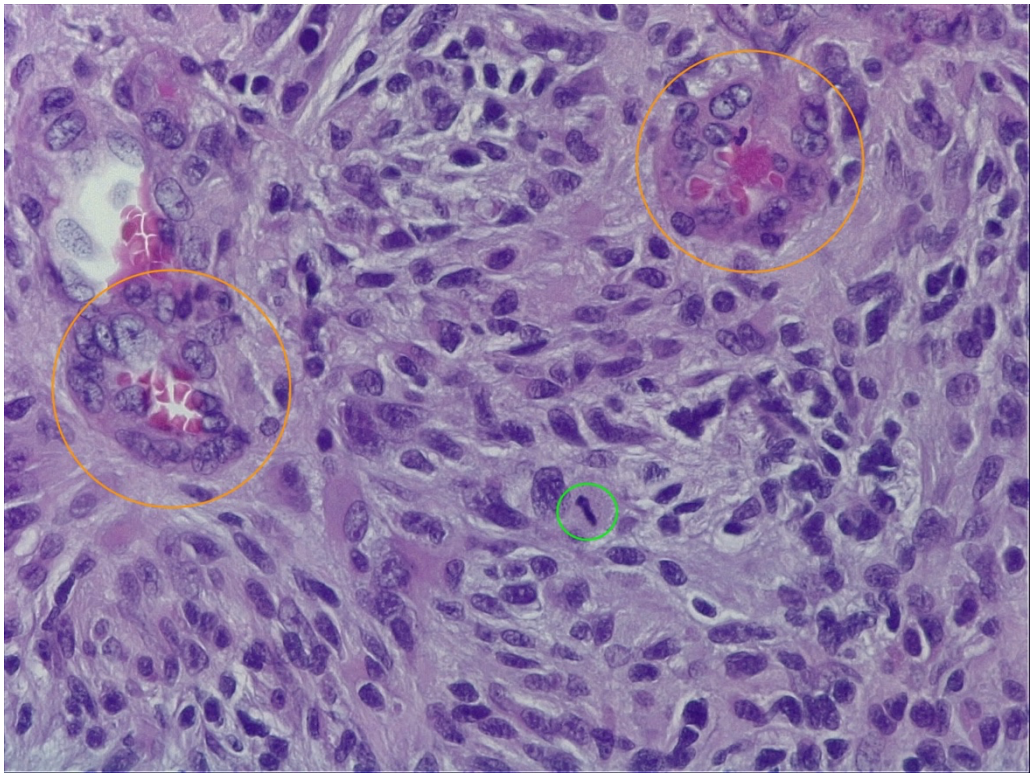


Figure 5. B. Representative histological image of glioblastoma showing cells with pleomorphic features and nuclear atypia, a mitosis (green circle) and glomeruloid appearance of microvascular proliferation (orange circles).

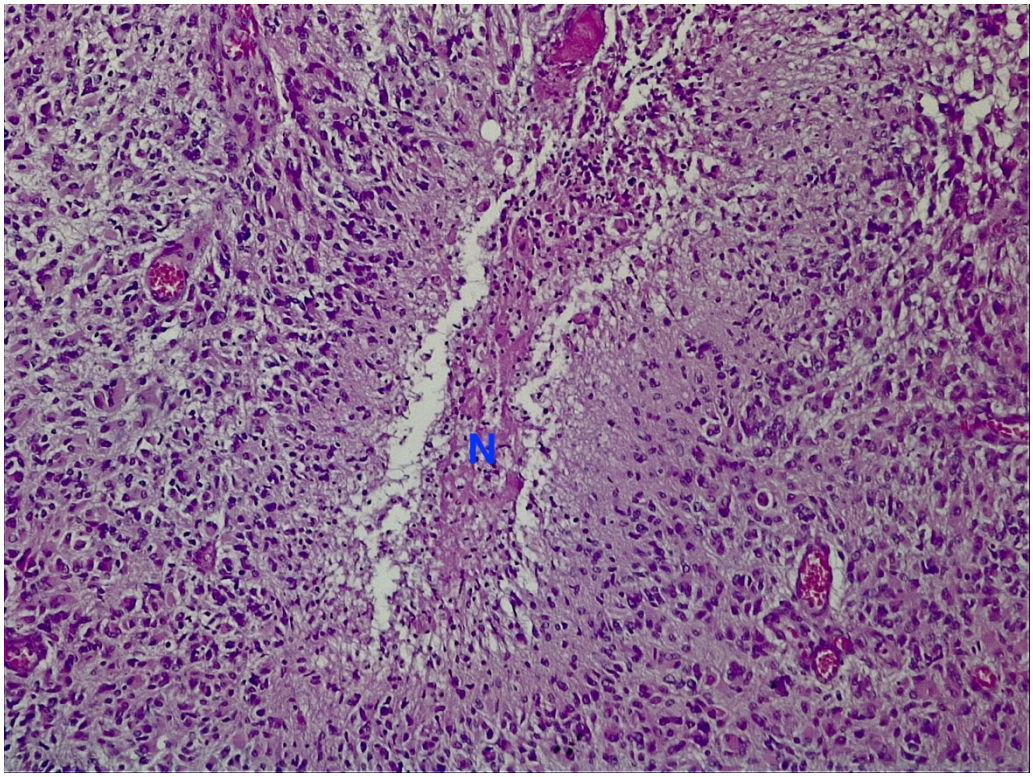


Figure 5. C. Representative histological image of glioblastoma showing palisading necrosis, characterized by necrosis (N) surrounded by dense accumulation of tumor cells.

Current Glioblastoma Treatment

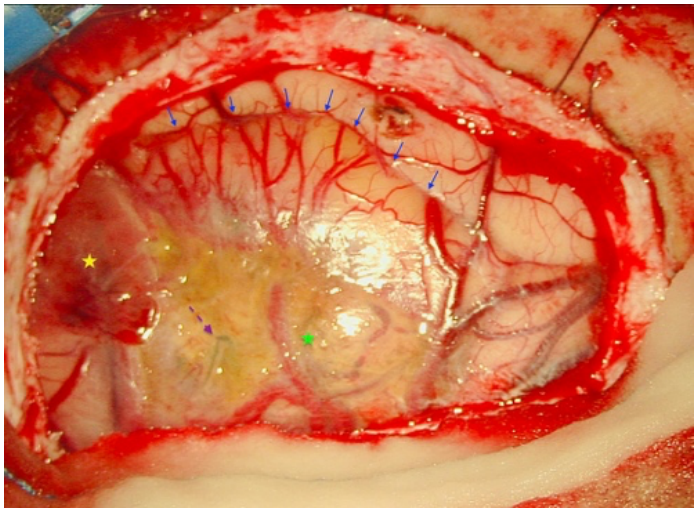


Figure 6 A. Operative image showing an IDHwt glioblastoma reaching the cortical surface of the brain. Blue arrows depict a sulcal border; yellow star shows an exophytic growth pattern; green star denotes superficial necrosis with a small "black vein" (dotted purple arrow).

The current standard-of-care of glioblastoma patient treatment is maximum safe resection followed by temozolomide-based chemoradiotherapy, the so-called Stupp protocol (5). Neurosurgical intervention for glioblastoma has several goals: removal of malignant tissue (also called 'debulking' or 'cytoreduction'), relieving mass effect, and acquiring tissue for histological diagnosis and molecular biological study. If safely possible, gross total resection of the contrast-enhancing part of the tumor should be achieved. Extent of resection is evaluated on MR imaging obtained within 48 hours after surgery, although methodological issues concerning tumor measurement remain unresolved (27-29). Large retrospective studies show that gross total resection results in better patient survival compared to biopsy only (30). There is no consensus at present concerning the effect of partial resection on glioblastoma patient survival. After surgery, external beam radiotherapy is applied. A 1-2 cm margin surrounding the clinical target volume, often including the FLAIR hyperintense zone, is irradiated to a total dose of 60 Gy delivered in daily fractions of 2 Gy each weekday. Temozolomide, an alkylating agent, is administered during radiotherapy (75 mg/m^2/day) and continued for six cycles after completion of the radiation therapy (first cycle 150 mg/m^2/day and escalated to 200 mg/m^2/day thereafter if tolerated). The application of the Stupp regimen adds a median overall survival benefit of 2.1 months, compared to radiotherapy alone (5). Despite this aggressive treatment, *IDH*-wildtype glioblastoma patients have a median overall survival of only 14.6 months from diagnosis; patients with *IDH*-mutant glioblastoma of 31 months. All the patients of the cohort studied in this thesis were treated with temozolomide-based chemoradiotherapy after surgery.

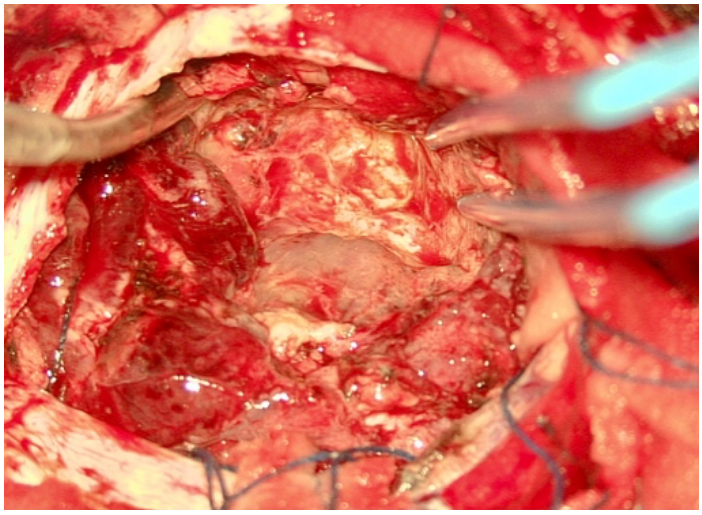


Figure 6 B. Operative image showing the neurosurgical microscopic view of the glioblastoma tumor core.

A new treatment modality for glioblastoma is alternating electric field therapy. Briefly, low intensity alternating electric fields (of intermediate frequency of 220kHz) are delivered to the brain via transducer arrays attached to the bald scalp. It has been shown that these fields can cause mitotic cell arrest and can also induce apoptosis, especially of rapidly dividing cells (31). According to the final analysis of a large prospective study published in 2017, adding *tumor-treating fields*® to the Stupp regimen prolonged median overall survival of glioblastoma patients with 4.9 months (32). Nevertheless, this treatment is at present not routinely applied in Belgium and in most of the industrialized countries. The cost of approximately 21.000 USD per patient per month may be an important factor to explain the delay for the routine implementation of this therapy, next to other, more practical, obstacles such as the necessity to apply the device 18 hours per day. None of the patients studied in this thesis were treated with tumor-treating fields®.

Neural Stem Cells, the Subventricular Zone and Glioblastoma Origin

Since 2001, the "Cancer Stem Cell Hypothesis" is the dominant theoretical framework in oncology (33). This theory implies a cellular hierarchy within oncological diseases, with the rarely occurring "Cancer Stem Cells" (CSCs) at the top of the pyramid. The CSCs rarely divide but give rise to precursor cells which, by rapid division, create high numbers of differentiated tumor cells; these in turn build up the bulk of the tumor (Fig. 7).

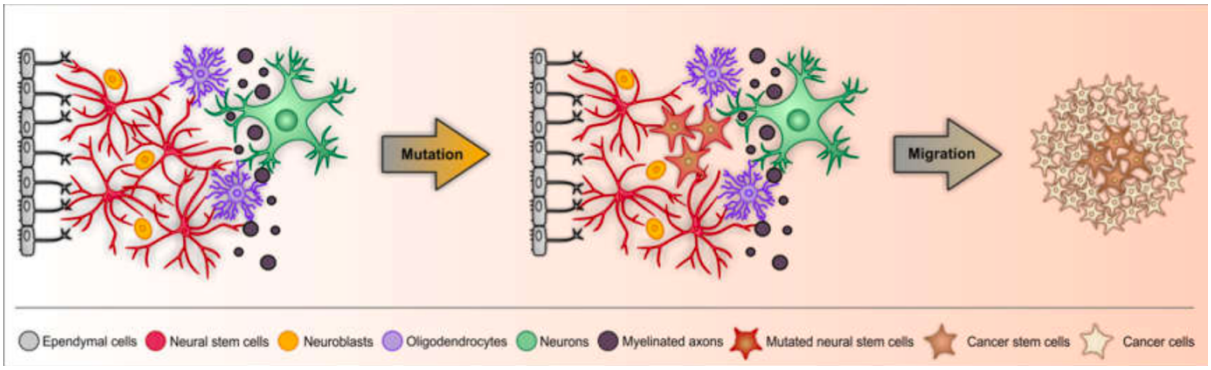


Figure 7. Schematic representation of the origin of brain tumor propagating cells based on the cancer stem cell theory (34).

If the very small subpopulation of CSCs is not eradicated, tumor recurrence is inevitable. Normal stem cells and CSCs share several essential characteristics: multipotency, rare occurrence and low self-renewal rate, a specialized and specific microenvironment which keeps a strict balance between proliferation and cell death, and similar signalling pathways. Applied to glioblastoma, this theory implies the existence of "Brain Tumor Propagating Cells" (BTPCs), which may originate from any (already differentiated) cell or from normal neural stem cells (NSCs). Although it is unlikely that a normal differentiated cell (e.g., an astrocyte) may dedifferentiate, it is not impossible. But accumulating evidence points towards NSCs as the origin of glioblastoma (34).

Although long disputed, it is now accepted that NSCs are present in the adult human brain. Two large niches for NSCs are described: the largest reservoir is the subventricular zone (SVZ), but the dentate gyrus of the hippocampus also contains NSCs (Fig. 8). Sanai et al. showed in 2004 that within the SVZ of the adult human brain a unique astrocyte ribbon exists containing NSCs (35). In 2018, Lee and colleagues delivered direct proof of BTPCs arising from the SVZ in both glioblastoma patients and glioblastoma mouse models (36). Of equal importance, these authors also showed that astrocyte-like BTPCs may leave the SVZ to travel large distances in the brain and ultimately form gliomas. So, the adult human SVZ is a reservoir of NSCs but most likely also of BTPCs (37,38). However, BTPCs are probably not exclusively found in the SVZ or the hippocampus, but also in other brain regions if the conditions of the microenvironment are appropriate for BTPCs. Aderetti et al. propose the concept of the 'hypoxic peri-arteriolar glioma stem cell niche', which integrates earlier descriptions of five glioma stem cell niches (39). The concepts of the SVZ glioma stem cell niche and the peri-arteriolar glioma stem cell niche are complementary, rather than mutually exclusive. It is likely that BTPCs migrate from one niche to another, given the complex interplay of signalling pathways and microtubes that is progressively being unravelled.

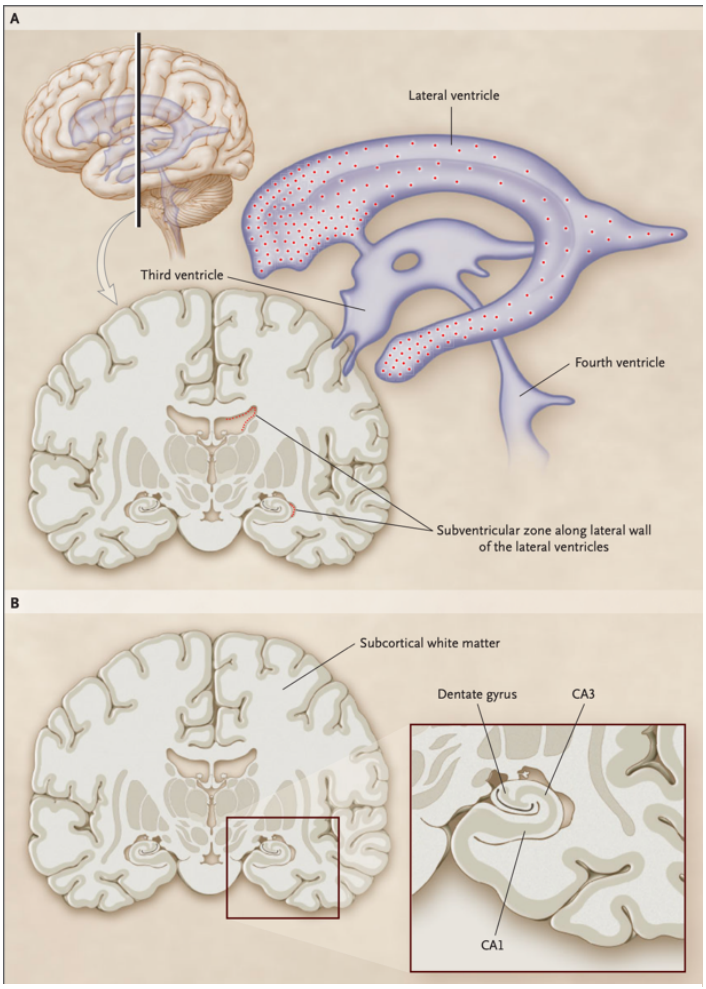


Figure 8. Schematic overview of the germinal regions of the adult human brain (37).

As stated before, the SVZ is the largest reservoir of NSCs in the adult human brain. To date, the astrocyte ribbon of the SVZ has been shown only at the level of the lateral borders of the lateral ventricles, including the atrium and the temporal horns. It was not found at the septal lining of the lateral ventricles nor at the level of the third and the fourth ventricle, although only six specimen from the third ventricle and three samples from the fourth were studied (35). The cytoarchitecture and cellular composition of the SVZ of the lateral ventricles have been described in detail (40). Briefly, using electron microscopy, the adult human SVZ was shown to contain four layers (I - IV). The layers are from medial to lateral: *I.* Ependyma; *II.* Hypocellular gap; *III.* Astrocyte ribbon, containing NSCs; *IV.* Transitional zone towards the brain parenchyma (Fig. 9). Regional differences in SVZ cytoarchitecture exist. Layer III (ribbon of astrocytes) was most prominent in the anterior horn and in the body of the ventricle but was present in the whole SVZ of the lateral ventricles. Between layer II and III, small clusters

of displaced ependymal cells were found. The exact meaning of this finding remains elusive (40).

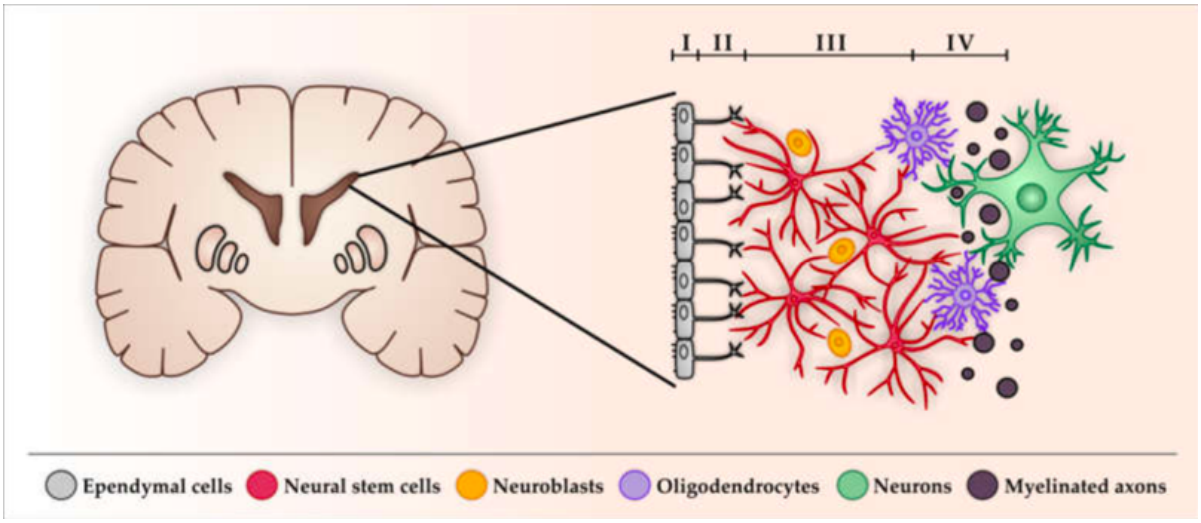


Figure 9. Schematic overview of the cytoarchitectonic layers of the adult human subventricular zone (34).

Glioblastoma Prognostic Factors

Before the introduction of temozolomide in clinical practice, retrospective clinical studies showed that patient age and the clinical condition of the patient at diagnosis (as measured in performance scores) were the most important prognostic factors in glioblastoma patient survival. In the glioblastoma patient cohort studied in this thesis too, age and Karnofsky Performance Score (KPS) were statistically significant prognostic factors (unpublished data; Fig. 10).

A. Age at diagnosis, with 65 years as cut-off point.

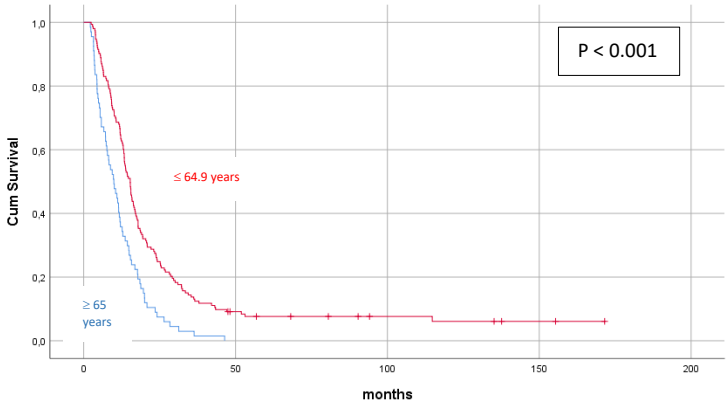
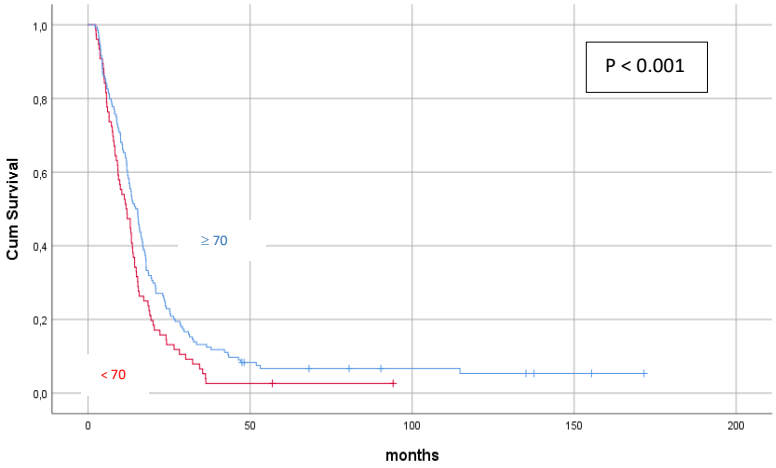


Figure 10 A. Kaplan-Meier overall survival curves of glioblastoma patients (n = 220) studied in this thesis according to age at diagnosis. Eleven patients were censored for survival analysis (+). (Unpublished data)

Figure 10 B. Kaplan-Meier overall survival curves of glioblastoma patients (n = 220) studied in this thesis according to Karnofsky Performance Score at diagnosis, with a score of 70 as cut-off point. Eleven patients were censored for survival analysis (+). (Unpublished data)



Although controversy remained, the general view prevailed in the 20th century that the extent of neurosurgical resection correlated with glioblastoma patient survival and offered a substantial survival advantage as compared to biopsy only (41). Radiotherapy was already early on established as a cornerstone of glioblastoma patient treatment (42). Currently, extent of resection and treatment with temozolomide-based chemoradiotherapy are considered the golden standard in glioblastoma treatment.

In the first decades of the 21st century, new prognostic parameters emerged. Review of large population-based datasets, led to the conclusion that in glioblastoma, as in other fields of oncology, a sex-based survival difference exists. Female glioblastoma patients have a survival advantage as compared to males, but the underlying mechanisms are still poorly understood (43-45).

In 2005 and 2008, molecular biological factors were revealed that are not only of academic interest but also of clinical importance in glioblastoma (10,46). The pivotal role of *IDH* mutations was already discussed (47). Patients with *IDH*-mutated glioblastoma have a significantly better prognosis than *IDH*wt glioblastoma patients (11).

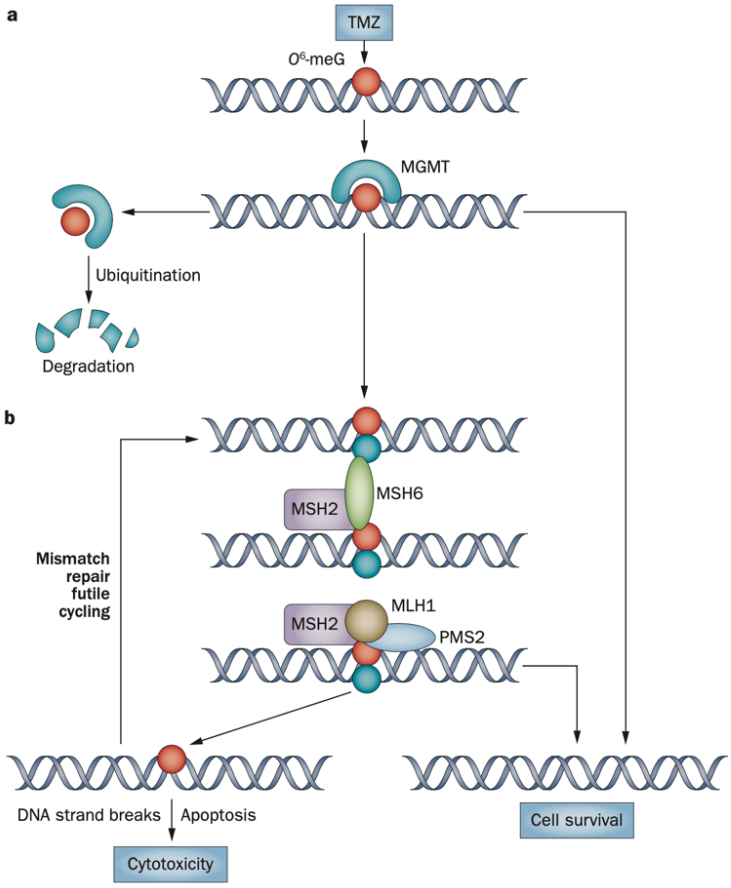


Figure 11. Schematic representation of MGMT-mediated DNA-repair (52). A. Repair of O^6-methylguanine (O^6-meG) by MGMT. B. Effects if O^6-methylguanine is not repaired.

Another molecular biological factor of high importance is *MGMT*p methylation. MGMT is a so-called DNA repair enzyme which acts by removing the alkyl group, if present, at the O^6 position of guanine. During this process, the enzyme is irreversibly inactivated ("suicide enzyme"). MGMT has an important role in protecting the cell DNA from potentially mutagenic changes in normal human cell biology. Temozolomide is an alkylating agent that induces methylation of cell DNA mainly at the N^7 position of guanine, but also at the N^3 and O^6 positions of adenine and guanine respectively (48). The effectiveness of temozolomide may be increased if the DNA repair mechanism by MGMT is inhibited (Fig. 11). Hegi et al. were the first to show that epigenetic silencing of the *MGMT*p results in a significantly better survival for glioblastoma patients, especially when treated with temozolomide (46). This survival benefit was confirmed in a meta-analysis of 11 studies which determined *MGMT*p methylation status by pyrosequencing assay (49). Epigenetic silencing of the *MGMT* gene results from hypermethylation of CpG islands in the promoter region of the gene (Fig. 12). As mentioned above, *IDH*-mutated gliomas are associated with a specific phenotype, the so-called G-CIMP, which results in a hypermethylated DNA status (15). Therefore, *IDH*-mutated glioblastoma is highly associated with the *MGMT*p methylation, explaining at least in part the better survival of *IDH*-mutated glioblastoma patients (13). But in *IDH*wt glioblastoma, on average only 40% of tumors will show *MGMT*p methylation (50). Importantly, consensus is currently lacking as to which technique should be used to determine *MGMT*p-methylation status and different assays may yield different results (51,52).

In this thesis, *MGMT*p methylation status was determined using quantitative methylation-specific polymerase chain reaction (qMSP). Therefore, this technique will be described succinctly (Fig. 12; Roman numerals in the following paragraph refer to the steps depicted in this figure). DNA was extracted from FFPE tissue samples, using the QIAamp DNA TTFP Tissue kit (Qiagen, Hilden, Germany). The role of an experienced neuropathologist is essential to select slides with high tumor concentration (a minimum concentration of 10% tumor cells is

generally accepted). Sampling of non-tumor cells or non-viable tumor regions (necrosis) could result in bias. Next, bisulfite conversion of unmethylated cytosine into uracil is performed: the bisulfite reaction deaminates unmethylated cytosine, but 5-methyl cytosine remains unaltered (I-II). In the following step (III), primers are used to specifically amplify alleles with converted (unmethylated sequences) or unconverted (methylated sequences) cytosines (IV). The amplified sequences were assessed via a quantitative real-time detection using the LightCycler 480 system (Roche, Basel, Switzerland). The β-actin gene (*ACTB*) is used as a reference gene to normalize the *MGMT* values. Criteria to consider an assay as methylated were as follows:

1. Quantification cycle (Cq) < 40 for MGMT assay.
2. Cq < 32 for ACTB assay.
3. A maximum difference of 1.5°C between the melting temperature and the positive control.
4. A maximum difference of 10 base pairs between the amplicon length from the length of the positive control.
5. Ratio value < 0.2, which was calculated as [(MGMT/ACTB) x 100)].

A ratio value of 2 was used as the threshold to consider an assay as 'strongly methylated', while a value of less than 0.2 was the threshold for lack of methylation. Finally, ratio values between 0.2 and 2 were considered as 'weakly methylated'.

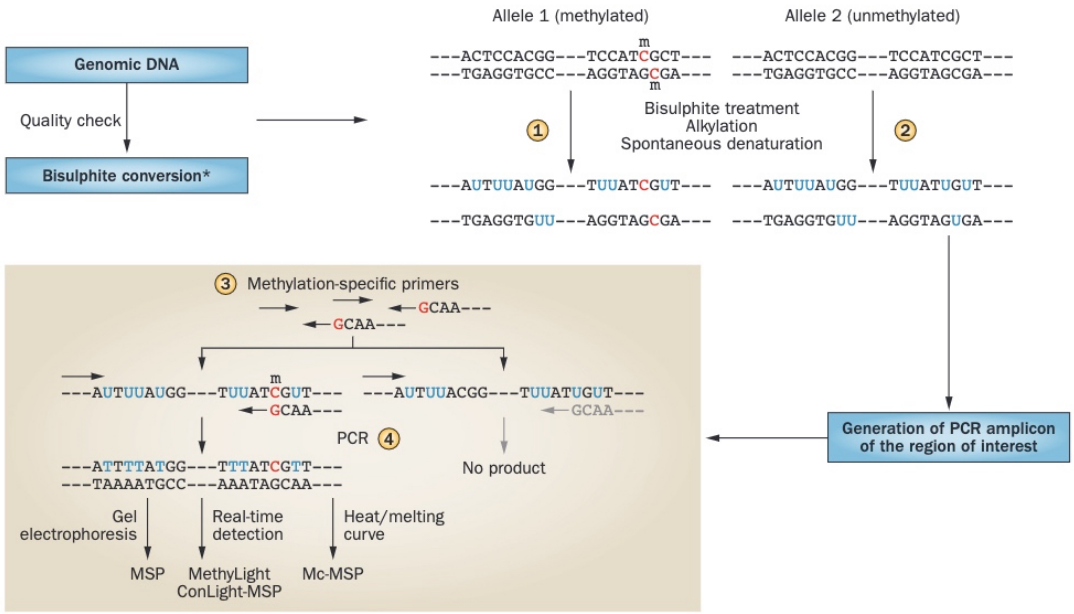


Figure 12. Methylation-specific polymerase chain reaction (52). Please see Roman numerals in text for explanation.

Recently, Gittleman and colleagues published a nomogram[4] to enable an individualized prognosis of *IDH*wt glioblastoma patients. Age at diagnosis, KPS, sex, extent of resection, treatment using concurrent temozolomide-based chemoradiotherapy and *MGMT*p-methylation status are the prognostic factors used to calculate one-year and five-years survival estimates for individual patients (53). *IDH* mutation and *MGMT*p methylation prove to be the strongest prognostic factors for glioblastoma patients' survival. Unfortunately, the majority of newly diagnosed glioblastoma patients will belong to the group with unfavorable molecular biological markers, namely the *IDH*wt group with *MGMT*p unmethylated tumors.

[4] https://gcioffi.shinyapps.io/Nomogram_For_IDH_Wildtype_GBM_H_Gittleman/

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OBJECTIVES AND OUTLINES

This thesis examines the influence on survival of glioblastoma subventricular zone contact, incidental subventricular zone irradiation, O^6-methylguanine-DNA methyltransferase gene promoter (*MGMT*p) methylation and partial resection of glioblastoma in a well-defined cohort of patients treated at Ghent University Hospital and AZ Delta Roeselare (formerly H. Hartziekenhuis) between 2003 and 2014. The ethical committees of both hospitals approved the study. Informed consent from individual patients or their next of kin was waived because of the retrospective study design which held no risk for the patient, the fact that more than 90% of patients had passed away at the time of database closure and because patients had agreed at diagnosis with storage of tumor tissue samples.

A list of all patients with the diagnosis of glioblastoma between 2003 and 2014 was extracted from the pathology database from both hospitals. Next, the pathology reports, patient charts and imaging were manually screened. The following exclusion criteria were used:

- Diagnosis of high-grade glioma, other than glioblastoma.
- Younger than 18 years at time of diagnosis.
- Infratentorial glioblastoma.
- Lost to follow-up.
- Treatment with best supportive care only.
- Shortened radiotherapy schedule (not 60Gy) and/or another chemotherapeutic agent than temozolomide.
- Preoperative diagnostic imaging impossible to retrieve and/or postoperative imaging lacking and/or only CT imaging available.
- Radiotherapy plans impossible to retrieve or to restore.

Demographic data were collected from the patient files. Date of death was cross-checked with the Belgian Cancer Registry. Stored tumor samples were collected from the database and re-examined by the pathologist, to reconfirm the diagnosis of glioblastoma. Representative formalin-fixed paraffine-embedded tumor samples were selected and analyzed for *MGMT*p methylation and presence of the isocitrate dehydrogenase (*IDH*) mutation. Glioblastoma tumor volumes were measured using semi-automated segmentation on the S7 planning software for neuronavigation (Medtronic Inc, Louisville, CO, USA). Hereto, 0.9 mm slice thickness contrast-enhanced Magnetization Prepared-Rapid Gradient Echo (MPRAGE) images, obtained for standard neuronavigation, were used. However, MPRAGE images were not always performed postoperatively.

So, we were able to study the aforementioned factors in a cohort of uniformly treated glioblastoma patients.

*MGMT*p methylation is a well-known prognostic and predictive factor in glioblastoma. However, using quantitative methylation-specific PCR, a zone of diagnostic uncertainty exists. We studied whether survival of patients with methylation test results in this zone differs from patients with unmethylated *MGMT*p (Publication 1: *Weak MGMT gene promoter methylation confers a clinically significant survival benefit in patients with newly diagnosed glioblastoma: a retrospective cohort study*).

We examined the role of the subventricular zone (SVZ) in glioblastoma from two different points of view. First, the prognostic role of contact of the contrast-enhancing part of glioblastoma with the SVZ was studied (Publication 2: *Subventricular zone contacting glioblastoma: tumor size, molecular biological factors and patient survival*). Second, we investigated the potential effect of incidental SVZ irradiation on the survival of *IDH*-wildtype glioblastoma patients (Publication 3: *Survival impact of incidental subventricular zone irradiation in IDH-wildtype glioblastoma*).

The effect of neurosurgical cytoreduction on the survival of glioblastoma patients, continues to stir intense scientific debate. We contributed to the debate (and the controversy) by examining the effect of partial tumor resection on glioblastoma patient survival (Publication 4: *Partial resection offers an overall survival benefit over biopsy in MGMT-unmethylated IDH-wildtype glioblastoma patients*).

PUBLICATION 1

Weak *MGMT* gene promoter methylation confers a clinically significant survival benefit in patients with newly diagnosed glioblastoma: a retrospective cohort study

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J Neurooncol. 2020; 146 (1): 55–62

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Epigenetic silencing of the O^6-methylguanine-DNA methyltransferase gene promoter (*MGMT*p) results in diminished expression of MGMT in tumor cells. Hegi et al. showed in 2005 that glioblastoma patients harboring tumors with hypermethylated *MGMT*p had a significantly better survival when treated with temozolomide-based chemoradiotherapy (1). Silencing of the *MGMT* gene occurs if the CpG islands in the promoter region are methylated. Different techniques are used to determine *MGMT*p methylation. Quantitative methylation-specific PCR (qMSP) is often applied in studies on the prognostic role of *MGMT*p methylation in glioblastoma because it is widely available, has a short turnaround time and is cheaper than pyrosequencing. But consensus on a fixed cut-off value to discriminate between methylated and unmethylated *MGMT*p using qMSP is lacking (2,3). As such, a "grey zone" of diagnostic uncertainty exists. We investigated if glioblastoma patients harboring tumors with a “low *MGMT*p methylation” also experience a survival benefit as compared to unmethylated glioblastoma.

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Journal of Neuro-Oncology
https://doi.org/10.1007/s11060-019-03334-5

CLINICAL STUDY

Weak *MGMT* gene promoter methylation confers a clinically significant survival benefit in patients with newly diagnosed glioblastoma: a retrospective cohort study

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Received: 22 September 2019 / Accepted: 3 November 2019

Abstract

Introduction Quantitative methylation specific PCR (qMSP) is a frequently used technique to assess *MGMT* gene promoter methylation in glioblastoma patients. The optimal technical cut-off value to distinguish methylated from unmethylated samples is nevertheless still undetermined. In literature, a "grey zone" of diagnostic uncertainty has been described.

Methods We performed a retrospective analysis of newly diagnosed glioblastoma patients treated according to the Stupp protocol. Epidemiological data were gathered from the individual patient files. *MGMT* gene promoter methylation status was determined on stored tumour samples using qMSP. A strong, weak or absent promoter methylation was determined based on Cq values (quantification value) of the *MGMT* and ACTB primers as well as a positive control sample.

Results In total, 181 patient files were reviewed and included for statistical analysis. *MGMT* promoter hypermethylation was detected in 38.7% of glioblastoma patients. The median overall survival of unmethylated and strongly methylated patients was 10.1 months and 19.7 months respectively. Furthermore, 11% of the total patient cohort had a weak *MGMT* gene promoter methylation. The median OS in this subgroup was 15.4 months, significantly better compared to the unmethylated cohort ($P < 0.001$). Multivariate Cox regression analysis showed weak *MGMT* promoter methylation as an independent prognostic parameter for overall survival.

Conclusion Glioblastoma patients with weak promoter methylation show a statistically significant longer overall survival when compared to clearly unmethylated patients. Patients with grey zone qMSP test results should receive additional molecular analysis in future to further direct individual therapy strategies.

Keywords Glioblastoma · *MGMT* gene methylation · qMSP · Prognosis

Introduction

Glioblastoma is the most malignant form of brain cancer with an estimated incidence of 3.19 per 100.000 person years [1]. It has a dismal prognosis with a median overall survival (OS) of 14.6 months, despite aggressive treatment [2]. The latter consists of maximal safe surgical resection followed

H. Pinson and G. Hallaert have contributed equally to this article.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11060-019-03334-5) contains supplementary material, which is available to authorized users.

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Published online: 07 November 2019

by concomitant chemoradiation and adjuvant chemotherapy according to the Stupp protocol [3]. This treatment protocol includes temozolomide as first-line chemotherapeutical agent.

Temozolomide is an alkylating agent belonging to the group of Imidazotetrazinones. In aqueous environment such as the human body, it is rapidly hydrolysed to MTIC (5-(3-dimethyl-1-triazenyl) imidazole-4-carboxamide). This intermediate is ultimately responsible for methylation of tumour DNA inducing double-strand DNA breaks and tumour cell death [4]. The methylation of cell DNA occurs mainly at the N^7-atom of guanine, but to a lesser extent as well at the N^3- and O^6-atoms of adenine and guanine respectively [4].

The human cell has three different defence mechanisms to counter act the methylation of DNA by temozolomide: (1) O^6-methylguanine-DNA methyltransferase (*MGMT*); (2) DNA-mismatch repair mechanisms; (3) base excision repair [4]. Epigenetic silencing of the *MGMT* gene is a known prognostic factor for newly diagnosed glioblastoma patients. As hypermethylation of the *MGMT* promoter region results in a diminished expression of *MGMT* in tumour cells, a higher susceptibility to the alkylating effects of temozolomide follows. The landmark article by Hegi et al. showed a significant survival benefit in patients with hypermethylated *MGMT* promoter regions when treated with temozolomide [5].

The promoter and enhancer region of the *MGMT* gene are working together as a cis-regulatory element of non-coding DNA. The promoter contains no classical TATA-box, but has a dense GC-rich sequence instead [6]. The enhancer is a small region downstream of the promoter capable of reducing the promoter activity by 98% [7]. Epigenetic silencing of the *MGMT* gene is a result of hypermethylation of CpG islands in the promoter region of the gene [8]. Nakagawachi et al. described two regions (Differentially Methylated Region (DMR) 1 & 2) of profound CpG hypermethylation in *MGMT* silenced cell lines corresponding to the above mentioned promoter and enhancer region of the gene [8]. Furthermore, Malley et al. found several CpG sites within the DMR 2-region critical for transcriptional control of the *MGMT* gene [9].

In clinical practice, different techniques are currently used to determine *MGMT* gene promoter methylation. Pyrosequencing has the best predictive value for survival in high grade glioma patients but is generally more expensive [10]. Quantitative methylation specific PCR (qMSP) is a cheaper alternative to pyrosequencing with still excellent prognostic performance. Moreover, the turnaround time is shorter and the technique is more widely available [11]. Therefore, qMSP unlike pyrosequencing has already been used in different prospective trials to determine the prognostic role of *MGMT* promoter gene methylation in glioblastoma patients [11]. The optimal cut-off value to be used in qMSP to dichotomize these tumours is nevertheless still a matter of debate [12]. Recent literature confirms the existence of a grey zone of ratio values close to the used cut-off value in which the actual methylation status might be uncertain [11, 12].

Here we investigated the possible correlation between OS of glioblastoma patients and *MGMT* promoter gene methylation using different cut-off values in qMSP.

Materials and methods

Patient selection and characteristics

This retrospective cohort study included all patients with supratentorial glioblastoma treated at two Belgian institutions between 2003 and 2014. The following inclusion criteria were used: (1) patients had a new diagnosis of glioblastoma; (2) no other concurrent tumours were present; (3) further treatment with 60 Gy external beam radiation therapy and concomitant temozolomide after biopsy or resection; (4) the patients were meant to receive at least six cycles of adjuvant temozolomide; (5) stored tumour tissue samples were present for further molecular analysis. Major exclusion criteria were treatment with best supportive care or a reduced and/or shortened radiation schedule; known progression from low grade glioma. In the early days after the study by Stupp et al. was published in 2005, some older patients with low performance scores and various comorbidities did receive lower radiation doses and/or shorter temozolomide treatments. These patients, who were not meant to receive six cycles of temozolomide, were excluded from our study as their treatment did not comply with the current standard of care and their survival is therefore not representative for today's practice.

Demographic parameters were gathered from the medical files of the patients. These parameters included sex, age at diagnosis, Karnofsky Performance Score (KPS) and surgical resection. Surgery was classified as biopsy only, subtotal resection (STR) or gross total resection (GTR) according to the presence (STR) or absence (GTR) of contrast enhancing tumour on postoperative imaging. Overall survival was measured from date of histological diagnosis to closure of the database or date of decease. The latter was confirmed by the Belgian Cancer Registry.

Furthermore, the *MGMT* gene promoter methylation status and IDH-1/2 mutations were assessed using the specimens harvested for histological diagnosis. The *MGMT* gene promoter methylation status was analysed using quantitative methylation specific PCR (qMSP). The presence of IDH-1/2 mutations was determined using next generation sequencing techniques (NGS).

The study protocol was approved by the ethics committee of both participating hospitals (Belgian Registration number B670201730765; AZD 17004; UZG 2016/1594). The obligation to obtain informed consent of the patients or their next-of-kin was waived as most of the patients had succumbed at the time of analysis.

MGMT gene promoter methylation status

The *MGMT* gene promoter methylation status was analysed using the qMSP technique as described elsewhere [13, 14].

Briefly, DNA extraction was performed on formalin-fixed paraffin-embedded (FFPE) tumour samples obtained during initial diagnostic work-up or surgical treatment using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). This resulted in 0.5–1.0 µg of purified tumour DNA. This DNA was afterwards exposed to bisulfite treatment in order to transform the unmethylated cytosine nucleotides into uracil using the EZ DNA Methylation Kit (Zymo Research, California, US).

The bisulfite treated DNA was used to perform a quantitative real-time methylation specific polymerase chain reaction (qMSP) using the LightCycler 480 system (Roche, Basel, Switzerland) [14]. The β-actin gene was used as a control to normalize the MGMT values (see Supplementary Material).

The primers used in the MSP assay were described earlier [14, 15]. The primers used for ACTB (located on chromosome 7) were TAGGGAGTATATAGGTTGGGGAAG TT (forward primer) and AACACACAATAACAAACA CAAATTCAC (reverse primer). The primers for methylated *MGMT* (chromosome 10) were TTTCGACGTTCG TAGGTTTTCGC (forward primer) and GCACTCTTC CGAAAACGAAACG (reverse primer). A qMSP assay was considered methylated if the following conditions were met: (1) $Cq < 40$ for the *MGMT* assay; (2) $Cq < 32$ for the ACTB assay; (3) the melting temperature (Tm) did not differ more than 1.5 °C from the positive control; (4) the amplicon length did not differ more than 10 base pairs from the positive control; (5) ratio value ($MGMT/ACTB \times 100) > 0.2$. The Cq value (quantification cycle) points to the first PCR cycle in which a positive fluorescence signal is detected; as such, a lower value indicates a higher DNA concentration at baseline. If the MGMT gene promoter was considered methylated, the samples were further dichotomized in weakly and strongly methylated variants using a different ratio value. In this study, a ratio value of 2 was used as cut-off value to consider a sample strongly methylated. The second value of 0.2 was used to identify weakly methylated tumours and to demarcate our own qMSP grey zone between 0.2 and 2.

Statistical analysis

To compare the means between different categorical groups a one-way ANOVA test was used if the following assumptions were met: (1) normal distribution of the dependent variable (using the Shapiro–Wilk test); (2) a homogeneity of variances (using the Levine test); (3) independence of variables. The Pearson chi-square test was used to compare two categorical variables based upon independent subgroups. For survival analysis, Kaplan–Meier curves were constructed and the Log Rank test was used to assess the probability of survival. Finally, Cox regression analysis was used to correct the survival analysis for confounding variables (age at diagnosis, KPS, surgery, *MGMT* gene promoter methylation status, cycles of temozolomide, IDH-1/2 mutations).

A two-sided cut-off value of 0.05 was used to determine statistical significance. The statistical analysis of the data was performed using SPSS (version 25, IBM, Armonk, NY, USA).

Results

In total, 181 patients met the inclusion criteria and their files were included for review. Mean age at diagnosis was 61.6 years; 33.7% of patients were female. The majority of patients (68.5%) had a KPS of 70 or higher. All patients underwent surgical therapy for their disease: 27.1% underwent only tumour biopsy for histological diagnosis, 43.1% received a subtotal resection and 29.8% a gross total resection. At the molecular level, 38.7% of tumours harboured weak or strong *MGMT* hypermethylation and 7.7% were determined as IDH-1 mutated. In 11% of cases, IDH-1 mutation status could however not be determined due to a lack of sufficient or qualitative DNA. Table 1 gives an overview of the different epidemiological parameters gathered according to *MGMT* methylation status. No statistically significant differences between the three groups could be detected concerning baseline characteristics. The main reasons why patients were unable to complete six cycles of adjuvant temozolomide treatment were disease progression, major treatment toxicity (mostly bone marrow failure) or death.

The median OS of the whole study cohort was 12.2 months. Age at diagnosis, KPS ≥ 70%, surgical resection, *MGMT* hypermethylation (weak and strong) and six or more cycles of temozolomide were statistically significant prognostic variables in univariate testing using the Cox proportional hazard model. The IDH-1/2 mutation did not reach statistical significance as a prognostic variable for OS. These results are summarized in Table 2.

Next, a multivariate model was created using the Cox proportional hazard model adding the dichotomized *MGMT* methylated tumours based on different cut-off

Table 1 Study population characteristics

Parameter	No methylation	Weak methylation	Strong methylation	P-value
n (%)	111 (61.3%)	20 (11%)	50 (27.6%)	–
Age at diagnosis (mean)	61	65	62	0.351[a]
KPS				
< 70	32 (28.8%)	5 (25%)	20 (40%)	0.296[b]
≥ 70	79 (71.2%)	15 (75%)	30 (60%)	
Surgery				
Biopsy	34 (30.6%)	2 (10%)	13 (26%)	0.430[c]
STR	46 (41.4%)	10 (50%)	22 (44%)	
GTR	31 (27.9%)	8 (40%)	15 (30%)	
IDH-1/2 mutation				
No	93 (83.8%)	18 (90%)	36 (72%)	0.359[c]
Yes	7 (6.3%)	1 (5%)	6 (12%)	
NA	11 (9.9%)	1 (5%)	8 (14%)	
Median TMZ cycles	3	6	6	0.028[d]
PFS (median)	5.4	11.2	10.5	< 0.001[e]
OS (median)	10.1	15.4	19.7	< 0.001[e]

NA not determined due to technical reasons

[a]One-way ANOVA

[b]Pearson Chi-Square test with degrees of freedom (df) = 2

[c]Pearson Chi-Square test with df = 4

[d]Kruskal–Wallis test

[e]Log Rank test with df = 2

Table 2 Different prognostic parameters in survival of glioblastoma

Parameter	Mean/proportion	Median OS	Hazard ratio	Wald test
Age at diagnosis (years)	61.6	–	1.019	P = 0.007
KPS				
< 70	31.5%	8.3	0.483	P < 0.001
≥ 70	68.5%	13.9		
Type of surgery				
Biopsy	27.1%	7.6	–	–
Subtotal resection	43.1%	12.4	0.594	P = 0.005
Gross total resection	29.8%	16.2	0.374	P < 0.001
MGMT gene promoter methylation				
No	61.3%	10.1	0.347	P < 0.001
Yes	38.7%	19.2		
Temozolomide				
< 6 cycles	56.4%	7.8	0.286	P < 0.001
≥ 6 cycles	43.6%	17.9		
IDH-1/2 mutation				
No	81.1%	12.2	0.592	P = 0.083
Yes	7.7%	21.3		

values for the qMSP ratio value. The results are summarized in Table 3. The median OS of patients without *MGMT* gene promoter hypermethylation was 10.1 months; for patients with weakly methylated tumours 15.4 months; for patients with strongly methylated tumours 19.7 months (see Fig. 1). Patients with weakly methylated tumours showed a significant survival benefit compared to the group with unmethylated tumours (P < 0.001). The median progression free survival (PFS) was 5.4, 11.2 and 10.5 months respectively.

Table 3 Multivariate Cox proportional hazards regression analysis

Parameter	B	Standard deviation	Hazard ratio	Wald test
Age at diagnosis	0.019	0.009	1.019	P = 0.033
KPS ≥ 70	− 0.801	0.196	0.449	P < 0.001
Subtotal resection (STR)	− 0.686	0.212	0.504	P = 0.001
Gross total resection (GTR)	− 1.147	0.245	0.317	P < 0.001
MGMT methylation—weak	− 0.831	0.289	0.436	P = 0.004
MGMT methylation—strong	− 1.339	0.222	0.262	P < 0.001
Temozolomide-6 or more cycles	− 1.144	0.183	0.318	P < 0.001
IDH-1/2 mutant	− 0.285	0.309	0.752	P = 0.356

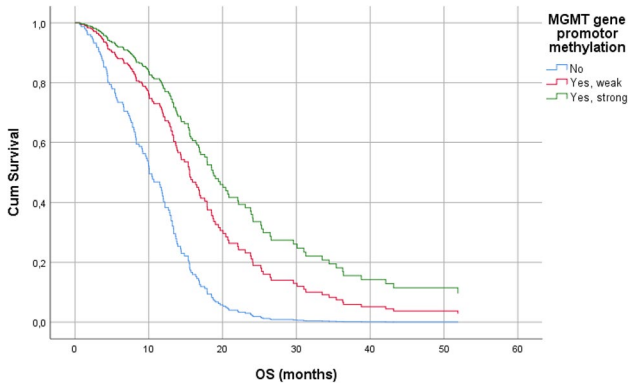

Fig. 1 Kaplan–Meier curve illustrating the differences in overall survival (OS) between the MGMT gene promoter methylation groups

Table 4 Survival analysis of different methylation groups (median OS in months)

Study	Methodology	Unmethylated	Grey zone	Methylated
Xia et al. [20]	MSP	17.0	20.0	34.1
Hsu et al. [21]	MSP	14.6	18.0	25.6
Hegi et al. [12]	qMSP	13.6	16.5	25.6
This study	qMSP	10.1	15.4	19.7

Discussion

Quantitative methylation specific PCR (qMSP) is a commonly used method to determine *MGMT* methylation status. In comparison to pyrosequencing, it is cheaper and widely available. Moreover, it has been used in various prospective, randomized trials to dichotomize glioblastoma samples in methylated and unmethylated groups [16–18]. The qMSP technique used to assess *MGMT* gene promoter methylation status in this study is adapted from a technique described elsewhere by Vlassenbroeck et al. [13]. Results of qMSP might be based on the quantification cycle (Cq-values) or on the copy numbers present in the sample.

Nevertheless, the optimal cut-off for this ratio value in qMSP is still a matter of debate. The technical cut-off based on copy numbers used in the original study by Vlassenbroeck et al. was founded on a bimodal distribution of the dichotomized tumour groups. Although technically valuable, both groups show a clinically significant overlap. This overlap has been termed the qMSP "grey zone" in which diagnostic uncertainty prevails [12]. Different trials have used a ratio value of 2 to identify *MGMT* gene promoter hypermethylation [16–18]. Contrary, trials looking for alternatives to temozolomide for *MGMT* gene promoter unmethylated patients have used a more stringent cut-off value of 0.6 to minimize inclusion of patients with methylated tumours [19]. This is important as the latter group substantially benefits from temozolomide treatment and should not be withheld today's standard-of-care. Hegi et al. recently tried to minimize the qMSP grey zone by defining optimal clinical cut-off points for future trials. The authors combined the results of earlier trials including 1725 patients using a slightly different "corrected" ratio value ($\log_2[(MGMT + 1)/\text{ACTB} \times 1000]$). The authors found a lower and upper limit of the grey zone of − 0.28 and 1.27 respectively [12]. An important remark has to be made; the group of Hegi et al. used absolute copy numbers to calculate their ratio value. This study used Cq-values so direct comparison might therefore not be possible.

Ambiguous results are a frequent problem in both qualitatively and quantitatively assessed MSP techniques for *MGMT* gene promoter methylation analysis. Accordingly, the existence of a (q)MSP grey zone as such has been illustrated in different studies (Table 4). Xia et al. used a qualitatively assessed technique (as opposed to the quantitative technique qMSP) and found an inconsistency rate of 12%. These samples could in other words not easily be labelled *MGMT* methylated or not. Further analysis showed that these inconsistent cases had an OS close to the unmethylated

group. Finally, the authors found prognostic heterogeneity within the inconsistent cases, possibly related to varying rates of gene methylation in these patients [20]. Similar results were found by Hsu and colleagues examining the prognosis of glioblastoma patients with faint MSP results. They found a prevalence of inconsistency of 15.5% with a median OS of 18 months in this group of patients [21]. Again, comparing the results with a paired qMSP analysis, the authors found a correlation with overall methylation level [21]. After optimizing the cut-off points for qMSP, Hegi et al. found their grey zone contained 8.1% of their test cohort with a median survival of 16.5 months. Finally, this study confirms that a subgroup of patients analysed with qMSP show worse survival than strongly methylated patients (qMSP ratio 2 or more), but perform still better than unmethylated patients (qMSP ratio 0.2 or less). We show that patients may still benefit from temozolomide treatment if they belong to the so-called grey zone. In this study, the latter group constitutes 11% of the total cohort and shows a median OS of 15.4 months.

This qMSP grey zone is in fact a heterogenous group of glioblastoma patients, methylated and unmethylated tumours, with overlapping and thus indiscriminating diagnostic test results. In a way, this zone contains false positives (unmethylated samples that seem methylated in qMSP) and false negatives (methylated samples that seem weakly or not methylated at all in qMSP). From a clinical point of view, the latter probably form no major therapeutic problem as even weakly methylated tumours might benefit from a temozolomide based adjuvant treatment. This was shown preclinically in cell lines by van Nifterik et al. and indirectly confirmed clinically by the study of Hegi et al. and by this study [12, 22]. One should therefore realise that *MGMT* expression is a continuum rather than an all or nothing phenomenon [22, 23]. The inclusion of unmethylated samples in the qMSP grey zone on the other hand poses a more important therapeutic problem as these patients might not benefit from treatment with temozolomide [19, 24]. The reason why these samples might seem (weakly) positive on qMSP is probably due to sample cell heterogeneity and suboptimal test sensitivity. Normal tissues, such as brain and liver cells, produce *MGMT* protein to protect and repair their DNA. Furthermore, tumour blood vessels, tumour-associated leukocytes and microglia express *MGMT* as well, possibly dominating the *MGMT* signal of glioblastoma cells in qMSP [23].

Mansouri et al. recently proposed a diagnostic algorithm for *MGMT* gene promoter methylation testing during the diagnostic work-up in newly diagnosed glioblastoma patients [11]. Using clinical relevant cut-offs, qMSP should be used to distinguish clearly unmethylated and methylated tumour samples, as this test is fairly cheap and widely available. Samples in the grey zone should be further analysed using an alternative diagnostic technique such as pyrosequencing. Here it may become of primary importance to identify the false positive and thus unmethylated cases, as these might benefit from alternative treatment regimens in future. On the other hand, this approach is very useful to include patients in future trials investigating alternative treatments for truly unmethylated tumours.

This retrospective study has several shortcomings. Obviously, the retrospective methodology implicates a risk of bias, confounding the interpretation of the results. This may equally apply to the rather small patient cohort of this study. Unfortunately, the study methodology was already designed before publication of the paper from the Hegi group, so different methods were used in comparison to their ratio value [12]. The baseline characteristics of the three methylation groups do not seem to differ significantly. However, attention should be paid to the fact that the weak methylation has a better KPS score in comparison to the strongly methylated group and the fraction of patients who underwent tumour debulking was higher. Furthermore, the relative proportion of IDH mutated tumours in the gross total resection group is higher compared to the other groups. IDH mutation status is a well-known prognostic parameter in glioma tumours, although not reaching statistical significance in this study cohort due to low incidence (Table 1). One of the major strengths of this study is that the results were gathered in a heterogenous clinical sample from day to day practice, improving external validity. We therefore confirm the results published by Hegi et al. gathered from a larger patient cohort, included in four different randomized trials, but subjected to various in- and exclusion criteria [12]. Next, the technique used for *MGMT* gene promoter methylation analysis is thoroughly known and often used in different prospective trials.

Conclusion

It is important to recognize the existence of a grey zone in qMSP analysis for *MGMT* gene promoter methylation in glioblastoma patients. In comparison to patients with clearly unmethylated tumours, these grey zone patients still show a better OS when treated with temozolomide based radiochemotherapy. Patients with ambiguous qMSP results should receive further molecular analysis using different techniques (preferably pyrosequencing) to definitely determine their methylation status. This may become ever more important in the age of precision medicine, and especially for patients with unmethylated tumours, as they might benefit from other treatments than or added to temozolomide in the future.

Funding This work was supported by grants from Ghent University Hospital, Centre for Oncology, from the "Stichting Luka Hemelaere" and "Fonds Arne Lannoy AKA Zorro."

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to be reported.

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PUBLICATION 2

Subventricular zone contacting glioblastoma: tumor size, molecular biological factors and patient survival

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Acta Oncol. 2020; 94 (7): 1–6

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The subventricular zone (SVZ) is the largest reservoir of neural stem cells in the adult human brain. Already in 1944, Globus and Kuhlenbeck[α] formulated the hypothesis of an etiological relationship between glial brain tumors and the "subependymal plate", as the SVZ was then called (1). Nowadays, growing evidence from both animal studies and human studies exists to support the hypothesis that the SVZ has an important role in glioblastoma, possibly in part by functioning as a niche for the glioblastoma stem cell (2,3). Several reports found that SVZ contact of the contrast-enhancing part of the tumor is a negative prognostic factor for glioblastoma patient survival (4,5). However, most publications were unable to include molecular biological factors in the survival analysis (6). We studied whether a survival difference between SVZ_{pos} and SVZ_{neg} patients exists when survival analysis is adjusted for molecular biological markers.

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[α] Perhaps Bailey and Cushing were the first to coin the idea in their book *A Classification of the Tumors of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis* published in 1926. However, Globus and Kuhlenbeck were the first to describe a full and comprehensive theoretical framework.

ACTA ONCOLOGICA
https://doi.org/10.1080/0284186X.2020.1794032

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ORIGINAL ARTICLE

Subventricular zone contacting glioblastoma: tumor size, molecular biological factors and patient survival

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ABSTRACT

Background: Several studies show that subventricular zone (SVZ) contact of glioblastoma at diagnosis is a negative prognosticator of survival. In this report, we study glioblastoma patient survival, molecular biological and MRI-based volumetric findings according to SVZ contact.

Patients and methods: We conducted a retrospective study of adult patients diagnosed with supratentorial glioblastoma and uniformly treated with temozolomide-based chemoradiotherapy after surgery. The patient cohort was dichotomized according to tumor contact with the SVZ at diagnosis as determined on preoperative MR imaging. Tumor volume was measured using semi-automated segmentation technique. MGMT-gene promoter methylation and IDH mutation status were determined on stored tumor tissue. Kaplan-Meier survival curves were constructed. Cox regression analysis was used to adjust for known confounding factors of glioblastoma patient survival.

Results: A total of 214 patients were included in the study of whom 68% belonged to the SVZ_{pos} group. Median tumor volume was significantly larger in the SVZ_{pos} group (33,8 mL vs 15,6 mL; $p < .001$). MGMT-unmethylated glioblastoma was more frequent in the SVZ_{pos} group (61.4% vs 44.9%; $p = .028$). The overall survival and progression-free survival were 12.2 months and 5.9 months for the SVZ_{pos} patient group but 16.9 months and 10.3 months for the SVZ_{neg} group (log-rank $p = .016$ and .007 respectively). In multivariate Cox survival analysis, SVZ contact proved a negative prognostic parameter, independent from age, KPS, extent of resection, MGMT-methylation and IDH mutation status.

Conclusions: This study confirms SVZ contact at diagnosis as an independent negative prognostic factor for glioblastoma patient survival. SVZ_{pos} glioblastoma had larger tumor size and a larger proportion of unmethylated tumors than SVZ_{neg} glioblastoma. Further research is needed to establish whether the observed differences are solely explained by a different molecular profile of SVZ_{pos} glioblastoma or by interaction of glioblastoma with the unique SVZ microenvironment.

ARTICLE HISTORY
Received 26 April 2020
Accepted 6 July 2020

Introduction

Glioblastoma is both the most frequent and most malignant primary brain tumor in adults. Almost all patients succumb to the disease, as reflected in a five-year survival rate of only 4.6% [1]. This tumor typically presents on MR imaging of the brain as an irregularly ring-shaped zone of contrast-enhancement with central necrosis and surrounded by edema. If the contrast-enhancing part of the tumor abuts the subventricular zone (SVZ) at diagnosis, the tumor is considered as SVZ-contacting glioblastoma (SVZ_{pos}; Figure 1). In recent years, the SVZ has regained attention as the potential source of brain tumor initiating cells (BTICs), a hypothesis already formulated in 1944 by Globus and Kuhlenbeck [2]. The evidence supporting the presence of BTICs in the SVZ is now rapidly growing and these cells probably originate from neural progenitor cells (NPCs) [3–5]. Several retrospective studies acknowledge SVZ contact at diagnosis as a negative prognostic factor in glioblastoma [6–9]. The negative influence of SVZ contact of glioblastoma seems to be independent from known prognostic factors such as age, Karnofsky Performance Score (KPS), extent of resection and molecular biological factors, specifically methylation of the *0^6-methylguanine-DNA-methyltransferase* (MGMT)-promoter and *isocitrate dehydrogenase* (IDH)-mutation status [7–9]. In this report, we conducted a retrospective study of a cohort of uniformly treated *de novo* glioblastoma patients with regard to survival and demographic, MRI-based volumetric and molecular biological differences between SVZ contact groups. The prognostic significance of SVZ contact was tested both in univariate and multivariate survival analysis, adjusting for independently validated prognosticators of glioblastoma patient survival [10].

Patients & methods

Patient selection

We performed a retrospective analysis of adult (18 years or older) patients treated for supratentorial glioblastoma in two

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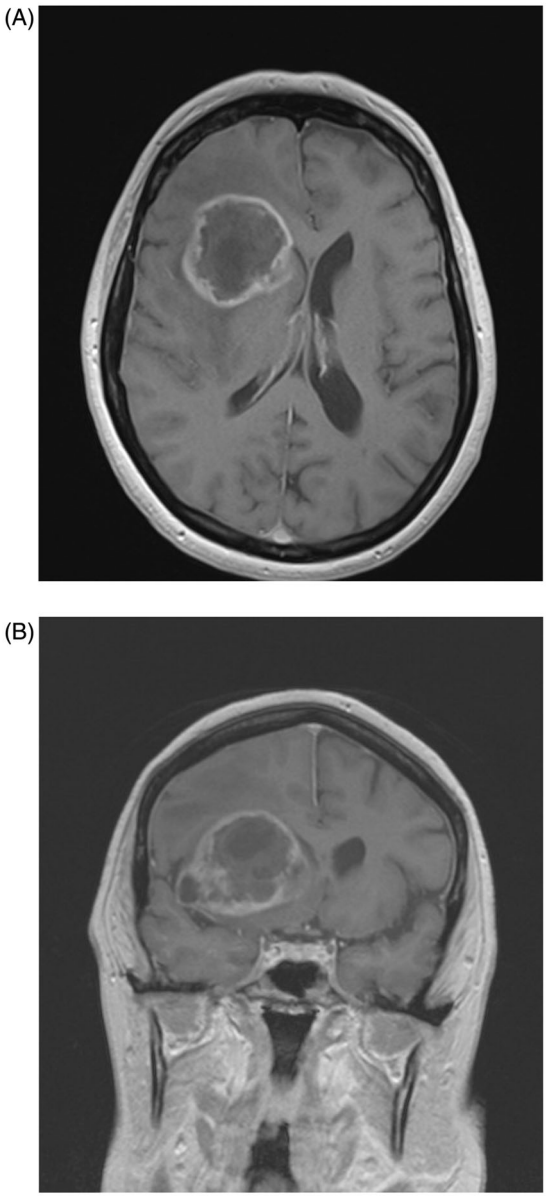

Figure 1. Contrast-enhanced axial T1 MR image (A) and matching coronal T1 image (B) illustrating a right frontal glioblastoma with SVZ contact.

hospitals in Flanders (Belgium) between 2003 and 2014. Patients were included only if they completed temozolomide-based chemoradiotherapy (60 Gy in 30 fractions) after surgery. Patients with a previous history of low-grade glioma or other brain tumors were excluded. If the preoperative imaging could not be retrieved, these patients were also excluded.

The following demographic parameters were collected from the written or electronic medical files: sex; age at diagnosis; Karnofsky performance score (KPS). Surgery was classified into biopsy only or resection, based on surgical intent. Resection was classified into subtotal resection (STR) or gross total resection (GTR) based on postoperative imaging, according to the method applied by Stummer et al. [11]. Briefly, if postoperative imaging showed the presence of contrast-enhancement of the size of one voxel or more, then surgery was classified as STR; if not, GTR was accepted. Overall survival (OS) was determined as the time between the date of histological diagnosis and the date of death. Progression-free survival (PFS) endpoint was determined by either the date of radiologic evidence of disease recurrence or progression or the date of change in treatment plan due to clinical disease progression. Patients who were still alive at the time of analysis were censored for OS. Patients without disease progression were censored for PFS at the time of the last registered follow-up visit.

This study was approved by the Ethics Committee of both participating hospitals (Belgian Registration number B670201730765; UZG 2016/1594; AZD 17004). Since the vast majority of patients had deceased at the time of analysis, the need for individual informed consent was waived by both committees.

Imaging and molecular biological factors

Contact of the contrast-enhancing part of the tumor with the SVZ was evaluated on preoperative MR imaging (SVZ_{pos} vs SVZ_{neg}). Preoperative tumor volume was measured on 3 D-T1 magnetization-prepared rapid acquisition gradient echo (MPRAGE) images with a slice thickness of 0.9 mm obtained for neuronavigation and using semi-automated segmentation technique [12]. These images were acquired on 1,5 T or 3 T magnetic resonance imaging systems (Siemens, Erlangen, Germany).

The neuropathologist selected a representative formalin-fixed paraffin-embedded tissue block from the tumor tissue archive for each case. All samples were reviewed and tested for IDH-1 and −2 mutation using next-generation sequencing techniques. MGMT promoter methylation was determined using semi-quantitative methylation-specific polymerase chain reaction (qMSP), as previously described [13].

Statistical analysis

Fisher's exact test was used to compare proportions between independent categorical variables of SVZ_{pos} and SVZ_{neg} groups and the independent samples *t*-test was applied for numerical variables, except for preoperative tumor volume. Mean difference in preoperative tumor volume between groups was assessed using a linear regression model, after log-transformation to improve normality of the measured tumor volumes. Kaplan-Meier survival curves for OS and PFS were plotted and compared between groups with the log-rank test; hazard ratios were also calculated using a univariate cox regression model. Next, a multivariate Cox regression model was fitted for survival analysis (OS and PFS), including known prognostic factors of survival in glioblastoma patients (age at diagnosis; KPS; biopsy vs resection; MGMT- methylation; IDH-mutation). In Cox regression models, numerical variables were not categorized [14]. Graphical methods were used to assess that the proportional hazards assumption was respected in Cox models for categorical variables. For numeric variables, a time-dependent covariable was introduced in the model and checked that it was not significant. All statistical analyses were performed using SPPS (v26, IBM,

Armonk, NY, USA). Statistical significance was set at $p < .05$ using two-tailed tests.

Results

In total, 399 patients were surgically treated for glioblastoma. Three patients were excluded because of cerebellar localization or the presence of other tumors; 43 were lost to follow-up; 93 patients were treated with shortened radiotherapy schedules or without temozolomide; diagnostic MR imaging could not be retrieved in 46 patients. So, 214 patients were included in the study of whom 12 were still alive at the time of database closure. The majority of patients (68%) belonged to the SVZ_{pos} group (Table 1). IDH mutation could not be determined due to technical reasons in 19.5% of patients while MGMT-methylation status is lacking in 8% of patients. Only the $IDH1^{R132}$ mutation was found. The difference in tumor size and in MGMT-methylation status between SVZ contact groups proved statistically significant (Table 1). The number of unmethylated tumors was higher in the SVZ_{pos} group while in the SVZ_{neg} group the numbers of methylated and unmethylated tumors were proportionally distributed (44.9% unmethylated and 46.4% methylated; Table 1). There was no difference in frequency of IDH-mutation between SVZ contact groups. Tumor volume could be determined in 177 patients. Median tumor volume was more than double in the SVZ_{pos} glioblastoma group (33.8 mL for SVZ_{pos} vs 15.6 mL for SVZ_{neg}; $p < .001$). There were no significant differences in age, KPS, female/male ratio or extent of surgical resection between SVZ contact groups.

Univariate survival analysis showed that age, KPS, extent of resection, MGMT-methylation status and IDH-mutation correlated significantly with glioblastoma patient survival albeit IDH-mutation only for PFS (Table 2). Preoperative tumor volume did not correlate with survival in univariate Cox regression model. SVZ contact was a significant prognostic factor for both OS (SVZ_{pos} median OS 12.2 months vs SVZ_{neg} median OS 16.9 months; $p = .016$) and PFS (SVZ_{pos} median PFS 5.9 vs SVZ_{neg} median PFS 10.3 months; $p = .007$). After adjustment for age, KPS, surgical resection, IDH-mutation and MGMT-methylation status, SVZ contact proved a statistically significant prognostic factor both for OS and PFS (Table 3, Figure 2).

Discussion

This study confirms SVZ contact as an independent negative prognostic factor in glioblastoma patient survival. Compared to SVZ_{neg} tumors, SVZ_{pos} glioblastoma has a double median tumor volume and comprises predominantly MGMT-unmethylated tumors. There were no significant differences between SVZ contact groups with regard to sex ratio, age, KPS, extent of resection or IDH-mutation.

Contrary to our results, a recent meta-analysis of 6 reports on MGMT-methylation status of SVZ_{pos} glioblastoma, showed no significant difference between SVZ contact groups concerning this important prognostic epigenetic factor [15]. This was also the case in the prospective study on SVZ contacting glioblastoma by Van Dijken et al. [7]. However, a 2018 report by Han et al. on predicting MGMT promoter methylation status based on preoperative MR imaging, showed similar results to this study, with a significantly higher number of unmethylated tumors in the SVZ_{pos} group compared to the SVZ_{neg} group (58.3% vs 36.4% resp.; $p = .012$) [16]. Literature does not allow to draw a definitive conclusion at present whether SVZ_{pos} glioblastoma has a different MGMT-methylation pattern than SVZ_{neg} tumors.

Importantly, despite the obvious higher number of unmethylated tumors in the SVZ_{pos} group, SVZ contact was an independent prognostic factor in multivariate survival

Table 1. Overview of demographic, surgical, molecular biological and volumetric characteristics as well as survival according to subventricular zone contact group.

Parameter	SVZ contact	No SVZ contact	*p*-value
N (%)	145 (68%)	69 (32%)	–
Sex			.223[a]
Female	55 (38%)	20 (29%)	
Male	90 (62%)	49 (71%)	
Mean age at diagnosis (years)	60	62	.162[b]
KPS (mean)	70	70	.115[b]
Surgery			.552[a]
Biopsy only	40 (27.6%)	15 (21.7%)	
STR	60 (41.4%)	28 (40.6%)	
GTR	45 (31.0%)	26 (37.7%)	
MGMT-methylation			**.028**[c]
No	89 (61.4%)	31 (44.9%)	
Yes	45 (31%)	32 (46.4%)	
unknown	11 (7.6%)	6 (8.7%)	
IDH-mutation			.276[c]
No	111 (76.6%)	44 (63.8%)	
Yes	10 (6.9%)	7 (10.1%)	
unknown	24 (16.5%)	18 (26.1%)	
Median tumor volume (mL)*	33.8	15.6	**<.001**[d]
Median overall survival (months)	12.2	16.9	**.016**[e]
Median progression-free survival (months)	5.9	10.3	**.007**[e]

[a]Fisher's exact test. [b]Independent samples *t*-test. [c]Fisher's exact test with exclusion of *NA* group. [d]Linear regression model of the log transformed volumes. [e]Log-rank test. *Available in 177 patients.
Significant *p*-values are highlighted in bold. KPS = Karnofsky Performance Score; STR = subtotal resection; GTR = gross total resection; MGMT = 0^6-methylguanine-DNA-methyltransferase; IDH = isocitrate dehydrogenase.

Table 2. Univariate survival analysis for several prognostic factors of glioblastoma patient survival.

		Overall survival			Progression-free survival		
Parameter	% of patients	Median (months)	Hazard ratio (95% CI)	Wald test *p*	Median (months)	Hazard ratio (95% CI)	Wald test *p*
Age at diagnosis (years)							
≥65	35,5%	11.7	1.021 (1.009–1.034)*	**.001**	6.2	1.009 (0.997–1.021)*	.161
<65	64,5%	14.3			6.7		
KPS							
≥70	70%	15.33	0.981 (0.971–0.991)*	**<.001**	7.7	0.987 (0.978–0.997)*	**.010**
<70	30%	9.4			4.6		
Preoperative tumor volume*	82.7%	–	0.997 (0.985–1.009	.588	–	1.005 (0.992–1.019)	.469
Surgery							
Biopsy only	25,7%	8.7	–	–	4.7		
STR	41,1%	13.0	0.673 (0.480–0.944)	**.022**	6.2	0.589 (0.417–0.832)	**.003**
GTR	33,2%	17.0	0.503 (0.371–0.682)	**<.001**	10.7	0.554 (0.403–0.760)	**<.001**
MGMT-methylation							
Yes	39%	20.9	0.361 (0.263–0.497)	**<.001**	12.0	0.471 (0.343–0.649)	**<.001**
No	61%	11.5			5.5		
IDH-mutation							
Yes	10%	23.8	0.612 (0.359–1.044)	.072	11.3	0.548 (0.319–0.939)	**.029**
No	90%	13.4			6.3		
SVZ contact							
Yes	68%	12.2	1.439 (1.067–1.941)	**.017**	5.9	1.523 (1.119–2.072)	**.007**
No	32%	16.9			10.3		

*Numerical variables were not categorized in Cox regression model.
Significant *p*-values are highlighted in bold. KPS = Karnofsky Performance Score; STR = subtotal resection; GTR = gross total resection; MGMT = 0^6-methylguanine-DNA-methyltransferase; IDH = isocitrate dehydrogenase; SVZ = subventricular zone.

Table 3. Multivariate Cox regression model of glioblastoma patient survival adjusted for age, KPS, extent of resection, MGMT-methylation and SVZ contact.

Parameter	Overall survival hazard ratio (95% CI)	Wald test *p*	Progression-free survival hazard ratio (95% CI)	Wald test *p*
Age	1.029 (1.014–1.045)	**<.001**	1.013 (0.998–1.028)	.093
KPS	0.982 (0.969–0.995)	**.008**	0.985 (0.973–0.997)	**.018**
Biopsy vs resection	0.503 (0.343–0.736)	**<.001**	0.594 (0.405–0.872)	**.008**
MGMT-methylated	0.294 (0.203–0.425)	**<.001**	0.442 (0.308–0.634)	**<.001**
IDH-mutated	0.685 (0.518–1.541)	.685	0.653 (0.368–1.158)	.145
With SVZ contact	1.464 (1.027–2.088)	**.035**	1.483 (1.037–2.121)	**.031**

Significant *p*-values are highlighted in bold. CI = confidence interval; KPS = Karnofsky Performance Score; MGMT = 0^6-methylguanine-DNA-methyltransferase; IDH = isocitrate dehydrogenase; SVZ = subventricular zone.

analysis. Our survival results are analogous to the results of the 2017 meta-analysis on SVZ contact and glioblastoma patient survival [6] and to those more recently obtained by Van Dijken et al. [7] and Mistry et al. [9]. In their 2019 study, Mistry et al. showed that SVZ_{pos} glioblastoma was associated with decreased survival and also with post-treatment hydrocephalus and leptomeningeal dissemination. This negative influence on glioblastoma patient survival of SVZ contact was also independent from ventricular entry during neurosurgical resection [9]. Moreover, in another report studying TCGA molecular data of glioblastoma, the same group could not find a distinct molecular biological profile of SVZ_{pos} glioblastoma [17]. Another group came to the same findings [8]. If the molecular signature of SVZ_{pos} tumors is not fundamentally different from SVZ_{neg} glioblastoma, possibly the interplay between glioblastoma cells and the complex and unique SVZ niche may hold the key to understanding how SVZ contact of glioblastoma influences patient survival [18]. For example, it has been shown in a mouse model that glioma cells invading the SVZ become radioresistant by influence of SVZ chemokines [19].

The current finding of significantly larger tumor size for SVZ_{pos} glioblastoma is consistent with previously published findings [7,8,15,20]. Nevertheless, this observation alone is insufficient to establish the SVZ origin of glioblastoma. A direct correlation between anatomical localization of glioblastoma and its origin is unlikely [20]. Mathematical glioma growth models demonstrate that tumors originating at a distance from the SVZ have a high likelihood of reaching the SVZ before detection [21,22]. SVZ_{pos} glioblastoma may just be the consequence of a highly malignant and rapidly growing tumoral lesion within the limited confinements of the brain rather than the indication of pure SVZ origin [22].

Most importantly, this study acknowledges SVZ contact as a negative prognostic factor for glioblastoma patient survival, independent from age, KPS, extent of resection, MGMT-methylation status and IDH mutation (Table 3, Figure 2). In other words, SVZ_{pos} glioblastoma is even more aggressive than SVZ_{neg} tumors. The SVZ niche in glioblastoma patients may become a therapeutic target in the future whether by radiating the SVZ or by targeting specific components of the SVZ microenvironment [5,18]. We join the call already made by Smith et al. in 2016 to increase translational and basic research on the SVZ and its role in glioblastoma [23].

This study has several shortcomings of which selection bias due to the retrospective study design may be the most important. Furthermore, corticosteroid use at diagnosis was not included in the analysis nor were the different treatment

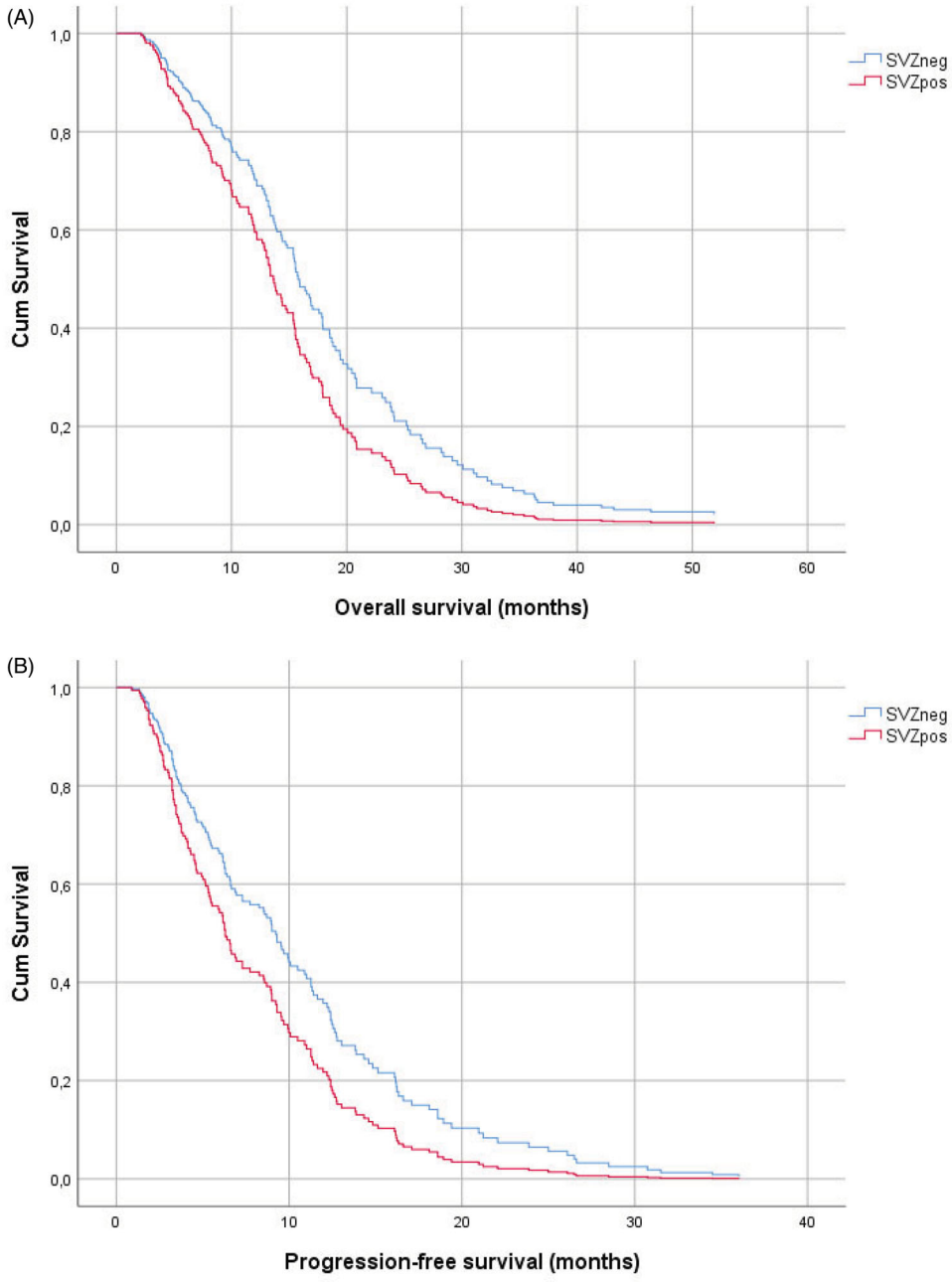

Figure 2. Kaplan-Meier curves of overall survival (A) and progression-free survival (B) in glioblastoma with (SVZ_{pos}) and without (SVZ_{neg}) subventricular zone contact.

modalities applied for disease recurrence or progression. Nevertheless, we present a large series of glioblastoma patients who were uniformly treated in first tier treatment. We were able to include MGMT-promoter methylation status and IDH-mutation in the study and adjust survival analysis for these and other well-known prognostic factors.

Conclusion

In this glioblastoma patient cohort, SVZ contact at diagnosis is a negative and independent prognostic factor. The SVZ_{pos} patient group had significantly more MGMT-unmethylated and larger tumors. More studies are needed to be able to draw a definitive conclusion whether differences in MGMT-methylation pattern exist between glioblastoma SVZ contact groups. The role of SVZ contact in glioblastoma needs to be examined further in larger patient groups in order to establish how SVZ contact influences patient survival. The SVZ emerges as a potential therapeutic target in glioblastoma treatment.

Acknowledgement

We gratefully acknowledge the help of Prof. em. Dr. M. Mareel with the study.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or

national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of both participating hospitals (Belgian Registration number B670201730765; AZD 17004; UZG 2016/1594).

Informed consent

The need for informed consent was waived by the ethical committees of both participating hospitals.

Author contributions

All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by Giorgio Hallaert and Harry Pinson. The first draft of the manuscript was written by Giorgio Hallaert and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by grants from the following nonprofit organizations: Ghent University Hospital, Center for Oncology; "*Stichting Luka Hemelaere*"; and "*Fonds Arne Lannoy AKA Zorro*".

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PUBLICATION 3

Survival impact of incidental subventricular zone irradiation in *IDH*-wildtype glioblastoma

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Acta Oncol. 2021; 60 (5): 613-619

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If the subventricular zone (SVZ) harbors the glioblastoma stem cell, then this brain region may emerge as a therapeutic target for glioblastoma patient treatment. The report by Evers et al. in 2010 was the first to study the correlation between incidental SVZ irradiation and high-grade glioma patient outcome (1). In a cohort of 55 patients, they found a significantly better progression-free survival if the bilateral SVZ received a dose of 43 Gy or higher. Several reports on the impact of SVZ irradiation on glioblastoma patient survival were published since. However, recent reviews on SVZ radiation could only conclude that the literature is conflicting, and definitive conclusions cannot be drawn at this point in time (2,3). Reports on SVZ radiation in the group of *IDH*-wildtype glioblastoma only are very rare. We investigated whether a correlation exists between incidental SVZ irradiation and patient survival in a cohort of *IDH*-wildtype glioblastoma patients.

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ACTA ONCOLOGICA
https://doi.org/10.1080/0284186X.2021.1893899

Taylor & Francis
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ORIGINAL ARTICLE

Survival impact of incidental subventricular zone irradiation in IDH-wildtype glioblastoma

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ABSTRACT

Background and purpose: The subventricular zone (SVZ) is an important niche for neural stem cells but probably also for brain tumor propagating cells, including the glioblastoma stem cell. The SVZ may become a target for radiation therapy in glioblastoma patients. However, reports studying the effect of irradiation of the SVZ on glioblastoma patient survival show conflicting results. We studied the correlation between incidental SVZ radiation dose and survival in a cohort of *isocitrate dehydrogenase*-wildtype (IDHwt) glioblastoma patients with inclusion of important survival prognosticators.

Patients and methods: In this retrospective analysis, only adult patients with supratentorial IDHwt glioblastoma were included who were treated with temozolomide-based chemoradiotherapy after surgery. The SVZ was contoured on the radiotherapy planning imaging. Cox proportional regression overall survival (OS) analysis was used to study the correlation between SVZ dose and survival. Age, Karnofsky Performance Score, extent of resection and O^6-*methylguanine-methyl-DNA-transferase* gene promoter (MGMTp) methylation were used as covariates in multivariate analysis.

Results: In total, 137 patients were included. Median OS was 13.3 months. The MGMTp methylation was present in 40% of cases. Ipsilateral SVZ (iSVZ) mean dose was 44.4 Gy and 27.2 Gy for the contralateral SVZ (cSVZ). Univariate survival analysis showed an inverse relationship between cSVZ mean dose and OS (HR 1.029 (1.003–1.057); $p = .032$). However, there was no correlation between cSVZ mean dose and OS in multivariate analysis. iSVZ dose did not correlate with survival.

Conclusion: In this cohort of 137 IDHwt glioblastoma patients, iSVZ did not correlate with OS. Higher cSVZ dose was inversely correlated with OS in univariate survival analysis but lost its significance in multivariate analysis, including MGMTp-methylation. Hence, the correlation between SVZ radiation and glioblastoma patient survival remains unclear. Carefully designed prospective studies are needed to provide unequivocal results on this controversial topic.

ARTICLE HISTORY
Received 21 December 2020
Accepted 18 February 2021

KEYWORDS
Glioblastoma; IDH-wildtype; subventricular zone; radiation therapy survival; MGMT-methylation

Introduction

Circumscribing the lateral walls of the lateral ventricles, the subventricular zone (SVZ) is the largest reservoir of neural stem cells (NSCs) in the adult human brain [1,2]. In humans, the SVZ contains a unique ribbon of astrocytes which are capable of self-renewal but also of differentiation into neural cell types and glial cells. The cancer stem cell (CSC) theory states that oncological diseases are hierarchically organized. Oncogenic mutations in stem cells lead to CSCs. These CSCs share several characteristics with normal stem cells, such as multipotency, low self-renewal rate, and a strictly regulated balance between proliferation and cell death, in which the local microenvironment of the stem cell niche has a crucial role [3]. Adapted to brain tumors, the CSC theory implies the existence of brain tumor propagating cells (BTPCs). In 2019, Lee et al. presented direct genetic evidence from both glioblastoma patients and mouse models that BTPCs develop from SVZ cells [4]. BTPCs divide and differentiate into proliferating glioblastoma cells which build up the bulk of the glioblastoma tumor mass. If the BTPCs are not eradicated, the tumor will inevitably return. This theory explains the high tumor recurrence rate in the vast majority of glioblastoma patients, despite maximum safe resection, radiation therapy, and concurrent temozolomide (TMZ)-based chemotherapy. Hence, targeting BTPCs or their microenvironment seems a logical next step in glioblastoma treatment [5]. Since BTPCs likely reside in the SVZ, this brain region may become a radiotherapeutic target in glioblastoma patients given that radiation therapy is already standard-of-care in glioblastoma treatment [6]. The past decade, several articles were published on the correlation between SVZ radiation and glioblastoma patient survival [7–20]. Three consecutive reviews of the available literature were unable to draw definitive conclusions from these reports [21–23]. Some of the major criticisms on these studies are the lack of inclusion

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Part of this work was presented at the 2019 meeting of the European Association of Neuro-Oncology in Lyon, France (P14.166).

of molecular biological factors in survival analysis, especially *isocitrate dehydrogenase* (IDH) mutation and *O^6-methylguanine-DNA-methyltransferase* gene promoter (MGMTp) methylation; relatively small sample sizes with subgroup analysis, limiting statistical power; and inclusion of other high-grade gliomas, next to glioblastoma, combined with lack of uniform first-tier treatment of patients at diagnosis [22]. Hence, reports studying the impact of SVZ radiation on survival of IDH-wildtype (IDHwt) glioblastoma patients with survival correction for MGMTp-methylation status are sparse. We wanted to evaluate the effect of incidental radiation to the SVZ on survival in a larger cohort of IDHwt glioblastoma patients who were uniformly treated with TMZ-based chemoradiotherapy after surgery [24]. MGMTp-methylation status was included in survival analysis.

Patients and methods

Patient selection

Adult patients (18 years and older) with supratentorial IDHwt glioblastoma were included in this retrospective study only if they completed the full radiotherapy course with concomitant TMZ chemotherapy after surgery. If patients did not complete six cycles of adjuvant TMZ, they were still eligible for inclusion. All patients were treated between 2003 and 2014 in two Flemish hospitals. Patients were excluded if the so-called Stupp protocol was not applied; if patients were lost to follow-up; or if radiotherapy planning data could not be retrieved or restored (Figure 1) [24]. Demographic parameters were collected from the patient charts or electronic medical records. Date of death was cross-checked with the Belgian Cancer Registry. This study was approved by the Ethics Committee of both participating hospitals (Belgian Registration number B670201730765; UZG 2016/1594; AZD 17004). The need for informed consent was waived because of the retrospective study design and because the study did not involve any risk to the patients. The STROBE guideline was used as a guide to write the manuscript.

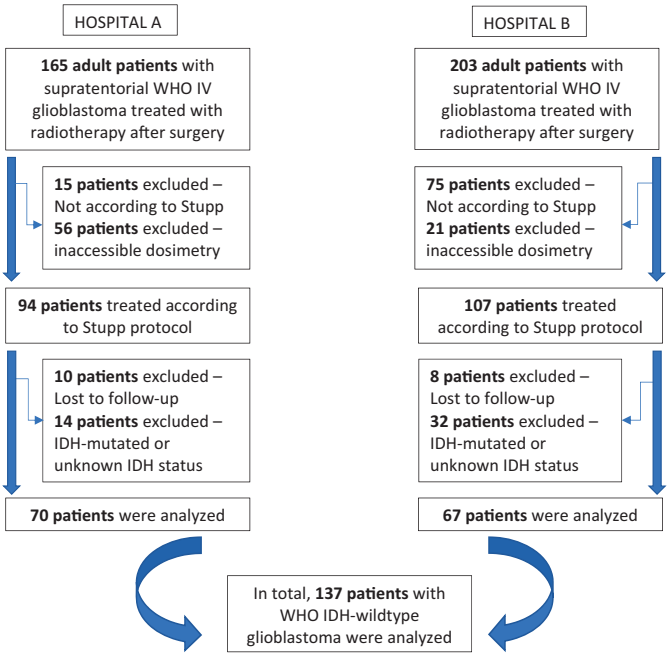

Figure 1. Selection criteria of the IDH-wildtype glioblastoma patient cohort.

Imaging, surgery, and molecular biological factors

The presence of SVZ contact at diagnosis was evaluated on post-contrast T1 magnetic resonance (MR) imaging. All MR images were acquired on 1.5 T or 3 T MR imaging systems (Siemens, Erlangen, Germany). If the contrast-enhancing part of the tumor abutted the ventricle, the tumor was classified as SVZ contacting glioblastoma. Surgery was dichotomized as biopsy vs. resection. Resection was further categorized as gross total resection (GTR) if there was no residual contrast-enhancement on postoperative imaging but as subtotal resection (STR) if there was. The neuropathologist selected an appropriate tissue specimen from the tumor database. Histological diagnosis was reconfirmed according to the 2016 World Health Organization (WHO) criteria. The presence of IDH1 or 2-mutation in exon 4 was analyzed using next-generation sequencing [25]. MGMTp-methylation was detected using semi-quantitative methylation-specific polymerase chain reaction (qMSP) as previously reported [26]. Finally, none of the patients were treated with tumor treating fields.

Radiotherapy: contouring and dosimetry

All patients were radiated using 3D conformal external beam therapy or intensity modulated radiotherapy. The radiation oncologist defined the GBM target volumes at the time of treatment planning. Gross tumor volume (GTV) was defined on planning computed tomography (CT) after co-registration with contrast-enhanced T1-weighted MR imaging. In general, a margin of 1–2 cm was used to generate the clinical target volume (CTV), taking into account anatomical borders and the FLAIR signal intensity. The planning target volume (PTV) was obtained by adding an additional 3–5 mm margin to the CTV. A median dose of 60 Gy in 30 fractions of 2 Gy was prescribed to the PTV. The SVZ was not contoured at the time of treatment and thus not intentionally irradiated. Ipsilateral and contralateral SVZs (cSVZs) were manually contoured on planning CT imaging at the time of this study. The SVZ was defined as a 5 mm zone surrounding the full extent of the lateral wall of the lateral ventricles and of the temporal horns (Figure 2), as anatomically and histologically described [2]. If the contrast-enhancing part of the tumor invaded the SVZ, then this region of the SVZ was not included in the contouring. The ipsilateral SVZ (iSVZ) was defined as the SVZ on the same side as the tumor. In case of multifocal or midline tumors, the iSVZ was defined as the side where the bulk of the tumor was located. Dose–volume histograms were calculated. Mean and quartile doses were obtained for the iSVZ and cSVZ.

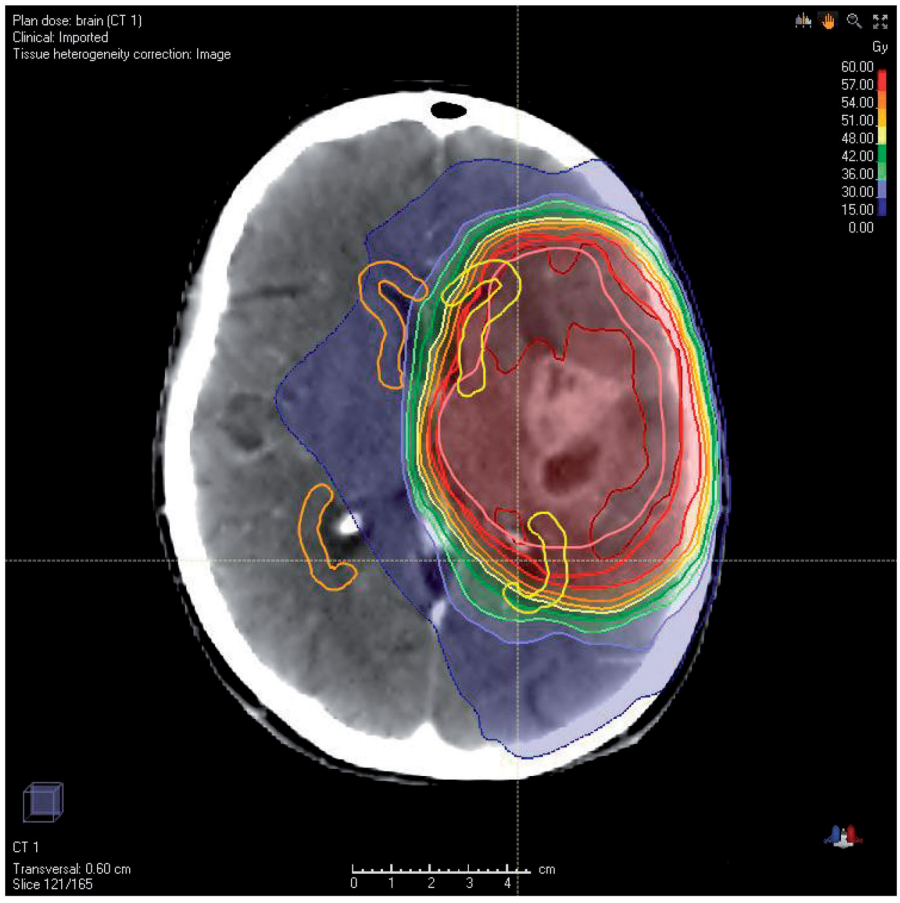

Figure 2. Representative illustration of two-dimension segmentation of the ipsilateral (yellow) and contralateral (orange) subventricular zone on radiotherapy planning CT imaging.

Statistical analysis

Progression-free survival (PFS) was defined as the time between the date of first surgery and the date of radiologic evidence of disease recurrence or progression or the date of change in treatment plan due to clinical disease progression, or patient death if this occurred before there was radiologic evidence of disease progression. Overall survival (OS) was calculated as the time between the date of the first surgery and date of death. Patients who were alive at the time of the analysis were censored for OS. Patients without disease progression were censored for PFS at the time of the last registered follow-up visit. The median survival times were calculated using the Kaplan–Meier estimates. Prognostic factors for PFS and OS were studied using univariate Cox proportional hazards analysis, including the mean doses of iSVZ and cSVZ. If SVZ dose showed a significant correlation with survival in univariate analysis, multivariate Cox proportional hazard models were subsequentially fitted adjusted for the following prognostic factors: age, Karnofsky Performance Score (KPS), extent of resection (biopsy vs. resection), and MGMTp-methylation (Figure 3). Although SVZ contact at diagnosis is described as a negative and independent prognosticator for glioblastoma patient survival [27,28], it was not included in multivariate survival analysis because of collinearity. Numerical variables were not dichotomized in Cox regression models [29]. For categorical variables, graphical methods were applied to check the proportional hazards assumption while for numerical variables the assumption was verified by means of introduction of a time-dependent co-variable in the model. All analyses were performed with SPSS (v26, IBM, Armonk, NY, USA). *P* values less than .05 were considered significant for two-sided testing.

Results

The study included 137 IDHwt glioblastoma patients of whom 62.8% were males (Table 1). The average patient age was 62.2 years. In 69.3% of cases, the tumor abutted the ventricle at diagnosis. Patients underwent surgical resection in 74.4% of cases but in 25.6% only a biopsy was performed. MGMTp-methylation was present in 54 patients (39.4%). Mean incidental radiation doses to the SVZ were 44.4 Gy and 27.2 Gy for the iSVZ and cSVZ, respectively (Table 1).

Eight patients were alive at the time of the database closure and were censored for OS. Median survival rates were 13.3 months for OS and 6.4 months for PFS. The following parameters proved statistically significant prognostic factors for both OS and PFS in univariate survival analysis: KPS; extent of resection; MGMTp-methylation (Figure 3); and SVZ contact at diagnosis (Table 2). Higher mean cSVZ dose was correlated with worse OS (HR 1.029; 95% CI 1.003–1.057; $p = .032$) in univariate survival analysis. Using a multivariate Cox regression survival model, adjusting survival for age,

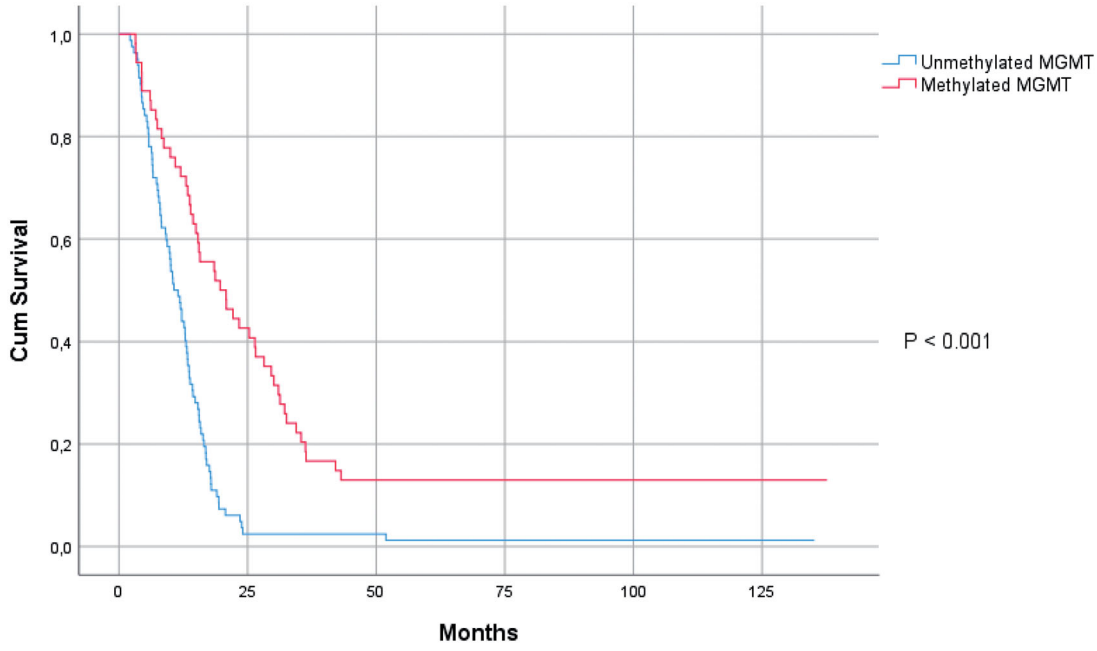

Figure 3. The Kaplan–Meier overall survival curves for IDH-wildtype glioblastoma patients according to methylation status of the MGMT gene promoter.

Table 1. Demographical, radiological, molecular and treatment characteristics of 137 IDH-wildtype glioblastoma patients.

Characteristic	
Sex, *n* (%)	
Female/male	51 (37.2)/86 (62.8)
Mean age, years (minimum–maximum)	62.2 (31–80)
Preoperative KPS $\geq$ 70, *n* (%)	95 (69.3)
Surgery, *n* (%)	
Gross total resection	44 (32.1)
Subtotal resection	58 (42.3)
Biopsy	35 (25.6)
Tumor laterality, *n* (%)	
Left	70 (51.1)
Right	59 (43.1)
Bilateral	8 (5.8)
SVZ contact at diagnosis, *n* (%)	95 (69.3)
MGMT-promoter, *n* (%)	
Methylated	54 (39.4)
Unmethylated	82 (59.9)
Unknown	1 (0.7)
Survival (months)	
Median overall survival (95% CI)	13.3 (12.0–14.7)
Median progression-free survival (95% CI)	6.4 (5.4–7.4)
Planning target volume	
Mean volume, mL (SD)	284.7 (108.1)
Median dose, Gy	60.1
Median dose quartiles, Gy (25th,75th)	60.0; 60.5
Ipsilateral SVZ	
Mean volume, mL (SD)	19.7 (7.4)
Mean dose, Gy	44.4
Mean dose quartiles, Gy (25th, 50th, 75th)	36.0; 46.9; 53.5
Contralateral SVZ	
Mean volume, mL (SD)	25.1 (8.5)
Mean dose, Gy	27.2
Mean dose quartiles, Gy (25th, 50th, 75th)	16.6; 25.6; 36.0

KPS: Karnofsky Performance Score; SVZ: subventricular zone; MGMT: *O^6-methylguanine-DNA-methyltransferase*; CI: confidence interval; SD: standard deviation.

KPS, extent of resection and MGMTp-methylation, cSVZ mean dose did not show a statistically significant correlation with OS (Table 3). iSVZ mean dose was not correlated with survival (Table 2). Age, extent of resection, and MGMTp-methylation proved independent prognosticators for OS in multivariate survival analysis (Table 3).

Discussion

The results from univariate survival analysis in this study of IDHwt glioblastoma patients show that incidental radiation dose to the cSVZ has negative impact on OS although the hazard ratio is very small (1.029). However, after adjustment of survival for age, KPS, extent of resection and MGMTp-methylation, cSVZ mean dose lost its significance. iSVZ mean dose did not correlate with survival.

Molecular biological factors play a key role in glioblastoma patient survival. MGMTp-methylation is a well-known epigenetic phenomenon in glioblastoma, silencing the MGMT gene and consequentially rendering the tumor cells more vulnerable to the alkylating effects of chemotherapeutics [26]. The presence or absence of the IDH-mutation is an essential molecular marker for all gliomas and since the publication of the 2016 WHO Classification of Central Nervous Tumors, IDHwt and IDH-mutated glioblastoma are to be distinguished [30]. In most studies on SVZ radiation in glioblastoma, however, molecular markers are missing. IDH-mutation was only considered in two previous reports [17,18]. The IDH-mutation status was missing in more than 90% of cases in the publication by Achari et al., but MGMT-methylation could be determined in 97% of cases [17]. The second report, by Muracciole et al., presented complete data for MGMT-methylation in an IDHwt glioblastoma patient population, similar to the cohort presented in this study [18]. Although the patient cohorts in the publications by Achari et al. and Muracciole et al. were relatively small (61 and 50 patients, respectively), both reports concluded that higher SVZ radiation dose had a negative impact on glioblastoma patient survival. Together with the 2014 publication by Elicin et al., ours is the fourth article that shows that increasing SVZ dose

Table 2. Univariate survival analysis.

Parameter	Overall survival		Progression-free survival	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
Age[a]	1.005 (0.972–1.039)	.784	0.982 (0.952–1.013)	.241
KPS[b]	0.979 (0.964–0.994)	**.007**	0.980 (0.965–0.994)	**.007**
STR (vs. biopsy)	0.627 (0.406–0.968)	**.035**	0.503 (0.320–0.791)	**.003**
GTR (vs. biopsy)	0.417 (0.261–0.668)	**<.001**	0.395 (0.240–0.650)	**<.001**
With SVZ-contact	1.476 (1.011–2.156)	**.044**	1.773 (1.203–2.611)	**.004**
MGMTp-methylated	0.362 (0.245–0.534)	**<.001**	0.432 (0.292–0.640)	**<.001**
Mean ipsilateral SVZ dose[c]	1.014 (0.987–1.043)	.313	1.006 (0.981– 1.031)	.657
Mean contralateral SVZ dose[c]	1.029 (1.003–1.057)	**.032**	1.009 (0.986–1.032)	.448

HR: hazard ratio; CI: confidence interval; KPS: Karnofsky Performance Score; STR: subtotal resection; GTR: gross total resection; SVZ: subventricular zone; MGMTp: *O*^{6}*-methylguanine-DNA-methyltransferase* gene promoter.
Statistically significant *p* values in bold.
[a]Per increment of one year.
[b]Per increment of 10.
[c]Per increment of 1 Gy.

Table 3. Multivariate overall survival analysis model for contralateral subventricular zone mean dose.

Parameter	Overall survival	
	HR (95% CI)	*p* Value
Age[a]	1.034 (1.014–1.054)	**.001**
KPS[b]	0.986 (0.971–1.001)	.074
Resection (vs. biopsy)	0.432 (0.278–0.669)	**<.001**
MGMTp-methylated	0.294 (0.196–0.443)	**<.001**
Contralateral SVZ dose[c]	1.016 (0.988–1.046)	.268

HR: hazard ratio; CI: confidence interval; KPS: Karnofsky Performance Score; MGMTp: *O*^{6}*-methylguanine-DNA-methyltransferase* gene promoter; SVZ: subventricular zone.
Statistically significant *p* values in bold.
[a]Per increment of one year.
[b]Per increment of 10.
[c]Per increment of 1 Gy.

may have a negative impact on glioblastoma patient survival [12]. The deleterious effect may be explained by the known side-effects of brain radiation, most importantly neurocognitive toxicity and lymphopenia [31]. Of course, also TMZ may cause moderate to severe lymphopenia. Furthermore, evidence shows the high vulnerability of NSCs to even low doses of radiation although a better long-term recovery for NSCs in the SVZ is assumed [32]. Hence, higher radiation doses to the SVZ may result in definitive destruction of the majority of NSCs and subsequentially in severely diminished restorative capacities of the brain. Contrary, the results from many previous publications, that a beneficial effect on survival of higher SVZ dose is present, are possibly biased by the lack of inclusion of the aforementioned molecular markers in survival analysis [7–11,13–16,22].

Another important consideration is the fact that there is at present no consensus in the literature on a cutoff value for SVZ radiation nor on contouring the SVZ [21–23]. Most publications use a high-dose cutoff because of the evidence that BTPCs are very likely to be radioresistant, possibly aided by the hypoxic microenvironment that is also present in the normal SVZ [33–35]. But even this 'high-dose' cutoff varies in the reports from 43 Gy to 59 Gy [22]. We chose not to categorize the SVZ mean doses but to include the doses as continuous variables in regression analyses, contrary to most other reports, in order to maximize statistical power and to avoid the bias of small subgroup analyses [29]. The lack of consensus on the methodology of contouring the SVZ is illustrated by the significant variability of published shapes and volumes of the SVZ [22]. Interestingly, a recent report showed the occurrence of significant SVZ volume shifts also during treatment, impacting dosimetric results [36]. Indeed, the published mean SVZ dose also varies widely across the literature [22]. We chose to perform the contouring of the SVZ according to the published cytoarchitectonic findings, comprising the full extent of the lateral ventricular system and including the temporal horns [1,2,22]. This approach explains why the SVZ volumes in this study are higher than in most previous publications and the SVZ mean doses are lower.

Contact of the contrast-enhancing part of the tumor with the SVZ is likely an independent negative prognostic factor for glioblastoma patient survival [27,28]. In this patient cohort, SVZ contact was present in almost 70% of cases and proved a significant prognostic factor for both PFS and OS (Table 2). Given the fact that traditional radiotherapy treatment protocols for glioblastoma have a 2–3 cm CTV margin, it logically follows that the iSVZ but also the cSVZ will receive by default higher radiation doses in the presence of SVZ contacting glioblastoma. Hence, SVZ contact could not be included in multivariate survival analysis because of collinearity as was the case in previous studies [10]. Moreover, we agree with Nourallah et al. that it is nearly impossible to distinguish between the prognostic effect of SVZ contact and a possible survival impact of SVZ radiation in a retrospective study [22]. This effect is present in every published study, including the current one.

Furthermore, the SVZ may be not the only niche of BTPCs in case of glioblastoma. Evidence suggests the existence of several niches for BTPCs and Aderetti et al. recently proposed the concept of the 'hypoxic peri-arteriolar glioma stem cell niche' [37]. Moreover, the concepts of the SVZ niche and the peri-arteriolar niche are complementary, rather than mutually exclusive. It is likely that BTPCs migrate from one niche to another, given the complex interplay of signaling pathways and microtubes that is progressively unraveled. But the existence of other BTPCs niches outside the SVZ, may explain why SVZ irradiation yields no therapeutic benefit in case of glioblastoma.

This study is prone to several similar shortcomings as were previous reports. Most importantly, the retrospective study design is subject to selection bias. Corticosteroid use was not studied and treatment for tumor recurrence was not standardized nor included in the study. The contouring of the SVZ could only be performed on planning CT and not on CT-MR fusion images. We could not overcome the problem of collinearity between SVZ contact and SVZ radiation dose. But we think the weaknesses of the study are proportionate to its strengths. Molecular biological factors were analyzed using sensitive and reliable methods [25,26] and MGMTp-methylation was included in multivariate survival analysis. The cohort of IDHwt glioblastoma patients is relatively large, especially when compared with the only other report studying the impact of SVZ radiation in IDHwt glioblastoma patients [18]. Finally, all patients received uniform first-tier treatment after surgery.

Conclusion

In this retrospective analysis of a cohort of uniformly treated IDHwt glioblastoma patients, higher cSVZ mean dose negatively impacted OS in univariate survival analysis, albeit with a very small hazard ratio, but lost its significance after adjustment of survival for age, KPS, extent of resection, and MGMTp-methylation. iSVZ mean dose did not correlate with survival.

Taken together, the results from SVZ radiation studies in glioblastoma are controversial at best. Adapting radiotherapeutic plans of IDHwt glioblastoma patients at this point in time to intentionally include or exclude the SVZ in the irradiated volume cannot be recommended. Unfortunately, one prospective trial (NCT02039778) studying the impact of deliberately radiating the SVZ in glioblastoma patients was terminated early because of poor patient accrual. Hopefully, the only ongoing prospective study (NCT02177578) of intentionally radiating the SVZ to 60 Gy in glioblastoma patients, will provide unequivocal results.

Acknowledgements

The authors gratefully acknowledge the help of Prof. em. Dr. M. Mareel with the study. We are in debt to the radiophysicists of AZ Delta and Ghent University Hospital for their help in retrieving and restoring the dosimetric data. We would like to specifically acknowledge the efforts made by F. Vanhoutte, PhD, H. Vanhauwaert, T. Vercauteren, and W. De Gersem, PhD.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of each hospital (Belgian registration number B670201730765; UZG 2016/1594; AZD 17004).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was financially supported by grants from the following non-profit organizations: Ghent University Hospital, Centre for Oncology; Lux Luka Foundation and Fonds Arne Lannoy AKA Zorro.

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Data availability statement

Research data are available on request.

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PUBLICATION 4

Partial resection offers an overall survival benefit over biopsy in *MGMT*-unmethylated *IDH*-wildtype glioblastoma patients

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Surg Oncol. 2020; 35: 515–9

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Ever since the beginning of modern neurosurgery, resection of glioblastoma has sparked intense debate. Already in 1928, it was shown by Dandy that glioblastoma, despite hemispherectomy, still recurs (1). The diffuse infiltrative nature of glioblastoma, growing along white matter fibers and following perivascular spaces, and its proximity to eloquent brain zones in a high number of patients, precludes this disease from being surgically curable. Since the year 2000 onwards, there has been a steady flow of reports showing a correlation between extent of resection and survival. Although methodological issues remain, there is accumulating evidence that gross total resection (GTR) results in better survival for glioblastoma patients (2,3). Most neurosurgical departments nowadays have adapted the principle of "maximum safe resection". But, unfortunately, in most glioblastoma patients obtaining GTR is *a priori* impossible. Consequently, the answer to the question if partial resection of glioblastoma, as compared to biopsy, yields a true survival benefit, is of high importance and even more in the molecular age of glioblastoma.

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Surgical Oncology 35 (2020) 515–519

Contents lists available at ScienceDirect

Surgical Oncology

journal homepage: http://www.elsevier.com/locate/suronc

Partial resection offers an overall survival benefit over biopsy in MGMT-unmethylated IDH-wildtype glioblastoma patients

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ARTICLE INFO

Keywords:
IDH-Wildtype glioblastoma
Overall survival
Partial resection
MGMT-methylation

ABSTRACT

Background

Isocitrate dehydrogenase (IDH)-wildtype glioblastoma patients with *O*^{6}*-methylguanine-DNA-methyltransferase* (MGMT)-unmethylated tumors have the worst outcome of all glioblastoma patients. The overall survival (OS) benefit of partial resection of glioblastoma compared to biopsy only remains controversial specifically in relation to molecular factors. In this report, we analyzed the effect of incomplete resection on OS compared to biopsy only in a cohort of IDH-wildtype glioblastoma patients who were uniformly treated with temozolomide-based chemoradiotherapy (TMZ-CR) after surgery.

Material & Methods

A retrospective study was conducted including only glioblastoma patients who were treated with TMZ-CR after surgery from two centers. Surgical groups were defined as biopsy only, partial resection (PR) or gross total resection depending on the presence of contrast-enhancing tumor on postoperative imaging. IDH-mutation was determined using next generation sequencing technique and MGMT-methylation was analyzed with semi-quantitative methylation-specific polymerase chain reaction. Next to descriptive statistics, univariate and multivariate survival analyses were performed using Kaplan-Meier estimates and Cox regression models.

Results

In total, 159 patients were included. 37 patients underwent biopsy only and 73 partial resections. 99 patients (62.3%) harbored unmethylated tumors. Median OS for the whole patient group was 13.4 months. In the subgroup of patients with unmethylated tumors, PR yielded a median OS of 12.2 months vs 7.6 months for biopsy patients ($P = 0.003$). PR proved an independent beneficial prognostic factor in multivariate Cox regression model, together with age, Karnofsky Performance Score and MGMT-methylation.

Conclusion

In IDH-wildtype glioblastoma patients with MGMT-unmethylated tumors, treated with chemoradiotherapy after surgery, PR yields a significant OS benefit compared to biopsy.

1. Introduction

The contemporary treatment of patients suffering from glioblastoma is based on the so-called "Stupp protocol": temozolomide-based chemoradiotherapy (TMZ-CR) after surgery [1]. Temozolomide is administered both during and after radiotherapy (60 Gy delivered in 30 fractions), if tolerated.

Molecular factors play a key role in the development and prognosis of glioblastoma. Methylation of the *O*^{6}*-methylguanine-DNA-methyltransferase* (MGMT) promoter is one of the most important prognostic factors in glioblastoma patient survival, in part by rendering the tumor tissue more susceptible to the cytotoxic effects of alkylating agents [2,3]. As from the 2016 WHO classification of tumors of the central nervous system, mutation of the *isocitrate dehydrogenase* 1 gene (IDH1) – and, rarely, of the IDH2 gene – is generally accepted as the molecular signature of "secondary glioblastoma" or IDH mutated glioblastoma [4,

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https://doi.org/10.1016/j.suronc.2020.10.016
Received 27 May 2020; Received in revised form 30 September 2020; Accepted 27 October 2020
Available online 1 November 2020

5]. IDH-wildtype and mutated glioblastoma do not only differ in molecular factors, but also in radiological features, patient demographics, clinical course and in prognosis: IDH-wildtype glioblastoma has a significantly worse prognosis than its IDH-mutated counterpart, with a median overall survival (OS) of 15 months compared to 31 months respectively [4,6]. Importantly, 90% of newly diagnosed glioblastoma are IDH-wildtype tumors and in more than half of these cases (about 60%) the MGMT gene promoter will be unmethylated [4,7]. In other words, the majority of newly diagnosed glioblastoma patients will have MGMT-unmethylated IDH-wildtype tumors.

Surgery constitutes a keystone element in glioblastoma treatment [8]. In the past years, several studies sought to demonstrate a resection threshold that would correlate with glioblastoma patient survival [9–13]. A meta-analysis of the relevant literature concluded in 2016 that gross total resection (GTR) offers a significant survival benefit over partial resection (PR) but the quality of the evidence according to the GRADE criteria was moderate to low [14]. In most neuro-oncological centers, the current consensus and practice is that of "maximum safe resection" for glioblastoma patients. However, the literature remains conflicting when it comes to the role of PR. Some reports show a survival advantage associated with PR while others see no benefit in PR over biopsy only [11,15,16]. So, the question remains if incomplete resection offers a survival benefit over biopsy for glioblastoma patients with unfavorable molecular markers who will be treated according to the Stupp protocol. Since surgical resection via craniotomy has higher complication rates than needle biopsy, the answer may play a pivotal role in glioblastoma patient counseling [17].

In this retrospective study, we evaluate the effect of partial resection on OS as compared to biopsy only in a cohort of IDH-wildtype glioblastoma patients who were uniformly treated with radiotherapy and temozolomide after surgery.

2. Material & methods

All glioblastoma patients treated in two Flemish hospitals between 2003 and 2014 were evaluated. In this retrospective study, only adult patients with IDH-wildtype supratentorial glioblastoma and who completed temozolomide-based chemoradiotherapy after surgery were enrolled. 3D-conformal beam or intensity modulated radiotherapy was applied, consisting of 30 fractions of 2 Gy. If tolerated, temozolomide was continued in the adjuvant setting for six cycles. Patients with a known malignant progression from low-grade glioma or with a previous history of any other brain tumor were excluded. Demographic parameters were retrieved from the patient files. OS was measured from the date of surgery to the date of decease. The Belgian Cancer Registry confirmed the date of death. Patients who were alive at the date of database closure (December 31, 2014) were censored for OS. The last date of follow-up collection was September 2017.

At the time of surgery, tumor tissue was stored in the tumor tissue archive. The neuropathologist selected and reviewed a representative formalin-fixed paraffin-embedded tissue block for each case. The pathological diagnosis of glioblastoma was reconfirmed according to the 2016 World Health Organization Classification. Semi-quantitative methylation-specific polymerase chain reaction (qMSP) was used to determine if the MGMT-promoter was methylated, as previously described [18]. Presence of the IDH1/2-mutation was tested using next-generation sequencing (NGS) techniques.

Preoperative as well as postoperative imaging was performed using 1,5 or 3T magnetic resonance imaging systems (Siemens, Erlangen, Germany). Postoperative imaging was performed within 72 h after surgery. Pre- and postcontrast T1 images were compared in order to be able to distinguish in the postoperative setting between blood products and residual contrast-enhancing tumor (RTV). Surgery was categorized into biopsy, PR or GTR. If postoperative imaging showed contrast-enhancement of the size of one voxel or more then surgery was classified as PR, as described by Stummer et al. [19]. Contrary, GTR was accepted when no residual contrast-enhancement was visible. If the 3D-T1 magnetization-prepared rapid acquisition gradient echo (MPRAGE) images (slice thickness of 0.9 mm) could be recovered, tumor volumes were measured using standard neuronavigation software (S7, Medtronic, Louisville, CO, USA) with the semi-automated segmentation technique [20].

Descriptive statistics are reported. Categorical variables were compared using the Pearson Chi square test. If the difference between surgical groups proved significant, pairwise comparisons were made using the Bonferroni correction. The Kruskal-Wallis test was applied for comparing numerical variables between surgical groups. Median OS estimates were calculated using the Kaplan-Meier method and compared with the log-rank test. Univariate Cox regression survival analysis was performed using the following prognostic factors: sex; age; Karnofsky Performance Score (KPS); MGMT-methylation status; surgical resection. Interaction variables were created for extent of resection and methylated MGMT and tested in univariate Cox regression. Next, a multivariate Cox regression model was fitted using prognosticators that proved statistically significant in univariate analysis. Graphical methods were used to assess that the proportional hazards assumption was respected. In Cox regression models, numerical variables were not categorized. P-values $<$ 0.05 using two-tailed test was defined as a statistically significant result. Data processing and statistical analyses were performed using SPSS (IBM SPSS, v26.0, Armonk, NY, USA).

The study was approved by the Ethics Committee of both hospitals (Belgian Registration number B670201730765; UZG 2016/1594; AZD 17004). Due to the retrospective study design and the fact that most patients had passed away at the time of database closure, the need for written informed consent was waived by both committees.

3. Results

In total, 159 patients were included in the study, and divided in three subgroups according to the type of surgery: biopsy only (37 patients), PR (73 patients) and GTR (49 patients). There were no missing data, except for preoperative tumor volume (Table 1). This was missing in 17% of patients because MPRAGE images could not be retrieved, mostly in patients from the 2003–2008 period. Only complete cases were used in analyses of preoperative tumor volume. Although the PR group had the highest median preoperative tumor volume, the differences in preoperative tumor volume between surgical groups were not statistically significant ($P = 0.131$). There were significantly more patients with favorable KPS in the GTR group as compared to the biopsy group (Table 1).

Median OS was 13.4 months for the entire patient cohort. Eight patients were censored for OS. Biopsy patients had a median OS of 8.3 months; PR patients of 13.7 months and GTR patients of 15.6 months (Table 2; Fig. 1). Patients with MGMT-methylated glioblastoma had a median OS of 19.7 months as compared to 11.8 months for patients with unmethylated glioblastoma (log-rank $P < 0.001$). In univariate Cox regression survival analysis, age, KPS, GTR, MGMT-methylation and the interaction variable for GTR and methylated MGMT were significantly associated with OS while sex, preoperative tumor volume, PR and the remaining interaction variables were not (Table 3). Within the group of unmethylated glioblastoma patients, partial resection yielded a significant OS advantage of 4.6 months but there was no significant OS difference between the PR and GTR patients (Table 2). Within the subgroup of methylated tumor patients, a significant OS difference was found between PR and GTR patients but not between biopsy and PR patients (Table 2; Fig. 1). A multivariate Cox regression model was fitted using type of resection, age, KPS and MGMT-methylation status as covariates. PR proved an independent beneficial prognostic factor compared to biopsy (HR = 0.53, $P = 0.003$). Methylation of the MGMT promoter was independently associated with a prominent reduction in hazard ratio (HR = 0.31, $P < 0.001$; Table 4).

Table 1
Synoptic overview of the characteristics, MGMT promoter methylation status and radiological findings of the entire cohort of IDH-wildtype glioblastoma patients and of the subgroups according to surgical classification.

Parameter	All patients (n = 159)	Biopsy (n = 37)	Partial Resection (n = 73)	Gross Total Resection (n = 49)	*P*-value
Sex, *n* (%)					0.627[a]
Female	59 (37.1)	13 (35.1)	25 (34.2)	21 (42.9)	
Male	100 (62.9)	24 (64.9)	48 (65.8)	28 (57.1)	
Age at diagnosis (years)					0.430[b]
Mean	61.5	60.7	61.1	62.5	
Minimum - Maximum	31–80	38–77	40–79	31–80	
Karnofsky Performance Score, *n* (%)					**0.010**[c]
≥70	111 (69.8)	19 (51.4)	52 (71.2)	40 (81.6)	
<70	48 (30.2)	18 (48.6)	21 (28.8)	9 (18.4)	
MGMT gene promoter, *n* (%)					0.912[a]
Unmethylated	99 (62.3)	24 (64.9)	44 (60.3)	31 (63.3)	
Methylated	60 (37.7)	13 (35.1)	29 (39.7)	18 (36.7)	
Preoperative tumor volume					0.131[b]
n (%)	132 (83)	31 (79.5)	61 (83.6)	40 (81.6)	
Median (mL)	26.4	21.5	32.4	22.9	
Minimum – Maximum (mL)	1.6–115.0	1.6–115.0	2.6–106.4	4.6–88.2	
Side, *n* (%)					**0.034**[d]
Left	84 (52.8)	13 (35.1)	45 (61.6)	26 (53.1)	
Right	69 (43.4)	21 (56.8)	25 (34.2)	23 (46.9)	
Bilateral	6 (3.6)	3 (8.1)	3 (4.1)	0 (0)	

[a] Chi square test.
[b] Kruskal-Wallis test.
[c] Chi square test between groups: biopsy - PR, $P = 0.057$; PR - GTR, $P = 0.207$; biopsy - GTR, $P = 0.004$; Bonferroni corrected $P < 0.017$.
[d] Chi square test between groups: biopsy - PR, $P = 0.036$; PR - GTR, $P = 0.166$; biopsy - GTR, $P = 0.048$; Bonferroni corrected $P < 0.017$.

Table 2
Comparison of Kaplan-Meier estimates of median overall survival of surgical groups in the whole patient cohort ($n = 159$) and according to MGMT-methylation status. 95% confidence intervals between brackets.

A. Comparison of the biopsy group with the partial resection group			
	Median overall survival (months)		
	Biopsy	Partial resection	Log-rank *P*
All patients	8.3 (5.72–10.88)	13.6 (11.71–15.62)	0.095
Methylated subgroup	13.8 (5.04–22.50)	18.8 (16.75–20.85)	0.804
Unmethylated subgroup	7.6 (5.59–9.67)	12.2 (9.49–14.91)	**0.002**
B. Comparison of the partial resection group with the gross total resection group			
	Median overall survival (months)		
	Partial resection	Gross total resection	Log-rank *P*
All patients	13.6 (11.71–15.62)	15.6 (14.20–16.94)	0.071
Methylated subgroup	18.8 (16.75–20.85)	31.0 (15.89–46.12)	**0.017**
Unmethylated subgroup	12.2 (9.49–14.91)	13.8 (10.9–16.78)	0.704

4. Discussion

Neurosurgical intervention is almost always necessary in glioblastoma management. After acquirement of tumor tissue, histopathological diagnosis can be established together with the study of molecular markers. Neurological symptoms due to mass effect can be relieved by

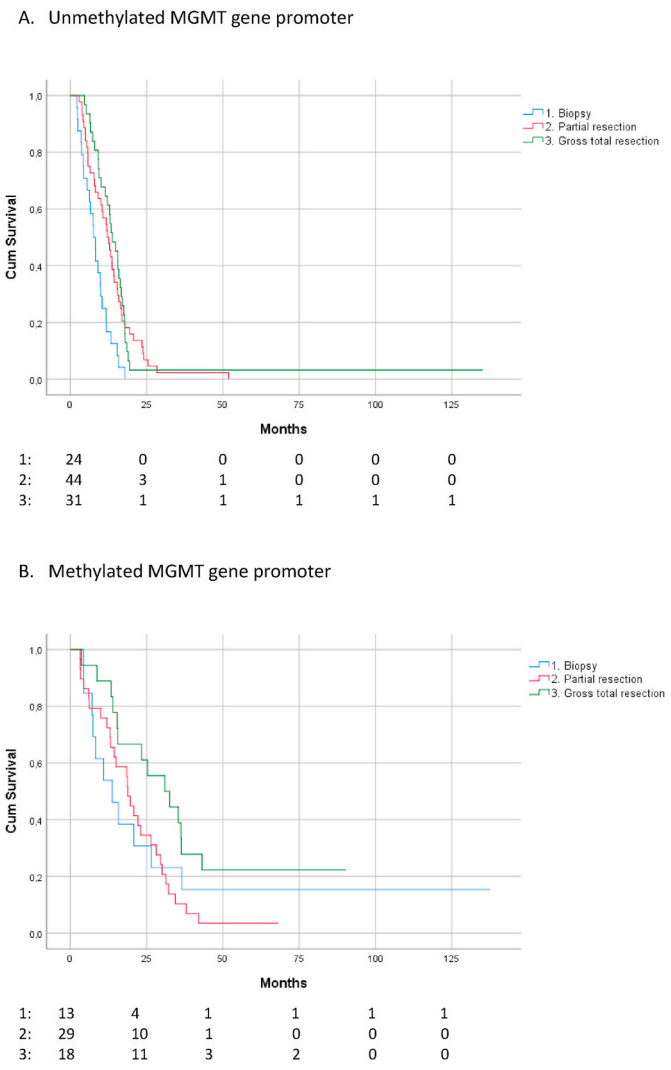

Fig. 1. Kaplan-Meier curves, with numbers at risk, for overall survival of IDH-wildtype glioblastoma patients according to extent of resection.
A Unmethylated MGMT gene promoter
B.Methylated MGMT gene promoter.

Table 3
Univariate Cox regression analysis of overall survival of IDH-wildtype glioblastoma patients. 95% confidence intervals between brackets.

Parameter	Hazard ratio	Wald test *P*
Age[a]	1.03 (1.01–1.04)	**0.002**
KPS[b]	0.98 (0.97–0.99)	**0.005**
MGMT-methylated	0.37 (0.26–0.53)	**< 0.001**
PR (vs biopsy)	0.70 (0.46–1.05)	0.081
GTR (vs biopsy)	0.50 (0.32–0.79)	**0.003**
Sex (male)	0.88 (0.63–1.23)	0.880
Preoperative Tumor volume[c]	1.0 (0.99-1.01)	0.724
I_M*B[d]	0.72 (0.39–1.32)	0.286
I_M*PR[e]	0.70 (0.46–1.06)	0.091
I_M*GTR[f]	0.35 (0.20–0.61)	**< 0.001**

KPS = Karnofsky Performance Score; MGMT = 0^6-methylguanine-DNA-methyltransferase; PR = partial resection; GTR = gross total resection.
[a] Per increment of one year.
[b] Per increment of 10.
[c] Per increment of 1 mL; data available in 83% of patients.
[d] Interaction variable for biopsy and methylated MGMT.
[e] Interaction variable for partial resection and methylated MGMT.
[f] Interaction variable for gross total resection and methylated MGMT.

Table 4
Multivariate Cox regression overall survival model of IDH-wildtype glioblastoma patients ($n = 159$). 95% confidence intervals between brackets.

Parameter	Hazard ratio	Wald test *P*
Age[a]	1.04 (1.02–1.06)	**< 0.001**
KPS[b]	0.98 (0.97–0.99)	**0.025**
MGMT-methylated	0.31 (0.22–0.46)	**< 0.001**
PR (vs biopsy)	0.53 (0.35–0.80)	**0.003**
GTR (vs biopsy)	0.39 (0.25–0.61)	**< 0.001**

KPS = Karnofsky Performance Score; MGMT = 06-methylguanine-DNA-methyltransferase; PR = partial resection; GTR = gross total resection.
[a] Per increment of one year.
[b] Per increment of 10.

tumor resection and steroid dependence may also be reduced. Although the highly infiltrative nature of glioblastoma excludes this tumor as a surgically curable disease, surgery has an important role in oncological control [14]. Recent studies confirmed GTR as an independent prognostic factor for glioblastoma patient survival, and still when molecular factors are considered [21–23]. Concerning PR and survival, however, the evidence is conflicting [11,15,16]. The results from this study show that PR in case of IDH-wildtype glioblastoma results in a significant OS benefit compared to biopsy only patients, specifically in the group of patients with MGMT-unmethylated tumors. Multivariate survival analysis showed that PR is an independent prognosticator, together with age, KPS and methylation of the MGMT gene promoter (Table 4).

Most of the patients with newly diagnosed glioblastoma will have IDH-wildtype tumors with unmethylated MGMT [2,5,7,8]. Obtaining GTR remains a major challenge for neurosurgeons and prospective studies show that GTR may be attainable in only 40% of glioblastoma patients [11,24]. A few years ago, a prospective study found no survival benefit for glioblastoma patients who underwent incomplete resection when compared to biopsy only [11]. Importantly, this study did not take the IDH mutation status into account. Based on the demographic characteristics of the study population, including young patients, it is likely that several patients with IDH mutated glioblastoma were included in the analysis. The possible random imbalance of patients with IDH-mutated glioblastoma between surgical groups, constitutes an important confounding factor in the survival analysis conducted in the report by Kreth and colleagues [11]. Our results acknowledge the conclusion of two recent retrospective studies [15,16]. The patient cohorts and study methodology of those reports are similar to the current study. An important difference is that chemoradiotherapy after surgery was not always uniformly applied in the study by Bette et al. [15]. But the beneficial prognostic effect of surgery was shown in subgroup analysis of the patients treated with chemoradiotherapy and was independent from MGMT promoter methylation status. The report by Sales et al. constitutes a survival analysis of 126 patients with unmethylated MGMT glioblastoma [16]. This is a subgroup analysis from the same initial patient cohort studied by Bette et al. Sales and colleagues found a significant correlation between RTV and survival in this group of patients with unfavorable tumor markers [16]. Our results confirm the conclusion from both reports: in the absence of favorable molecular markers, surgical resection constitutes a statistically significant survival benefit compared to biopsy even if GTR is not obtained. Taken together, the current results and those from the aforementioned reports, considering both MGMT-methylation status and IDH-mutation in survival analysis, weaken the conclusion by Kreth et al.

The current OS results and the analyses of the interaction variables for PR and GTR seem to indicate that resection might have a differential effect according to methylation status (Tables 2 and 3; Fig. 1). However, in the whole patient group as well as in the MGMT-methylation status-based subgroups increasing extent of resection was systematically associated with an OS benefit, but statistical significance was not always reached. This may be explained by the overlap of some confidence intervals which, in turn, could be attributed to the relatively small patient groups and subsequentially lower statistical power. So, definitive results on the presence of a differential effect of surgery related to MGMT-methylation status cannot be drawn from this study. Also, other studies did not show such an effect [11,21,22]. The fact that a 4.6 months OS benefit by PR in the subgroup of MGMT-unmethylated glioblastoma patients is statistically significant (Table 2), underlines the importance of resection, even partial, in this patient subgroup.

Extent of resection was dichotomized in this study into PR and GTR according to the methodology by Stummer et al. and as applied in other reports [11,19,22]. This method has the advantage of simplicity and reproducibility but does not allow to identify a threshold of RTV. In some cases, PR will have been a deliberate choice made by the neurosurgeon and the patient, but in others, the finding of a remnant of contrast-enhancing tumor will have been unexpected. Because of the retrospective design of this study, the reasons for PR could not be ascertained with certitude. It is well known that the perception of glioblastoma resectability varies widely between neurosurgeons, even amongst experts [10,25]. The perception of resectability is influenced by several factors: anatomical tumor localization, including proximity to eloquent zones; patient-related clinical issues; but also, surgeon-related factors including the perceived risks and benefits of neurosurgical resection [25]. This is illustrated by a recent study that compared two large Dutch neuro-oncological centers [26]. Despite the fact that both centers treated comparable glioblastoma patient populations and the fact that in both centers state-of-the-art techniques were available, including awake brain mapping, one center performed significantly more often only biopsy. This may likely be attributed to the aforementioned perceived risks and benefits of (partial) neurosurgical resection. In our opinion, the current study and many previous reports show the benefit of (partial) surgical resection of glioblastoma, even in the era of molecular factors [12–16,19,21–23].

Preoperative tumor volume could be measured on MPRAGE images in most patients (Table 1). Using semi-automated segmentation technique, we tried to diminish observer variability [12,20]. In statistical analysis, preoperative tumor volume was kept as a continuous variable rather than dichotomized, in order to avoid loss of statistical power and confounding [27]. Preoperative tumor volume did not correlate with OS, contrary to the results from previous studies [12,15]. Some differences between these studies and the present one should be noted. In the report by Bette et al. not all patients were treated with chemoradiotherapy and preoperative tumor volume was dichotomized [15]. After inclusion of postoperative chemoradiotherapy in multivariate analysis, preoperative tumor volume lost its statistical significance. The study by Grabowski et al. excluded biopsy patients, resulting in a patient cohort with surgical resection patients only, in contrast to the patient cohort studied here which includes also biopsy patients [12]. Moreover, the relationship between patient survival and glioblastoma tumor volumes is more complex and may depend on volume ratios of different segments rather than on the total tumor volume [28].

This study has several shortcomings. First, the retrospective study design may have introduced selection bias. Next, corticosteroid use at diagnosis was not included in the analysis. Also, therapy for tumor progression or recurrence was neither standardized nor included in the analysis. A threshold for RTV could not be established. This study also has important strengths. First, we present a glioblastoma patient cohort uniformly treated with TMZ-CR after surgery. Second, categorization of patients after surgical resection was straightforward which improves the external validity of the study. Third, we were able to determine molecular factors using sensitive and reliable techniques, notably qMSP and NGS [3,18,29].

5. Conclusion

The debate about the impact of neurosurgical tumor resection on glioblastoma patient survival started already in the beginning of the 20th century. While in 1923 Harvey Cushing reported on glioblastoma

that "the general idea prevails that these tumors represent hopeless surgical lesions" [30], nowadays the practice of maximum safe resection is supported by evidence. Our study adds to the evidence by showing that also in the group of glioblastoma patients with unfavorable molecular markers, partial resection does offer a significant overall survival advantage compared to biopsy only.

Funding

This work was supported by grants from the following non-profit organisations: Ghent University Hospital, Center for Oncology; *Lux Luka Foundation*; and *Fonds Arne Lannoy AKA Zorro*.

CRediT authorship contribution statement

Giorgio Hallaert: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Visualization, Funding acquisition. **Harry Pinson:** Validation, Formal analysis, Data curation, Writing - review & editing. **Dimitri Vanhauwaert:** Resources, Writing - review & editing. **Caroline Van den Broecke:** Resources, Writing - review & editing. **Dirk Van Roost:** Conceptualization, Supervision, Project administration. **Tom Boterberg:** Conceptualization, Project administration, Resources, Writing - review & editing, Funding acquisition. **Jean-Pierre Kalala:** Conceptualization, Project administration, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest. None of the authors has any commercial interest in the techniques or materials described in this study.

Acknowledgement

We gratefully acknowledge the help of Prof. em. Dr. M. Mareel with the study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.suronc.2020.10.016.

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DISCUSSION & FUTURE PERSPECTIVES

The publications in the previous chapters lead to the following conclusions. Glioblastoma patients suffering from tumors with weakly methylated O6-methylguanine-DNA-methyltransferase gene promoter (*MGMT*p), have a better overall survival than patients with fully unmethylated tumors. Contact of the contrast-enhancing part of the tumor with the subventricular zone (SVZ) is a negative prognostic factor, but irradiation of the SVZ does not have a straightforward influence on glioblastoma patient survival. Partial resection of isochitrate dehydrogenase wild type (*IDH*wt) tumors results in better overall survival for glioblastoma patients treated with chemoradiotherapy, compared to patients with biopsy as the only surgical procedure. In the following paragraphs, the role of weak *MGMT*p methylation, the SVZ and of partial resection in glioblastoma patient survival will be further discussed. A brief reflection on retrospective study design and the consequences for the quality of evidence is also desirable because the research presented in this thesis is largely retrospective. Finally, this chapter closes with some short reflections on future glioblastoma therapies.

1. The Role of the Subventricular Zone

Although the subventricular zone (SVZ) has been known since the mid 1950s as the origin of neuroblasts during the embryological development of the human brain, it was as late as 2004 before the presence of neural stem cells (NSCs) in the SVZ was shown in the adult human brain (1). Nowadays, the SVZ is accepted as the largest reservoir of NSCs in adults. Quiñones and colleagues described the cytoarchitecture of the SVZ in detail (2). The stem cells are present in the lateral walls of the lateral ventricles but seem to be unequally distributed. The similarities between NSCs and brain tumor propagating cells (BTPCs) are striking (3). So, the obvious hypothesis arose (or was “rediscovered” – as was described in the introduction of this thesis) that the glioblastoma cell(s) of origin, may be located in the SVZ. Two possibly related clinical aspects were soon studied: is there a prognostic value of the spatial relationship between the SVZ and the contrast-enhancing part of glioblastoma and does incidental SVZ irradiation influences glioblastoma patient survival (4,5)?

Lim and colleagues published their findings on glioblastoma and SVZ contact in a study of 53 patients. They showed that SVZ-contacting glioblastoma was significantly more frequently multifocal at diagnosis and that relapses occurred at greater distance from the original lesion (4). A relationship with the NSC containing SVZ was deemed likely. Next, Chachaina et al. showed one year later in a case-control study, that the survival of patients with SZV-contacting glioblastoma was significantly worse compared to patients harboring tumors without SVZ contact (6). Several reports followed and a 2017 meta-analysis acknowledged that SVZ contact of glioblastoma at diagnosis is a negative prognostic factor (7). However, only few studies were able to include *IDH* mutation status or *MGMT*p methylation in the survival analysis. These molecular biological factors have been established as strong prognosticators of glioblastoma patient survival. Our study showed that SVZ contact at diagnosis is an independent negative prognostic parameter, even when *IDH* mutation and *MGMT*p methylation are included in multivariate survival analysis (8).

The exact reason why SVZ contact results in a worse prognosis for glioblastoma patients remains unknown. Quite obviously, the fact that the tumor volume of SVZ contacting glioblastoma is larger than its non-contacting counterpart, led to the hypothesis that gross total resection (GTR) was less frequently obtained in this group, thus explaining the worse prognosis (9). But our study and others show that SVZ contact is a negative prognosticator independently from extent of resection. Another hypothesis was that decreased survival is indirectly associated with SVZ contact in glioblastoma: higher frequency of ventricular opening during neurosurgical resection, could result in higher incidences of hydrocephalus, leptomeningeal tumor dissemination and distant parenchymal recurrences, resulting in death. However, Mistry et al. recently rebutted this hypothesis in their 2019 publication (10). Next, the presence of a SVZ-contacting glioblastoma specific biological profile may explain worse survival. There seems to be no difference in presence of *IDH* mutation and/or *MGMT*p methylation between SVZ contacting and non-contacting glioblastoma, but data are still sparse (9). Interestingly, in our study *MGMT*p methylation was significantly less frequently found in SVZ contacting glioblastoma. Also, the very likely presence of BTPCs in the SVZ glioblastoma does not offer a straightforward explanation for the worse prognosis associated with SVZ contacting glioblastoma. Again, a separate biological profile would be expected. However, to date, a distinct biological profile of SVZ contacting glioblastoma has not been shown (11). More data are needed to decisively show if the molecular features of SVZ contacting glioblastoma differ from non-contacting tumors.

Although the explanation for the worse prognosis of SVZ-contacting glioblastoma remains elusive, some centres soon identified the SVZ as a possible therapeutic target in glioblastoma. Evers and colleagues were the first to report their findings on the influence of SVZ irradiation on glioblastoma patient survival (5). Initial reports were promising but later on conflicting results were published. Two recent reviews concluded that no clear conclusions could be currently drawn (12,13). Our study in *IDH*wt glioblastoma patients showed that higher incidental SVZ dose did not correlate with survival in multivariate survival analysis, including molecular biological factors. There are several important methodological issues between SVZ irradiation reports that render their comparison cumbersome. First, while apparently straightforward, consensus on contouring the SVZ is lacking, resulting in notable differences in SVZ volumes and doses between studies. None of the previous publications mentions if the part of the SVZ that was invaded by the glioblastoma, was contoured or not. Second, most reports did not include *MGMT*p methylation and IDH mutation in survival analysis. Importantly, SVZ contacting glioblastoma results in collinearity: the fact that the tumor abuts the SVZ will inevitably result in higher SVZ doses, regardless of the radiotherapy technique applied. This bias could be counteracted by studying the effect of SVZ irradiation in non-contacting glioblastoma only. Or, even more strict, in glioblastoma where the clinical target volume (CTV) does not include any part of the SVZ. Since most glioblastomas are large at diagnosis, this will be difficult to achieve. Nourallah et al. studied one hundred randomly selected glioblastoma patients from their institution and found that only in 3% of cases the CTV did not include the SVZ at all (13).

Furthermore, some reports, including our own, indicate that higher SVZ dose might be harmful to the patient and results in shortened patient survival (14-16). Perhaps this possible deleterious effect is due to known side-effects of radiotherapy and temozolomide,

such as lymphopenia. Alternatively, higher radiotherapy doses on the SVZ may also obliterate the NSCs, thus weakening the restorative capacities of the brain already injured by glioblastoma. Combining the observation of possible negative effects of SVZ irradiation with the observation of SVZ contact as a negative prognostic factor, may lead to the hypothesis of a causal correlation between both. Indeed, the prognostic effect of SVZ contact has only been studied and described in patient groups who were all treated with radiotherapy. By default, as already mentioned, patients with SVZ contacting glioblastoma will have received higher radiotherapy doses to both the ipsilateral as well as the contralateral SVZ. Although not impossible, the hypothesis that the worse survival of patients with SVZ contacting glioblastoma could be due to higher SVZ radiotherapy doses is unlikely. If this effect would be so apparent, then it would have been also present in most, if not all, SVZ irradiation studies to date. But the opposite holds true. Also, this hypothesis is contradicted by dose-response studies in glioblastoma, of which the report by Walker et al. was one of the earliest (17,18). Patients receiving 60 Gy radiotherapy after surgery, had significantly better survival than those receiving 50 Gy and than those patients receiving no radiotherapy. Another argument against this hypothesis is the fact that recent dose-escalation studies in glioblastoma did not found worse survival (19). Although Kim et al. did not specifically study SVZ contact and irradiation, the CTV (and a fortiori the PTV) of radiotherapy treatment plans of most of the glioblastoma patients will have included some part of the SVZ.

Future perspectives

- ◊ Glioblastoma patient nomograms should include SVZ contact as an independent negative prognostic factor.
- ◊ More research is needed on possible molecular biological differences between SVZ-contacting and non-contacting glioblastoma, e.g., prospective registry of glioblastoma patients and analysis of bulk tumor data (gene mutations, copy number alterations, methylation patterns, gene and protein expression).
- ◊ An international consensus statement on contouring the SVZ is necessary.
- ◊ Well-designed prospective studies on SVZ irradiation are needed, adjusting survival for molecular biological factors.

2. The Role of Partial Resection

Neurosurgeons traditionally apply several terms to describe the extent of tumor resection in glioma surgery: ‘supratotal resection’ (SuTR); ‘gross total resection’ (GTR); ‘near total resection’ (NTR); ‘debulking'; ‘partial resection’ (PR) and ‘biopsy’. However, these terms lack a clear definition and are sometimes used interchangeably.

The concept of ‘brain biopsy’ is well-known: the surgical act of removing a small amount of tumor tissue, usually via a stereotactic procedure using a dedicated cannula (often called “needle”), with the purpose of establishing histopathological diagnosis and enabling genetic analysis of the tissue but without the intent of cytoreduction. So, a biopsy induces only insignificant changes to the tumor volume, even if an "open biopsy" is performed. All other procedures have the intent of cytoreduction. Figure 13 shows the terms arranged according to extent of resection (EOR) achieved. Most commonly, the term GTR is used to denote the complete removal of contrast-enhancing glioblastoma tumor tissue. The term recognizes that discussion between observers may exists concerning a small volume of tissue that may represent either true tumor or blood products (vide infra). Unfortunately, a definition of “a small volume” is lacking. NTR means that a small rim of tumor remains. SuTR means that the resection extends beyond the contrast-enhancing borders on MR imaging to include also (parts of) the T2 or FLAIR hyperintense zones surrounding the tumor (20,21). In a way, the pinnacle of supratotal resection is the lobectomy: not only the tumor is removed but the entire brain lobe in which it resides e.g., the non-dominant right frontal lobe. Finally, PR means that only part of the tumor has been removed, whether intentionally or not. The term ‘debulking’ means that the bulk of the tumor was removed; the word ‘bulk’ is a synonym for ‘the larger part’. The free use of these terms, lacking a clear definition, in scientific papers complicates interpretation and increases the risk of bias.

Importantly, the neurosurgeon's judgement and estimation of EOR during surgery is not sufficiently reliable, not even among experts (22-24). The only exception may be the lobectomy, if the tumor is significantly smaller than the lobe itself. Only postoperative MR imaging can objectively show the extent of tumor removal. In order to avoid early inflammatory changes, which may result in gadolinium uptake mimicking residual contrast-enhancing tumor tissue, MR imaging should be obtained within 72 hours after surgery, preferably within 48 hours (25,26). While postoperative MR imaging itself is an objective parameter, the interpretation of the imaging is not. It may be notoriously difficult to distinguish between early inflammation or blood products and true residual glioblastoma tumor tissue. Observer variability remains an important but underreported issue concerning postoperative glioblastoma MR imaging (27,28). Therefore, the term ‘GTR’ is most commonly used in neurosurgical literature which acknowledges that a small (although not consistently defined) amount of contrast-enhancing tissue may be present, whether or not true tumor tissue.

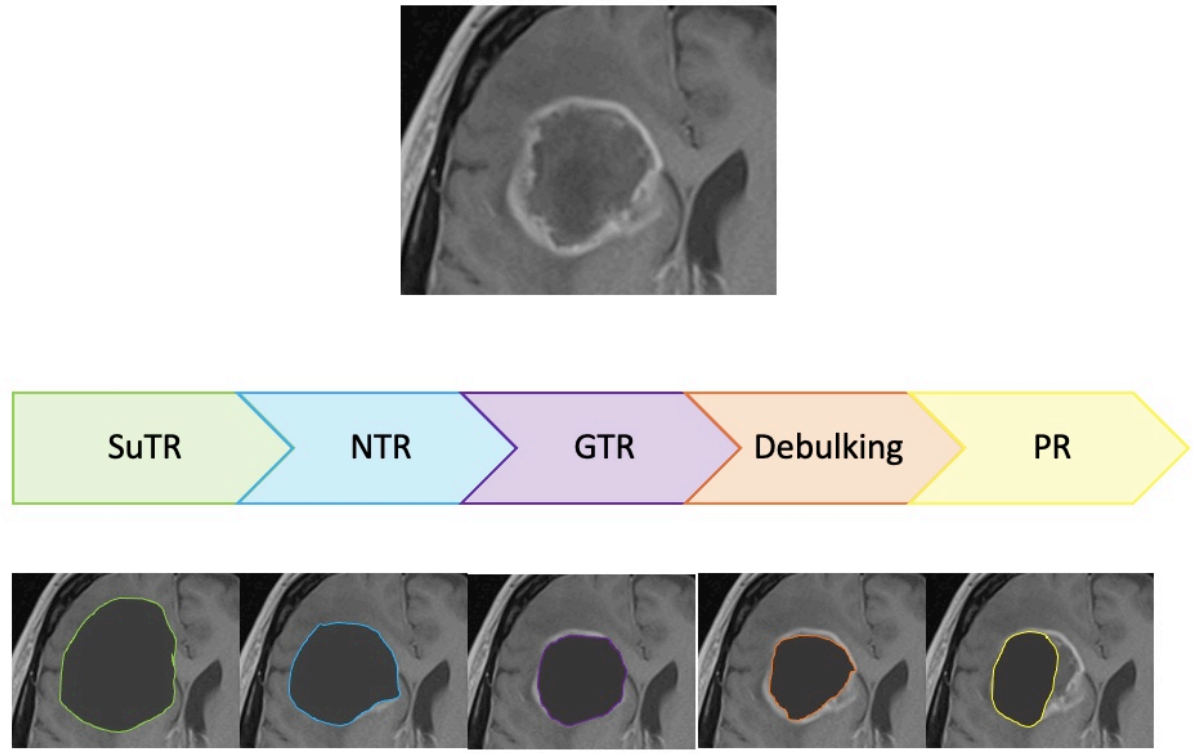


Figure 13. Schematic overview of the most commonly used terms in neurosurgical literature concerning glioblastoma resection.

The central hypothesis of cytoreduction is the positive correlation between tumor tissue removal (completely or partially) and patient survival. In 1999, Hess reported truthfully that "little scientifically credible evidence is available to support the assertion that aggressive surgical resection prolongs survival" in glioblastoma (29). Two years later, the first of several important studies on the correlation between EOR and glioblastoma patient survival was published by Lacroix et al. (30). Many others followed (31-36). Glioblastoma resection focuses on the contrast-enhancing part of the tumor. The zone of intense contrast-enhancement of glioblastoma is due to the disruption of the blood-brain barrier by the tumor, so the contrast agent leaks out of the lumen of the vessels and accumulates in the extravascular extracellular space. This represents the region of most 'dense' tumor and highest concentration of malignant cells. All aforementioned studies come to the conclusion that complete resection of contrast-enhancing tumor prolongs glioblastoma patient survival. Stummer and colleagues deliver the strongest statistical evidence in favor of glioblastoma resection in their report (33). They present an analysis of EOR based on the highly controlled 5-aminolevulinic acid study for which the patient data were prospectively collected. Complete resection of contrast-enhancing tumor resulted in a 5.3-month prolonged patient survival compared to incomplete resection. Or, in other words, the presence of gadolinium-enhancing tissue on postoperative MR imaging, before the start of chemoradiotherapy, is a negative prognostic factor for glioblastoma patients. However, all these studies lacked inclusion of molecular biological markers because these were not yet unravelled, let alone included, at the time of data collection. In 2018 and 2020, two retrospective studies showed the beneficial impact of maximum resection of contrast-enhancing tumor on glioblastoma patient survival,

independently from molecular biological factors (37,38). The study by Molinaro et al. also suggests a beneficial influence of supramarginal resection, including the FLAIR hyperintense zone surrounding the tumor (so-called 'FLAIRectomy'). This zone comprises not only oedema, but also viable malignant cells although in lower numbers than in the contrast-enhancing part of the tumor. However, glioblastoma is not a surgically curable disease.
At present day, the survival benefit associated with - safely accomplished - GTR in case of glioblastoma is generally accepted. Importantly however, prospective studies show that in glioblastoma, GTR will be obtained only in about 40% of patients despite advances in neurosurgical techniques such as neuronavigation, fluorescence techniques (Fig. 14), and awake brain mapping. (39,40). So, in more than half of the newly diagnosed glioblastoma patients, PR will be the maximum achievable neurosurgical intervention.

Neurosurgical literature remains conflicting concerning the effect of partial tumor resection on glioblastoma patient survival, although most neurosurgeons agree that PR may be offered to patients as a palliative measure, especially to relieve symptoms induced by the mass-effect of the tumor and/or related to raised intracranial pressure (41). The focus of the discussion was sharpened after the publication of the prospective study by the German Glioma Network in 2013. Kreth et al. found a moderate survival benefit associated with GTR but no survival benefit for PR as compared to biopsy-only (39). They suggested that, in the age of chemoradiotherapy and molecular markers, a biopsy-only management may be a better alternative than PR for glioblastoma patients if GTR is deemed unattainable. This conclusion follows logically from their study results: on the one hand, PR (in their paper called 'incomplete resection') did not offer a survival benefit compared to biopsy-only and, on the other hand, craniotomy had a tenfold higher complication rate than needle biopsy (12.1% vs 1.4% respectively). After all, glioblastoma tumor resection will by advised by the neurosurgeon only if the survival benefit outweighs the risks and possible complications – *primum non nocere*. And, of course, if there is substantial scientific evidence supporting the correlation between PR and prolonged survival.

It seems common sense that complication rates associated with craniotomy for high-grade glioma are much higher as compared to those associated with (stereotactic) biopsy. Although a uniform reporting method for biopsy-related complications is lacking, a recent meta-analysis concluded that the mortality rate associated with stereotactic biopsy varies from 0.7% to 4% and the overall complication rate from 3% to 13% (42). As expected, cerebral haemorrhage was the most frequent complication. Concerning craniotomy for primary malignant brain tumors, a 2018 study of 7376 patients, operated on in the period 2005-2009, showed a mortality rate of 2.6%; a major complication rate of 12.9%; and an overall complication rate of 16.4% within 30 days after surgery (43). These figures put the tenfold higher risk associated with craniotomy in the series by Kreth et al. into perspective (39). So, a cautious interpretation on surgery-related risks is warranted, but it is correct to state that, on average, a craniotomy for high-grade glioma nowadays carries a moderate higher morbidity and mortality rate than biopsy.

The controversy surrounding the effect of PR on survival of glioblastoma patients arises from several issues. There is no consensus on which parameter is best to express residual tumor burden: EOR or direct RTV measurement (36,44). Nor is there a consensus on glioblastoma volume measurement methodology (45). Percentage EOR is calculated as follows:

$$\%\ EOR = \frac{(preoperative\ tumor\ volume\ -\ RTV)}{preoperative\ tumor\ volume} \times 100$$

So, EOR is dependent of the preoperative tumor volume. Therefore, a 99% EOR may denote a totally different volume in a small versus a large glioblastoma. As already mentioned, observer variability is an important issue (27,46,47). Next to these methodological concerns, most of the publications on PR predate the currently deemed essential molecular biological factors in glioblastoma (48). Finally, many reports include glioblastoma patient cohorts that were not uniformly treated after surgery.

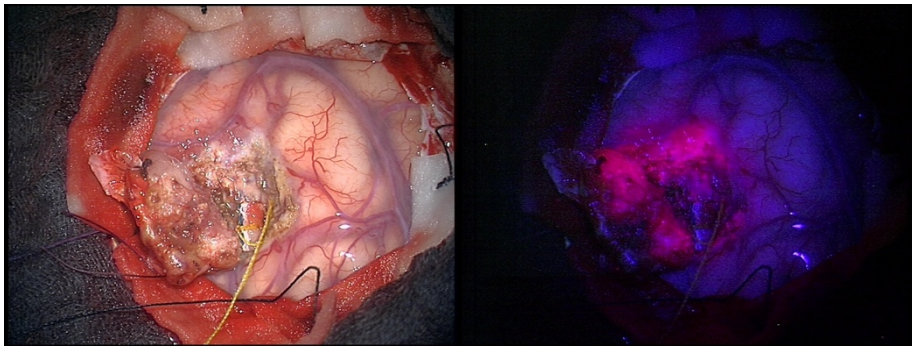


Figure 14. A representative neurosurgical microscopic image using blue light (right side), demonstrating the typical pink appearance of glioblastoma when operated using 5-ALA (aminolevulinic acid). The picture on the left side shows the same image using white light.

In their prospective study on the use of 5-aminolevulinic acid (ALA)-derived fluorescence in glioblastoma surgery, Stummer and colleagues used a straightforward method to distinguish between GTR and PR, bypassing most of the aforementioned problems (33). If postoperative MR imaging showed residual contrast-enhancement of 1 voxel or more, surgical resection was categorized as 'incomplete'. The study by Kreth and colleagues used the same method and our retrospective study was designed accordingly (39,49). There are several advantages to this approach: it is easy applicable, it minimizes observer variability, and it avoids the necessity of specific volumetric software and interpretation of the imaging data. It enhances the external validity of the study. However, the major disadvantage of this method is that the PR group will be very heterogenous in terms of RTV and EOR. On the one hand, there will be cases with 80% EOR or more; on the other hand, whether intentionally or not, some cases will have an EOR of only 50% or even lower. Obviously, this method does not allow to identify an EOR or RTV threshold. Interestingly, Stummer et al. also performed volumetric assessment of RTV (33). The median RTV was 1.5 mL whereas maximum RTV was 33 mL. Best survival, as compared to survival of patients with complete resection, was associated with smallest RTV while worst survival was found in the patient group with highest RTV. However, survival differences between different RTV groups were not statistically significant, possibly because of small sample sizes. Our unpublished results (comprising only a small cohort of 93 patients in whom immediate post-operative imaging with 0.9 mm thickness MPRAGE images were available) show a statistically significant inverse correlation between contrast-enhancing tumor volume at the start of chemoradiotherapy and glioblastoma patient survival. For every increase of tumor volume with 1 mL, the mortality hazard is expected to increase multiplicatively with 1.7% (unadjusted hazard ratio = 1.017; 95% confidence interval = 1.005 – 1.029; *P* = 0.006). If a cut-off for RTV

of 2 mL is applied, as suggested by Grabowski et al., the survival curves of both groups show a statistically significant difference in favour of the lower tumor volume group (Fig. 15).

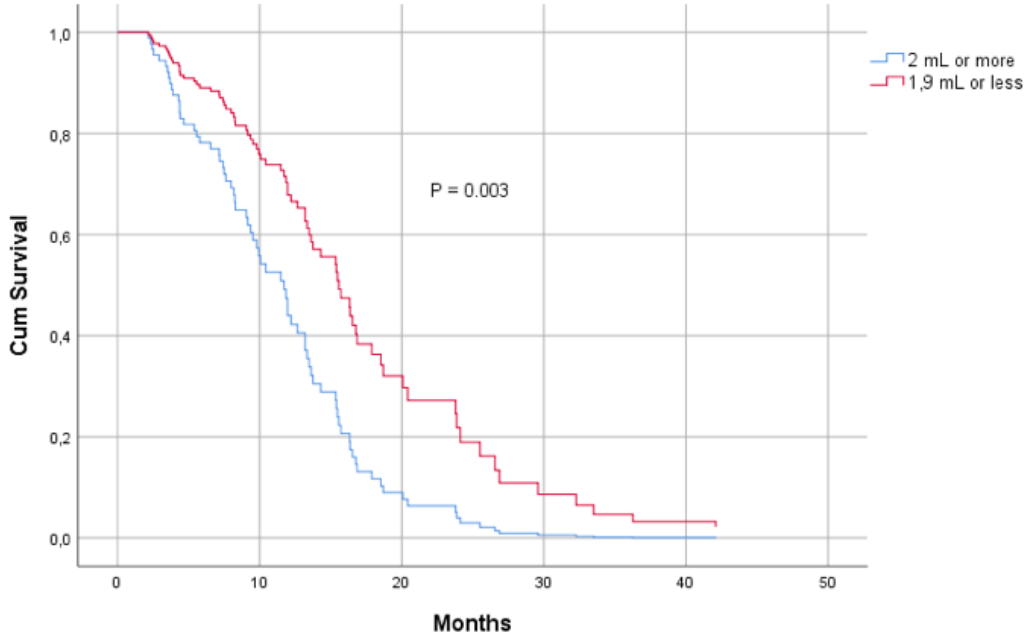


Figure 15. Overall survival curve of glioblastoma patients dichotomized according to tumor volume at the start of chemoradiotherapy with a cut-off of 2 mL. (Cox regression survival model, adjusted for age, KPS and MGMTp methylation. Six patients were censored for survival.)

Taken together, despite many shortcomings and statistical difficulties, extent of resection studies in glioblastoma from the past fifteen years strongly suggest a proportionate and inverse correlation between RTV and patient survival (50). Very recently, the Berger group from San Francisco published a large retrospective study showing a survival benefit associated with extent of resection in glioblastoma across all molecular subgroups (38). Hence, neurosurgical cytoreduction to prolong glioblastoma patient survival is supported by evidence. But this survival benefit must be weighed against the risks of acquiring a new neurological deficit. This remains a difficult topic for the shared-decision process for both the neurosurgeon and the patient alike. Ultimately, the obtained extent of glioblastoma resection depends on many factors (Fig. 16). However, maximum safe resection is at present a sound and scientifically based *leitmotiv*.

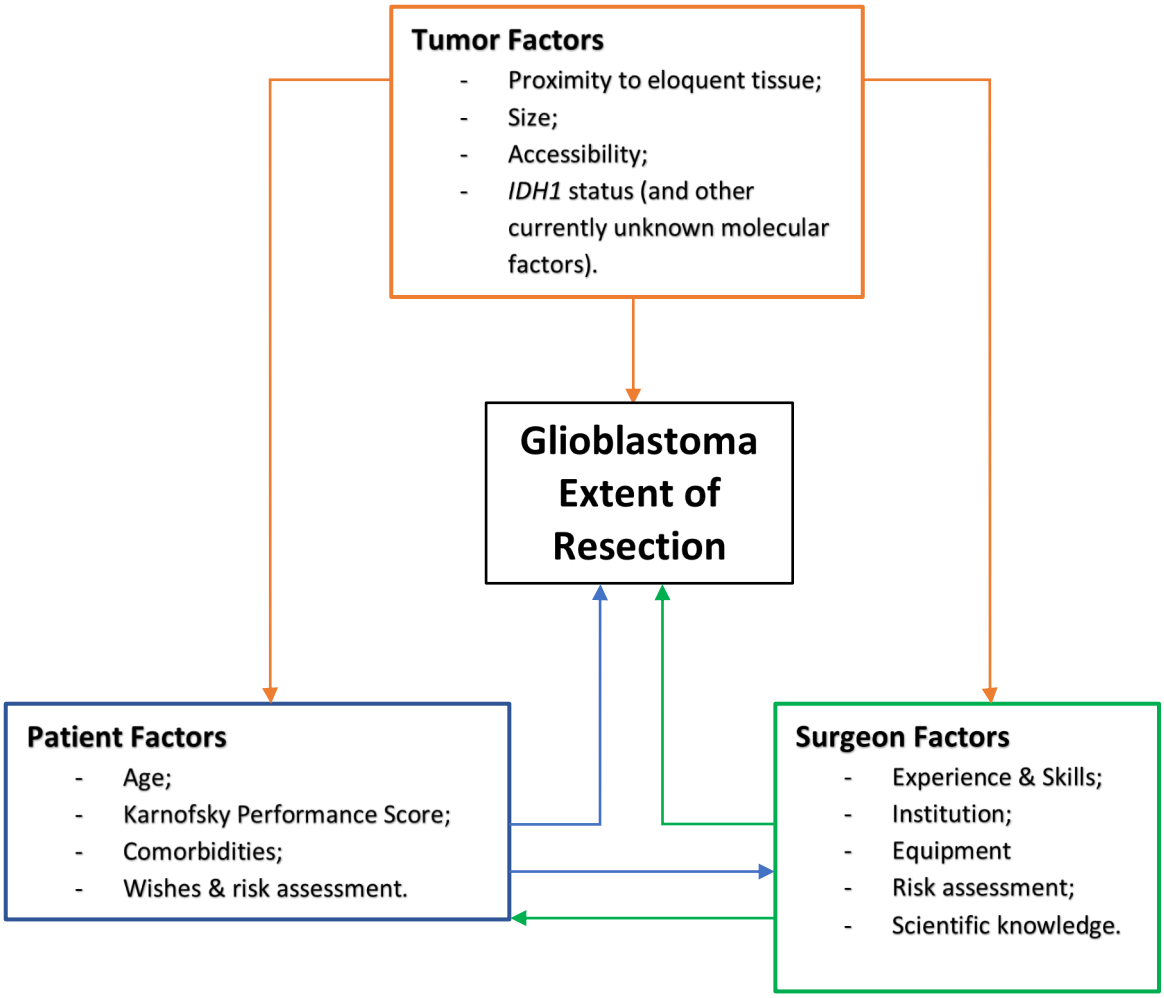


Figure 16. Schematic overview of influencing factors of extent of glioblastoma resection.

Future perspectives

- ◊ Since randomized controlled trials to test the effect of extent of glioblastoma resection on survival should be rejected on ethical grounds, prospective glioblastoma registries provide a valid alternative to study this important topic in a prospective manner.
- ◊ The international neuro-oncological community should develop
  - Internationally accepted definitions of the commonly used terms in glioblastoma resection.
  - Internationally accepted guidelines on the methodology of glioblastoma volumetry before and after neurosurgical resection, including observer variability.
- ◊ Glioblastoma patient counseling should include a thorough discussion of the risks and benefits involved in neurosurgical resection; the leitmotiv of maximum safe resection is supported by evidence.

3. The Role of O^6-methylguanine-DNA-methyltransferase Gene Promoter Methylation

The presence of O^6-methylguanine-DNA-methyltransferase gene promoter (*MGMT*p) methylation in glioblastoma tissue, is a strong predictor of the beneficial effect of alkylating drugs (notable temozolomide) in the corresponding patients. As such, it is also a strong molecular biological prognostic factor, next to *IDH* mutation (51). In this glioblastoma patient cohort too, *MGMT*p methylation resulted in the highest hazard ratio reduction.

Several techniques are available to determine *MGMT*p methylation (Table 2 & Fig. 17), each with its specific (dis-) advantages (52,53). Unfortunately, different techniques may also lead to different results. Nowadays, methylation-specific polymerase chain reaction (MSP) and pyrosequencing are applied in more than 70% of cases (54). The MSP technique has been explained briefly in the introduction. Compared to pyrosequencing, MSP is cheaper, has a shorter turn-around time, and is more widely available. It was used also in this thesis to determine *MGMT*p methylation status. But an important concern in using MSP is the lack of consensus on the optimal cut-off value to dichotomize between methylated and unmethylated *MGMT*p.

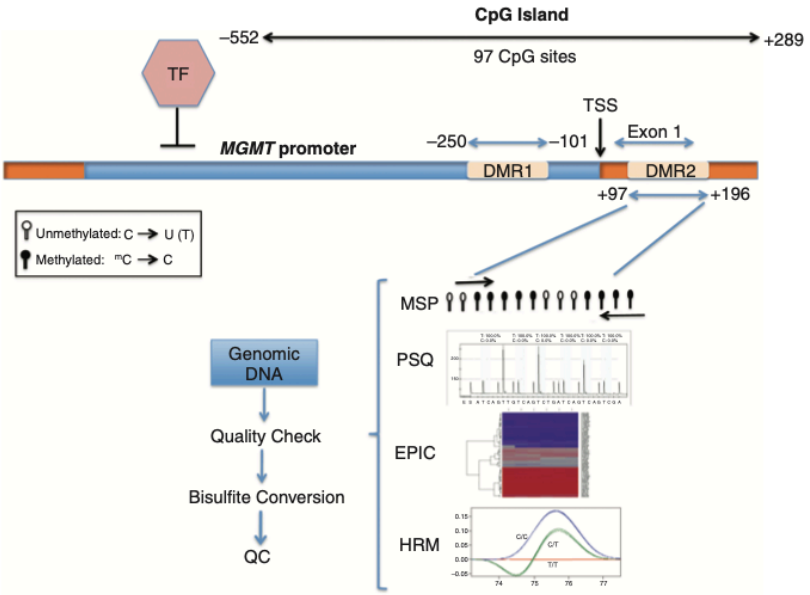


Figure 17. *MGMT* promoter region and commonly used methylation assays (53).

Vlassenbroeck and colleagues identified already in 2008, the existence of a “grey zone” of diagnostic uncertainty correlating with the upper and lower limit of the 95% confidence interval surrounding the cut-off point of methylation using MSP (55). It has been shown that glioblastoma patients with methylation test results within this grey zone, may have a better survival than fully unmethylated glioblastoma patients (56). This thesis confirms the aforementioned finding for patients suffering from glioblastoma with ‘weak’ *MGMT*p-methylation.

Table 2. Overview of the three most used techniques to determine *MGMT* promoter methylation (52,53).

Technique	**Turnaround Time**	**Advantages**	**Disadvantage**	**Estimated cost per sample (USD)**
Quantative MSP	2 days	Quantitative; High throughput; Cut-off point validated in clinical studies	Unreliable results with mosaic methylation patterns; (Poor reliability in FFPE tissues)	$10-20
Pyrosequencing	5 days	Quantitative	Expensive; Unclear cut-off point; not validated in clinical trials; High throughput core facility required; Longer time to results	$10-30
EPIC*	5 days (or more)	Also testing of other biomarkers (1p/19q; G-CIMP); Compatible with different sample preparations	Expensive; Unclear cut-off point; not validated in clinical trials; High throughput core facility required; Longer time to results	$500-700

MSP = methylation-specific polymerase chain reaction; FFPE = formalin-fixed paraffine-embedded;
*refers to the methylation array platform by Illumina, Inc. (Illumina HumanMethylation EPIC Beadchip)

The observation of a possible survival advantage for patients with test results in the grey zone, is important from both an academic and a clinical point of view. When applied to clinical studies enrolling patients with unmethylated glioblastoma, the presence of a grey zone may permit unwilling and unknowing inclusion of patients with weakly methylated glioblastoma. Hence, a significant bias may occur. From the clinical point of view, it is important to realize that some patients with test results in the grey zone, may still experience significant benefit from treatment with temozolomide. Since some authors advocate to withhold temozolomide from patients with unmethylated glioblastoma, especially in the elderly, patients may be at risk to be denied access to a potentially life-prolonging treatment (57).

The solution to these concerns is the development of international consensus guidelines on *MGMT*p methylation testing in glioblastoma both concerning the assays to be used and cut-off values. These guidelines should cover which test is to be used as first-tier; which cut-off value is used to signify “truly methylated” and “truly unmethylated” tumors and the grey zone in between; which test subsequentially should be used to further examine the grey zone test results. We agree with the proposed algorithm by Mansouri et al., to use MSP as the first test

and to use pyrosequencing next (not EPIC because of the higher costs involved, at least at this moment) if the test results belong to the grey zone surrounding the MSP cut-off value (53). We would suggest using 0.2 and 2.0 as lower and upper limit (Fig. 18). The swift technical evolution in medical genetics will probably allow for faster, more sensitive and more specific but also cheaper techniques. Specifically, next generation sequencing may allow this progress in the near future also for methylation profiling and perhaps obliterating the grey zone of uncertainty.

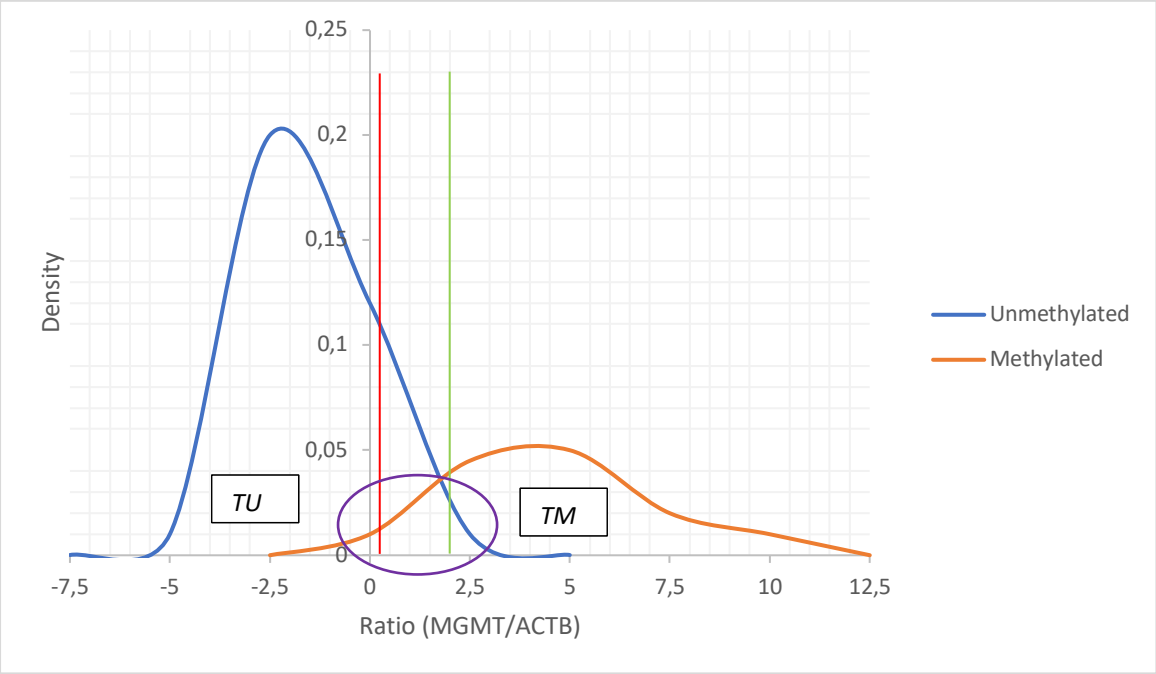


Figure 18. Schematic representation of the "grey zone" of diagnostic uncertainty using qMSP for determination of *MGMT*p methylation. The red vertical line shows the proposed lower cut-off limit of 0.2. The green vertical line depicts the upper limit of 2.0. The purple circle highlights the "grey zone".
TU = truly unmethylated; TM = truly methylated.

Of equal importance is the question if all glioblastoma patients should be tested nowadays for *MGMT*p methylation in routine clinical practice. Since MGMTp methylation is both a predictive as well as a prognostic factor, it is an important and useful parameter to include during patient counselling. If the patient is confronted with a *MGMT*p-unmethylated glioblastoma, she/he should be informed that the effect of chemoradiotherapy will very likely be modest at best and that his/her prognosis is worse than that of patients with *MGMT*p-methylated tumors, as was shown in the seminal study by Hegi et al. in 2005 (58). In the important subgroup of elderly patients (70 years or older), two studies showed that for patients with unmethylated glioblastoma, treatment with temozolomide is futile or even harmful (59,60). In case of unmethylated glioblastoma in an elderly patient, withholding temozolomide may be the better treatment option: side-effects are avoided, maintaining a better quality of life, without sacrificing survival. In younger patients with unmethylated glioblastoma, withholding temozolomide is more controversial. In their 2015 report, Hegi and Stupp contemplate the reasons why almost every glioblastoma patient is treated 'upfront' with temozolomide, despite absence of *MGMT*p methylation (57). The validation of the

technique used to determine the *MGMT*p-methylation status is an important issue as is the presence of the grey zone. Oncologists are worried that they may incorrectly deny patients access to a potential life-prolonging treatment based on unreliable test results. As stated above, this valid concern may be solved by an international consensus on *MGMT*p methylation testing, including cut-off values. Another important argument is the fact that an alternative treatment for unmethylated glioblastoma patients is currently lacking. But Hegi and Stupp turn this argument upside down by arguing that development of new treatments is delayed because all glioblastoma patients still receive temozolomide.

Future perspectives

- ◊ Development of international guidelines on *MGMT*p testing: techniques, cut-off value and grey zone; development of a testing algorithm (cf. Mansouri et al.).
- ◊ Next generation sequencing may revolutionize methylation testing and eliminate the diagnostic uncertainty.
- ◊ Glioblastoma patient counseling should include discussion of *MGMT*p-methylation results and possible prognostic and therapeutic consequences.

4. The Role of the Onco-Functional Balance

Historically speaking, patient performance status was introduced in the late 1940s by Karnofsky and Burchenal to describe the ability of a patient to carry on his or her normal daily tasks or the need for care (61). They developed the 'Karnofsky Performance Scale' (KPS) which is now an accepted standard in medical literature, especially in the field of oncology. Importantly, 70% demarcates a critical point on this scale: above 70% the patient is not only able to care for himself, but also to perform normal daily activities, including work. But at 70% or below, the patient is unable to work or to carry out day-to-day tasks. The oncological patient with a KPS score of 70 or lower will have worse survival than a similar patient with a higher KPS score. This holds true for glioblastoma patients (vide supra, introduction) and was also clear from our own patient cohort (please see Fig. 10 B). But, in a way, the KPS is a rather rough assessments and misses more subtle, but important, features that also have a major impact on quality of life (61). Specifically for glioblastoma patients, KPS was not developed to detect neurological deficit. Nevertheless, glioblastoma patients may experience symptoms that severely impact their quality of life but without corresponding low KPS.

These symptoms may be self-evident, e.g., motor deficit or aphasia, but may also pose a diagnostic challenge, especially in case of cognitive dysfunction. Cognition is a critical hallmark of the human brain. Classically, five cognitive domains are described: executive functions; learning & memory; perceptual-motor function; language; and attention (62). Glioma patients, and certainly glioblastoma patients, are particularly prone to cognitive dysfunction. Several reports have shown the presence of severe impairments in at least one cognitive domain at tumor diagnosis, before surgery or antimitotic treatment started (63,64). Medication (antiepileptic drugs or steroids) may influence cognition, but glioblastoma itself also causes cognitive dysfunction. Of course, glioblastoma location may have a severe impact on cognition. Interestingly however, different locations may result in analogous symptoms. This observation is hard to explain using the dominating "localizationist" view of brain that prevailed during the 19th and 20th century, starting with the works and observations of Paul Broca and Carl Wernicke. Since the 1990 landmark publication by Mesulam, the paradigm has shifted towards viewing the brain as a dynamic system of functional neural networks (65). Nowadays, the paradigm of the human brain 'connectome' dominates neurosciences (66). This paradigm also explains the influence of growth rate on neurocognitive functioning of glioma patients. Wefel and colleagues showed in 2016 that more severe cognitive dysfunction was present in patients suffering from IDHwt malignant gliomas than in patients with IDH mutated tumors, and that tumor volume inversely correlated with cognition only in patients with IDHwt tumors (67). These observations strongly suggest that brain plasticity, based on neural networks, is hindered by fast-growing tumors but allows for compensation in slower evolving lesions. Unfortunately, glioblastoma treatment, especially resection, may also harm cognitive function. Already in 2012, Johnson et al. published an important report that shows that neurocognitive impairment after glioblastoma resection is an independent negative prognostic factor (68). Recently, Rijnen and colleagues showed that it may be possible to preoperatively identify glioblastoma patients at risk for neurocognitive decline after surgery (64). Also, in some cases neurocognition may improve after surgery but this is rather exceptional.

These considerations should be incorporated in the conversation between the patient and the neurosurgeon. While tumor resection may significantly prolong the patient's life, it may present a trade-off with quality of life. Therefore, the shared-decision making process is indeed the pinnacle of patient-centered care (69). Each patient has the right to be fully informed and to decide which deficits and/or risks are acceptable to him. For instance, a hemianopia may be very acceptable to one patient but can constitute an insurmountable problem for another. The same holds true for cognitive deficits. Most neurosurgeons focus attention on the risk of 'major' neurologic deficit and pay less attention to neurocognition. Although many practical obstacles exist, routine preoperative neurocognitive testing of glioblastoma patients may be useful to elaborate on cognitive risks of surgery, next to providing a baseline of neurocognition at diagnosis. This way, the neurosurgeon and the patient can explore the optimal balance between oncology (quantity of life) and functionality (quality of life). Of course, cognitive impairment may already impede an in-depth and nuanced discussion in some patients which renders the decision-making process only more difficult (62).

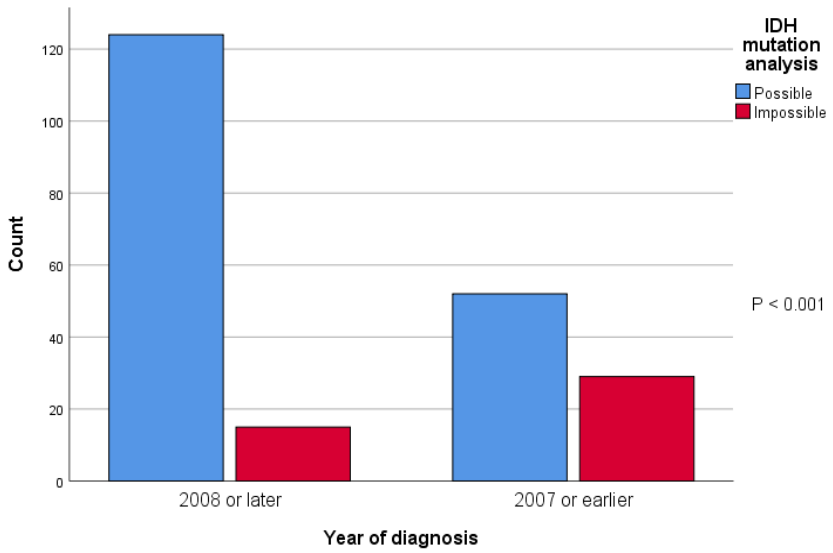
5. The Role of Retrospective Study Design

The studies presented in this thesis are best classified as historical (or retrospective) cohort studies. The clinical data were not prospectively collected at the time of the patients' diagnosis and treatment. However, several important parameters were determined *de novo*, notably the molecular biological factors (*IDH* mutation status and *MGMT*p methylation), the contouring of the SVZ and measurement of glioblastoma tumor volumes. Retrospective studies suffer from inherent flaws and shortcomings, as does science itself. However interesting a topic, a theoretical in-depth analysis of retrospective study design is beyond the scope of this thesis. But the most important setbacks of retrospective studies relevant to this thesis will be discussed in the following paragraphs as will be the measures taken to attempt to control them. Also, it is important to acknowledge that rightful ethical considerations render randomized controlled trials in glioblastoma patients difficult, especially concerning extent of resection: this is mirrored by the fact that most studies on this topic are indeed retrospective studies.

Bias and confounding are two major concerns. In short, bias creates a false association between parameters (type I error, or false positive) while confounding describes a true association between factors that is, however, potentially misleading. The most relevant types of bias relevant to this thesis are selection and information bias. These types of bias are closely related to the interesting topic of dealing with missing data.

Retrospective studies are prone to selection bias. Specifically for this thesis, it means that the selection of patients was one way or the other potentially dependent on the outcome of patients. Selection bias may be differentiated further into several subtypes, of which misclassification bias and attrition bias are important in this thesis.
Glioblastoma patients in this thesis were not randomly selected: only patients who were treated after surgery with chemoradiotherapy were selected (cf. inclusion criteria, chapter 2). In other words, all weaker patients, who only received supportive care, or a shortened radiotherapy schedule, were a priori excluded.
A risk of misclassification bias exists: this systematic error occurs when a patient is included in a wrong category of patients or should not have been included in the study at all. For this thesis, the risk of including patients with a diagnosis other than glioblastoma was minimized using several methods. First, glioblastoma patients were selected from the histopathological databases of the hospitals, not from imaging databases or patient charts. Next, all pathology reports were thoroughly reviewed. If there was any doubt about diagnosis, these cases were excluded. Third, stored tumor tissue of all included cases was reexamined by the neuropathologist before sending them out for molecular analysis. So, the possibility that a patient suffering from another type of tumor than glioblastoma was included, is very low.
Importantly, misclassification of glioblastoma subtype may also occur concerning *IDH* mutation status. This issue was addressed by determining the presence of an *IDH* mutation using sequencing, which has higher accuracy than IHC. Unfortunately, *IDH* mutation status was impossible to determine in several cases, due to low DNA quality. The longer the FFPE samples were stored, the more likely that technical failure for sequencing arose. This was not the case for *MGMT*p methylation, since qMSP allows reliable results also on tissue samples of inferior quality (Fig. 19). So, the data on *IDH* mutation were missing in a predictable way,

A. *IDH* mutation; *P* < 0.001



B. *MGMT*p methylation; *P* = 0.307

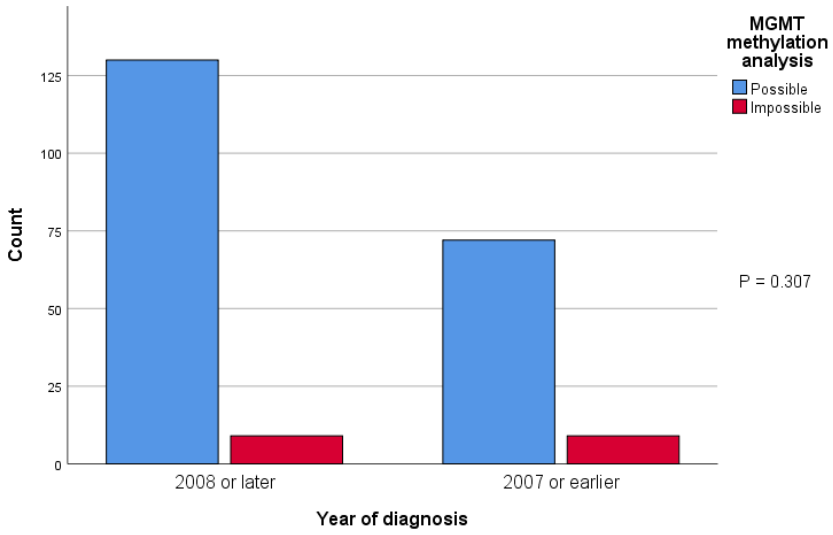


Figure 19. Bar charts representing the correlation between technical analysis failure of molecular biological factors and the date of diagnosis (dichotomized between 2007 or earlier and 2008 or later). A chi square test was performed to verify if the difference between the number of failed analyses in the earlier vs the later years is statistically significant.

although not depending on *IDH* mutation itself, which is -confusingly- known in statistical literature as 'missing at random' (70). 'Complete case analysis' for *IDH* mutation was chosen for publications 3 & 4, inevitably leading to a smaller patient cohort and loss of statistical power. However, compared to analogous studies concerning *IDH*wt glioblastoma patients, the publications in this thesis have similar or larger patient cohorts. It should be noticed that, from a statistical point of view, the publication on weak *MGMT*p methylation (publication 1) could be considered as a study on avoiding misclassification of glioblastoma patients according to *MGMT*p methylation status.
Also, inclusion of patients who did not receive 60 Gy radiotherapy or who were not treated with temozolomide was minimized by detailed chart review, and of course review of the radiotherapeutical plans. If dosimetry plans could not be retrieved, or restored, these patients were excluded for analysis in the study concerning SVZ irradiation (publication 3). These data were missing due to random technical issues and can be considered as data 'missing completely at random' (70).
Finally, information bias and more specifically measurement bias and observer bias, may play an important role in this thesis, especially concerning glioblastoma tumor volume measurement and contouring of the SVZ. Several measures were taken to reduce measurement errors:

1. Measurements were performed by experienced observers blinded for each other's results.
2. Random double checks were performed by each observer for the results of the other observer
3. Dedicated clinical software was used for both glioblastoma volume measurements as well as for contouring the SVZ. Unfortunately, the contouring of the SVZ had to be performed on CT imaging only because imaging fusion of radiotherapeutic planning CT imaging with MR imaging could not be restored due to technical reasons.
4. In case of volumetric analysis of glioblastoma, only MPRAGE imaging with 0.9 mm slice thickness was used.

Both intra and inter observer variability are well-known sources of bias but are notoriously difficult to correct or prevent. There is some evidence that semi-automated segmentation techniques may reduce observer variability in volumetric analysis of glioblastoma (36,45). This technique was applied in this thesis.

Confounding is a specific concern in cohort studies when risk factors are not distributed randomly in the population. Therefore, it is important to apply statistical methods to adjust for known confounders, e.g., using proportional hazard regression for survival analysis. Of course, this methodology can only be used for known confounders or confounders for which data have been collected. Previous glioblastoma studies concerning SVZ contact, SVZ irradiation and partial resection, often lacked molecular biological factors in multivariate survival analysis. Since *IDH* mutation and *MGMT*p methylation are strong prognosticators for glioblastoma patient survival, exclusion of these parameters from survival analysis may lead to erroneous conclusions. In this thesis, confounding was reduced by applying Cox proportional hazards model next to Kaplan-Meier survival analysis, because it is important to compare adjusted and unadjusted survival rates. Importantly, survival analysis was adjusted for known prognosticators, including molecular biological factors. However, some variables were not included because they could not be reliably collected from the patient charts, e.g., steroid use at diagnosis.

Although the retrospective study design of this research inherently suffers from shortcomings, this thesis also has important strengths which are proportionate to the weaknesses. Bias and confounding are well-known problems in medical literature and several methods have been developed to critically assess publications concerning the quality of the evidence they deliver. The GRADE guidelines (Grading of Recommendations Assessment, Development, and Evaluation) provide an excellent and internationally accepted framework of rating quality of evidence (71). The GRADE guidelines rate the final level of confidence in four categories: “high”, “moderate”, “low” or “very low” (Figure 20). Applying these guidelines, publications 1 & 2 of this thesis should be considered as evidence of “moderate” quality, while the risk of bias is higher in publications 3 & 4, so these studies present “low” quality evidence (72).

GRADE’s approach to rating confidence in effect estimates (quality of evidence)

1. Establish initial level of confidence		2. Consider lowering or raising level of confidence		3. Final level of confidence rating
		Reasons for considering lowering or raising confidence		
Study design	Initial confidence in an estimate of effect	↓ Lower if	↑ Higher if	Confidence in an estimate of effect across those considerations
Randomized trials →	High confidence	Risk of Bias Inconsistency Indirectness Imprecision Publication bias	Large effect Dose response All plausible residual confounding and bias • Would reduce a demonstrated effect or • Would suggest a spurious effect if no effect was observed	High ⊕⊕⊕⊕ Moderate ⊕⊕⊕◯ Low ⊕⊕◯◯ Very low ⊕◯◯◯
Observational studies →	Low confidence			

GRADE = Grading of Recommendations Assessment, Development and Evaluation

Figure 20. GRADE’s approach to quality of evidence and to rating confidence in effect estimates (72).

6. Some Reflections and Speculations on Future Glioblastoma Treatment

Compared to advances in treatment of other oncologic diseases, progress in glioblastoma treatment is unfortunately slow. In fact, since 2005, the American Food and Drug Administration (FDA) approved only three new treatments for glioblastoma: temozolomide, bevacizumab and tumor-treating fields. This paragraph focuses on some promising future therapies, from the neurosurgeon's, the radiotherapist's, and the oncologist's perspective. But ultimately, the most important perspective remains that of the patient.

Safely maximizing extent of glioblastoma resection, remains a major neurosurgical challenge, despite the availability of several ancillary techniques. 5-Aminolevulinic acid (5-ALA) – based fluorescence is an effective and safe technique in helping to achieve GTR in glioblastoma resection and is nowadays generally accepted (73,74). Interestingly, 5-ALA may not only be applied as an optical imaging agent but may also serve as a potential therapeutical adjunct. Intracellular porphyrins, induced by 5-ALA, are photosensitizers, and may be used for photodynamic therapies. One small study, using laser diffusors to apply per operative photodynamic therapy for patients with resection of recurrent glioblastoma, showed promising results (75). Since 5-ALA is widely used in surgical neuro-oncology, its application as a therapeutic method, if proven safely and effective, is only a small step further. As already mentioned, the proximity of the tumor to eloquent regions of the brain may limit extent of resection or even render it impossible. Evidence is lacking in neurosurgical literature whether awake craniotomy with brain mapping would not only maximize EOR but also patient survival while preserving neurological function. To answer this important question, the colleagues from Erasmus Medical Center Rotterdam designed a multicenter prospective randomized controlled trial, in which the department of Neurosurgery of Ghent University Hospital participates (76). Of course, glioblastoma patients with new or recurrent tumors may be deemed unsuitable for surgery due to various reasons. In these patients, laser interstitial thermal therapy may prove to be an alternative method for achieving cytoreduction (77,78). In summary, a laser-tip probe is introduced into the centroid of a glioblastoma and by heating the tissue, a thermal lesion is induced. Importantly, real-time MR thermometry is applied to continuously monitor the ablation zone. But the diffuse infiltrative nature of glioblastoma, impedes this tumor from being surgically curable.

This thesis casts serious doubts if irradiation of the SVZ would improve glioblastoma patient survival. However, the SVZ may become a target for other treatment modalities since its role in glioma and glioblastoma is progressively unraveled. Recently, the research group from Liège, Belgium, showed that CXCL12 (CXC motive chemokine 12) has an important role in attracting glioblastoma stem cells towards the SVZ. It may also protect against the effects of radiotherapy (79). Research shows that CXCL12 is an important regulator of the post-radiotherapy glioblastoma microenvironment (80). Since the proximity of the SVZ to the lateral ventricular system, these pathways may become future targets of pharmacotherapies, attempting to block CXCL12 (81). Furthermore, the recent discovery of the nasal/olfactory lymphatic route, an important part of the lymphatic drainage system of the brain, provides an excellent opportunity for intranasal drug administration, reaching the SVZ and the brain via the cerebrospinal fluid, and obliterating the need for invasive ventricular access (82).

Radiotherapeutical innovation in glioblastoma focuses on dose distribution rather than on dose escalation. The well-known concept of 'dose sculpting' or 'dose painting', to customize the delivered radiation dose distribution, is revisited in glioblastoma (83). Based on laboratory animal experiments, a prospective study[5] is currently conducted by the departments of Radiotherapy-Oncology and Nuclear Imaging of Ghent University Hospital, using metabolic imaging (PET) to guide dose painting in glioblastoma patients (84). This way, more metabolic active tumor zones, will receive higher doses without exceeding a total of 60 Gy. There are high expectations that this technique will significantly improve patient survival, without inflicting higher toxicity.

Currently, a lot of attention is raised about the possibility of immunotherapy in glioblastoma (85). A few topics will be highlighted.
The crucial role of *IDH* mutations has been emphasized repeatedly. A few months ago, the results of a first-in-humans phase I trial concerning an *IDH*1^{R132H}-specific peptide vaccine were published by Platten et al. (86). In 33 patients suffering from *IDH* mutated high grade gliomas, the vaccine proved safe and immunogenic. Although the patient cohort was too small to draw definitive conclusions, the efficacy of the vaccine was promising with an overall response rate of 84.4%. Of course, this type of vaccine will only be effective in *IDH* mutated glioblastoma. Dendritic cell vaccines, however, are applied in *IDH*wt and mutated glioblastoma and have been developed since 2001 (87-89). But vaccination as a single-modality immunotherapy is probably not sufficiently effective, based on the negative results from several phase II and III trials (85). Since the report of a case of drastic glioblastoma regression after treatment with chimeric antigen receptor (CAR)-engineered T cells, this research has drawn a lot of attention – and high hopes (90). Contrary to the success of CAR-T cell therapy for hematological malignancies, the clinical effect in solid tumors and glioblastoma was reserved for selected patients but still encouraging (91). As stated by Lim et al., the future success or failure of CAR-T cell therapy in glioblastoma depends on the identification of sufficiently expressed tumor-specific antigens which are also stable (85).

Finally, precision oncology and targeted therapies gain interest in glioblastoma, although demonstration of efficacy is lacking (92). Although still incompletely understood, advances in next-generation sequencing technology have transformed the knowledge of the genomic landscape of glioblastoma. An exponential acceleration of this knowledge revolution is to be expected, bringing forth new treatment modalities. An innovating treatment option may be found in targeting microRNAs (miRNA), although clinical trials are yet to be undertaken (93). Very briefly, miRNAs are small (19-22 nucleotides) non-coding RNA molecules. They have an important role in posttranscriptional regulation of messenger RNAs by sequence-specific repression (94). Nowadays, more than 2000 miRNAs have been discovered in the human genome, playing a role in gene regulation and the development of diseases, including cancer, comprising glioblastoma (93). It has been shown experimentally that several miRNAs are involved in the regulation of MGMT expression, amongst which miRNA-181d shows the most potential. Zhang and colleagues showed that the expression level of miRNA-181d was inversely correlated with MGMT expression level (95). Furthermore, high expression of miRNA-181d was associated with significantly improved overall patient survival in three

[5] *Positron-emission tomography (PET)-based dose painting radiation therapy in glioblastoma*

independent cohorts of glioblastoma patients. Upregulation of this specific miRNA in the tumors of patients suffering from unmethylated glioblastoma may result in better response to chemoradiotherapy and increased survival. This would constitute a major breakthrough especially for *IDH*wt glioblastoma patients with unmethylated tumors. Target specificity and tissue toxicity, however, pose major obstacles for clinical implementation of miRNA, as well as the difficulty of delivering the miRNA to their messenger RNA target since they will have to cross the blood-brain barrier. Perhaps direct ventricular access or the already mentioned nasal/olfactory lymphatic route could provide solutions, but this remains mere speculation for now. The role of miRNAs as a biomarker in glioblastoma is also very promising (95). The departments of Radiotherapy-Oncology and Neurosurgery are currently engaged in a clinical pilot study to determine the presence of circulating miRNAs in the serum of glioblastoma patients. The assumptions that these miRNAs will not only be present at diagnosis of glioblastoma but will also fluctuate according to tumor control or relapse, remain to be proven.

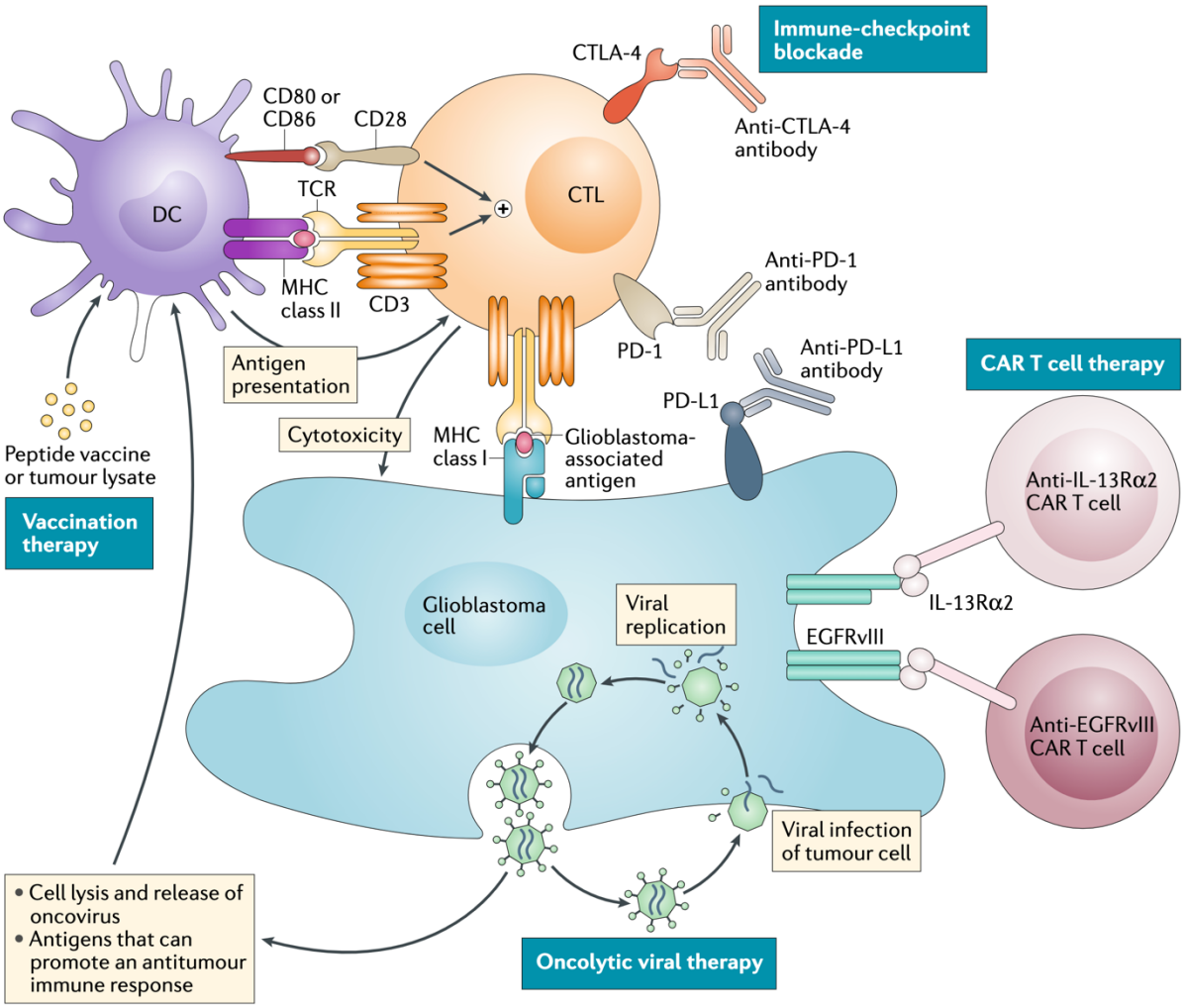


Figure 21. Illustration of the contemporary immunotherapy modalities for glioblastoma treatment (85).

CAR = chimeric antigen receptor; CD = cluster of differentiation; CTL = cytotoxic T lymphocyte; CTLA-4 = cytotoxic T lymphocyte protein 4; DC = dendritic cell; EGFRvIII = endothelial derived growth factor receptor variant III; IL-13Rα2 = Interleukin 13 receptor subunit α2; MCH = major histocompatibility complex; PD-1 = programmed cell death protein 1; PD-L1 = programmed cell death 1 ligand 1; TCR = T-cell receptor.

Conclusion

In conclusion, this thesis allows for identification of weak *MGMT*p methylation as a positive prognostic factor for glioblastoma patient survival while SVZ contact of glioblastoma at the time of diagnosis is to be accepted as a negative prognostic factor, although the underlying mechanisms remain unclear at present. Publication 1 & 2 of this thesis present evidence of "moderate" strength according to the GRADE guidelines. Further, the results from publications 2 & 3 in this thesis suggest that in *IDH*wt glioblastoma patients, treated with chemoradiotherapy after surgery, SVZ irradiation does not yield a beneficial survival effect, but partial glioblastoma resection will result in better overall survival, as compared to biopsy only. The quality of evidence delivered by these two studies (publication 3 & 4) is lower and should be considered as "low" according to the GRADE guidelines. Glioblastoma treatment, specifically resection, often imposes an important choice on the patient and the neurosurgeon, comprising a trade-off between oncology and function. Glioblastoma patients are in urgent need of new and innovating treatment modalities, but the laboratory experiments do not transform clinical practice yet.

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SUMMARY

Despite aggressive treatment with temozolomide-based chemoradiotherapy after surgery, the prognosis of glioblastoma patients remains dismal with a median overall survival of only 15 months. In this thesis, several prognostic factors of glioblastoma patient survival are discussed, next to age at diagnosis and patient condition.

Two molecular biological factors have an important prognostic effect in glioblastoma patients. First, the isocitrate dehydrogenase (*IDH*) mutation plays a pivotal role in current glioma and glioblastoma classification. Patients suffering from *IDH*-mutated glioblastoma have a significantly better prognosis than I*DH*-wildtype glioblastoma patients. Unfortunately, only 10% or less of newly diagnosed glioblastoma patients will belong to the IDH-mutated group. Second, the epigenetic phenomenon of methylation of the O^6-methylguanine-DNA-methyltransferase gene promoter (*MGMT*p), renders the tumor tissue more sensitive to the alkylating effects of temozolomide because *MGMT*p methylation silences the gene and DNA-repair by MGMT is lowered or even stopped. So, glioblastoma patients with *MGMT*p methylation will have a significantly better survival. The widely used test to establish the *MGMT*p methylation status, quantitative methylation-specific PCR (qMSP), does not allow for a simple dichotomization between "methylated" and "unmethylated" tumors but leaves a "grey zone" of diagnostic uncertainty. Patients with test results within this grey zone, but showing 'weak' methylation of glioblastoma tissue, will still have a significantly better survival than patients with fully unmethylated tumors, as is described in this thesis. An international consensus should be established concerning both the cut-off values for qMSP testing in glioblastoma as well as an internationally accepted testing algorithm. This way, future glioblastoma survival studies will be standardized concerning *MGMT*p testing, and bias will be avoided. Moreover, from the clinical point of view, this consensus and standardization will prevent that patients with test results within the grey zone could be at risk of missing out on therapeutic opportunities.

Although considered impossible for decades, it was proven in the beginning of the 21st century that the adult human brain contains neural stem cells (NSCs) both in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ), which is the largest reservoir of NSCs. The role of the SVZ in glioblastoma is poorly understood. As this thesis also shows, patients suffering from SVZ-contacting glioblastoma have a significant worse survival although the explanation for this observation remains elusive for now. The correlation with the presence of NSCs seems obvious and there is mounting evidence for the presence of brain tumor propagating cells (BTPCs) in the SVZ in case of glioblastoma. Failure to eradicate these cells will inevitably result in tumor recurrence, which indeed is the fate of the overwhelming majority of glioblastoma patients. Therefore, it seems logical to irradiate the largest reservoir of these BTPCs, namely the SVZ, since radiation therapy is of course already an essential part of glioblastoma patient treatment. However, reports on the survival impact of SVZ irradiation in glioblastoma deliver conflicting results. Some publications, as is the case with the report discussed in this thesis, show no effect or even detrimental effects of higher SVZ radiation doses on glioblastoma patient survival. Carefully designed prospective studies are needed to overcome the limitations of the retrospective studies concerning SVZ

irradiation. The presence of normal NSCs in the SVZ, and their vulnerability to radiation, remains a worrisome point of attention.

Gross total resection (GTR) has been shown to result in a significant longer glioblastoma patient survival, although these studies are prone to methodological issues especially concerning tumor measurement. However, neurosurgical intervention that creates new neurological deficit, will unfortunately cancel out the beneficial effects of GTR. Partial resection of glioblastoma, not only as a palliative measure to relieve symptoms due to mass-effect, but also to prolong survival, remains highly controversial especially in the 'molecular age' of glioblastoma. A report included in this thesis, shows that in case of patients with *IDH*-wildtype unmethylated glioblastoma, partial resection results in a significant, but modest, better overall survival, as compared to patients who were treated with biopsy only. This may offer some hope to those patients with unfavorable molecular markers at the onset of their disease.

Next to age at diagnosis, the condition of the patient and *IDH*-mutation, also weak *MGMT*p-methylation and partial tumor resection are favorable prognostic factors for glioblastoma patient survival. While the impact of SVZ radiation on glioblastoma patient survival remains highly controversial, SVZ-contacting glioblastoma at diagnosis applies as an unfavorable prognostic factor.

SAMENVATTING

De behandeling van patiënten lijdend aan glioblastoma bestaat in 2021 nog steeds uit resectie gevolgd door bestraling en chemotherapie, met name temozolomide. Ondanks deze agressieve aanpak, blijft de prognose van deze patiënten bijzonder somber: de mediane overleving bedraagt slechts ongeveer 15 maanden. Behalve de leeftijd en de algemene conditie van de patiënt bij diagnose, bestaan er nog verschillende prognostische factoren waarvan enkele in dit proefschrift nader onderzocht werden.

Er worden op heden twee moleculairbiologische factoren beschreven die de prognose van patiënten met een glioblastoma sterk beïnvloeden. De eerste is de mutatie van het isocitraat-dehydrogenase (*IDH*). Deze *IDH*-mutatie is dermate belangrijk, dat de gehele glioma-classificatie sinds 2016 deels werd gefundeerd op deze afwijking. Ook voor het glioblastoma geldt dat een *IDH*-gemuteerde tumor een duidelijk langere overleving betekent voor de patiënt. Helaas behoren slechts 10% of minder van de patiënten die met de nieuwe diagnose van glioblastoma geconfronteerd worden tot deze groep. De tweede factor is een epigenetische factor, met name de methylatie van de promotor van het O^6-methylguanine-DNA-methyltransferase-gen (*MGMT*-gen). Wanneer deze methylatie in het glioblastoma aanwezig is, dan werkt het DNA-hersteleiwit MGMT minder efficiënt of zelfs helemaal niet meer, waardoor het tumorweefsel veel gevoeliger wordt aan de alkylerende invloed van temozolomide. Op zijn beurt, zorgt dit voor een significant langere overleving van de patiënt. Echter, bij de techniek die vaak wordt toegepast om de aanwezigheid van deze methylatie te bepalen, kwantitatieve methylatie-specifieke PCR (qMSP), komt een zgn. "grijze zone" voor, waarbinnen er diagnostische onzekerheid heerst. Daarbij is het belangrijk, zoals in dit proefschrift bestudeerd werd, dat zelfs patiënten met een ‘zwak’ gemethyleerd glioblastoma een significant langere overleving vertonen dan patiënten waarbij deze methylatie volledig afwezig is. Patiënten met testresultaten binnen deze grijze zone, zouden therapeutische kansen kunnen verliezen. Ook wetenschappelijk onderzoek naar de overleving van patiënten met glioblastoma en *MGMT*-promoter-methylatie kan door het bestaan van deze diagnostische onzekerheid vertroebeld worden. De oplossing voor deze problematiek bestaat erin om een internationale consensus te bereiken met betrekking tot referentiewaarden voor qMSP en een algoritme op te stellen m.b.t. de verschillende technische analyses voor *MGMT*-promoter-methylatie-bepaling.

Ondanks decennialange wetenschappelijke overtuiging van het tegendeel, werd begin deze eeuw onweerlegbaar aangetoond dat er ook in volwassen menselijke hersenen neurale stamcellen aanwezig zijn en wel in de gyrus dentatus van de hippocampus en in de subventriculaire zone (SVZ). Deze zone speelt een belangrijke, maar onvoldoende begrepen rol bij het glioblastoma. Patiënten bij wie de tumor bij diagnose contact maakt met de SVZ, hebben een duidelijk slechtere prognose dan de groep patiënten bij wie dit niet het geval is, hetgeen ook in dit proefschrift opnieuw werd aangetoond. De exacte reden(en) hiervoor zijn nog onvoldoende gekend, hoewel vele auteurs menen dat er een verband moet bestaan met de aanwezigheid van neurale stamcellen in de SVZ. Meer nog, recente publicaties tonen aan dat, hoogstwaarschijnlijk, de oorsprong van het glioblastoma moet gezocht worden in stamcellen die maligne ontaarden. Zolang deze 'glioblastoma-stamcellen' niet uitgeroeid zijn, zal de tumor onvermijdelijk recidiveren, hetgeen inderdaad het lot is van bijna alle

patiënten die lijden aan glioblastoma. Aangezien radiotherapie reeds een vast en gevalideerd onderdeel is van de standaard glioblastoma-behandeling, lijkt het logisch om het grootste potentiële reservoir van glioblastoma stamcellen, de SVZ, ook te bestralen. Echter, de studies betreffende de invloed van bestraling op de SVZ spreken elkaar tegen. Sommige publicaties, waaronder de studie die in dit proefschrift gepresenteerd wordt, tonen aan dat bestraling van de SVZ geen of zelfs een negatief effect op de overleving van glioblastoma patiënten kan hebben. Enkel zorgvuldig ontworpen prospectieve studies kunnen de belangrijke tekortkomingen van de verrichte retrospectieve studies overstijgen en meer duidelijkheid omtrent dit belangrijke onderwerp te scheppen. Maar voorzichtigheid blijft geboden, gezien bestraling ook de neurale stamcellen zal aantasten.

Neurochirurgische resectie van glioblastoma blijft één van de pijlers van de behandeling. Hoewel er m.b.t. de metingen van tumorvolumes methodologische discussies blijven bestaan, tonen vele studies aan dat een totale resectie van het aankleurende tumorgedeelte van glioblastoma aanleiding geeft tot een belangrijke verlenging van de overleving van de patiënt. Kanttekening hierbij is dat het veroorzaken van nieuwe neurologische schade dit voordeel tenietdoet, zelfs als een volledige resectie bekomen wordt. De effecten van een partiële resectie van het glioblastoma op de overleving van de patiënt, meer dan louter het reduceren van het tumormassa-effect, zijn controversieel zeker in het licht van de reeds besproken moleculairbiologische factoren. In dit proefschrift wordt een studie gepresenteerd die aantoont dat partiële tumorresectie bij glioblastoma patiënten met *MGMT*-ongemethyleerde *IDH*-wildtype-tumoren, resulteert in een significante, hoewel bescheiden, verlenging van de overleving in vergelijking met patiënten die louter een biopsie ondergingen. Dit biedt enige hoop aan patiënten die hun ziekte starten met een ongunstig tumoraal genetisch profiel.

Behalve leeftijd en conditie van de patiënt, en de aanwezigheid van een *IDH*-mutatie, worden ook de aanwezigheid van een ‘zwakke’ *MGMT*-promoter-methylatie en een partiële resectie van de tumor als prognostisch gunstige factoren bij patiënten met glioblastoma weerhouden. Contact van het glioblastoma met de SVZ bij diagnose is daarentegen een ongunstige prognostische factor terwijl de rol van bestraling van de SVZ bij deze patiënten onduidelijk blijft.

ZUSAMMENFASSUNG

Im Jahre 2021 besteht die Behandlung von Patienten mit einem Glioblastom weiterhin aus einer Resektion mit anschließender Bestrahlung und Chemotherapie, letztere vorwiegend mit Temozolomid. Trotz dieses aggressiven Ansatzes bleibt die Prognose von Glioblastom-Patienten sehr düster. Die mediane Überlebenszeit beträgt nur etwa 15 Monate. Neben dem Alter und dem Allgemeinzustand des Patienten zum Zeitpunkt der Diagnose, gibt es mehrere prognostische Faktoren, von denen einige in dieser Dissertation weiter untersucht wurden.

Derzeit werden zwei molekularbiologische Faktoren beschrieben, die die Prognose von Patienten mit Glioblastom stark beeinflussen. Die erste ist die Mutation der *Isozitratdehydrogenase* (IDH). Diese IDH-Mutation ist derart wichtig, dass seit 2016 die gesamte Gliomklassifizierung teilweise auf dieser Variablen basiert. Im Falle des Glioblastoms bedeutet ein IDH-mutierter Tumor ein deutlich besseres Überleben für den Patienten. Leider gehören nur etwa 10% der mit Glioblastom diagnostizierten Patienten dieser Gruppe an. Der zweite Faktor ist ein epigenetischer, nämlich die Methylierung des Promotors des *O^6-Methylguanin-DNA-Methyltransferase* (MGMT)-Gens. Wenn diese Methylierung im Glioblastom vorhanden ist, arbeitet das DNA-Reparaturprotein MGMT weniger effizient oder es hört sogar auf, zu arbeiten, wodurch das Tumorgewebe viel empfindlicher für die alkylierende Wirkung von Temozolomid wird. Dies wiederum verbessert das Überleben der Patienten erheblich. Bei der häufig verwendeten Technik zur Bestimmung des Vorhandenseins dieser Methylierung, der quantitativen methylierung-spezifischen PCR (qMSP), tut sich jedoch eine sogenannte Grauzone auf, innerhalb derer es eine diagnostische Unsicherheit gibt. In diesem Zusammenhang ist es wichtig, wie in dieser Arbeit untersucht wurde, dass auch Patienten, deren Glioblastom nur "schwach methyliert“ zu sein scheint, ein signifikant besseres Überleben vorweisen als Patienten, bei denen diese Methylierung gänzlich fehlt. Somit bleiben den Patienten mit Testergebnissen innerhalb obiger Grauzone womöglich wertvolle therapeutische Optionen vorenthalten. Die diagnostische Unsicherheit kann außerdem die wissenschaftlichen Untersuchungsergebnisse zum Überleben von Patienten mit Glioblastom und MGMT verzerren. Die Lösung für dieses Problem besteht darin, einen internationalen Konsens bezüglich der Referenzwerte für qMSP zu erzielen und einen Algorithmus zu erarbeiten, der hinsichtlich der verschiedenen technischen Analysen für die Bestimmung der Methylierung des MGMT-Promotors zu befolgen wäre.

Trotz jahrzehntelanger gegenteiliger wissenschaftlicher Überzeugung, wurde zu Beginn dieses Jahrhunderts unwiderlegbar nachgewiesen, dass neuronale Stammzellen auch im erwachsenen menschlichen Gehirn vorhanden sind, nämlich im Gyrus dentatus des Hippocampus und in der subventrikulären Zone (SVZ). Diese Zone spielt beim Glioblastom eine wichtige, aber nicht ausreichend verstandene Rolle. Patienten, deren Tumor zum Zeitpunkt der Diagnose Kontakt mit der SVZ hat, haben eine deutlich schlechtere Prognose als Patienten ohne einen solchen Kontakt, was auch in dieser Dissertation gezeigt wird. Die genauen Gründe hierfür sind noch unzureichend bekannt, obwohl viele Autoren der Ansicht sind, dass ein Zusammenhang mit den neuronalen Stammzellen in der SVZ bestehen dürfte. Tatsächlich zeigen neuere Veröffentlichungen, dass entartete Stammzellen

höchstwahrscheinlich den Ursprung des Glioblastoms bilden. Solange diese Stammzellen nicht ausgerottet werden, ist ein Tumorrückfall unausweichlich, was in der Tat das Schicksal fast aller Patienten ist, die an einem Glioblastom erkrankt sind. Da die Strahlentherapie bereits ein fester und validierter Bestandteil der Standardbehandlung für Glioblastome ist, erscheint es logisch, auch das größte potenzielle Reservoir an Glioblastomstammzellen, nämlich die SVZ, zu bestrahlen. Die Studien zum Einfluss der Bestrahlung auf die SVZ sind jedoch widersprüchlich. Einige Veröffentlichungen, einschließlich der in dieser Dissertation vorgelegten Studie, zeigen, dass die Bestrahlung der SVZ keine oder sogar negative Auswirkungen auf das Überleben von Glioblastom-Patienten haben kann. Nur sorgfältig konzipierte, prospektive Studien können die erheblichen Mängel der retrospektiv durchgeführten Studien überwinden, um mehr Klarheit zu diesem wichtigen Thema zu verschaffen. Vorsicht ist jedoch geboten, da die Bestrahlung auch die neuronalen Stammzellen angreift.

Die neurochirurgische Resektion des Glioblastoms bleibt eine der Säulen der Behandlung. Trotz methodischer Debatten bezüglich der Messung des Tumorvolumens, zeigen viele Studien, dass eine vollständige Resektion des anfärbenden Anteils des Glioblastoms das Überleben des Patienten signifikant verlängert. Es sollte beachtet werden, dass das Verursachen neuer neurologischer Schäden diesen Vorteil zunichtemacht, selbst wenn eine vollständige Resektion erreicht wird. Die Auswirkungen einer teilweisen Resektion des Glioblastoms auf das Überleben des Patienten, noch abgesehen von einer Verringerung der Raumforderung, sind, insbesondere angesichts der bereits diskutierten molekularbiologischen Faktoren, umstritten. In dieser Arbeit wird eine Studie vorgelegt, die zeigt, dass eine partielle Tumorresektion bei Patienten mit MGMT-unmethylierten IDH-Wildtyp-Glioblastomen, im Vergleich zu Patienten, bei denen lediglich eine Biopsie durchgeführt wurde, zu einer signifikanten, wenn auch bescheidenen Verlängerung des Überlebens führt. Dies gibt den Patienten mit einem von Anfang an ungünstigen tumorgenetischen Profil, eine gewisse Hoffnung.

Neben einem jüngeren Alter und einem guten Allgemeinzustand, erweisen sich beim Patienten mit Glioblastom das Vorhandensein einer IDH-Mutation sowie einer "schwachen Methylierung“ des MGMT-Promotors, außerdem eine zumindest teilweise Resektion des Tumors als günstige prognostische Faktoren. Im Gegensatz dazu ist der Kontakt des Glioblastoms mit der SVZ bei der Diagnose ein ungünstiger prognostischer Faktor, während die Rolle der SVZ-Bestrahlung bei diesen Patienten unklar bleibt.

RESUME

Malgré un traitement agressif par chimioradiothérapie à base de témozolomide après la chirurgie, le pronostic des patients atteints de glioblastome reste sombre avec une survie globale médiane de seulement 15 mois. Dans cette thèse, plusieurs facteurs pronostiques sont discutés, à côté de l'âge au moment du diagnostic et de l'état du patient.

Deux facteurs biologiques moléculaires ont un effet pronostique important chez les patients atteints de glioblastome. Premièrement, la mutation de *l'isocitrate déshydrogénase* (IDH) joue un rôle central dans la classification actuelle des gliomes et des glioblastomes. Les patients souffrant de glioblastome à mutation IDH ont un pronostic significativement meilleur que les patients atteints de glioblastome de type sauvage IDH. Malheureusement, seulement 10% ou moins des patients atteints de glioblastome nouvellement diagnostiqué appartiendront au groupe ayant subi une mutation IDH. Deuxièmement, le phénomène épigénétique de la méthylation du promoteur du gène *O^6-méthylguanine-ADN-méthyltransférase* (MGMTp), rend le tissu tumoral plus vulnérable aux effets alkylants du témozolomide parce que la méthylation MGMTp fait taire le gène et la réparation de l'ADN par MGMT est abaissée ou même arrêté. Ainsi, les patients atteints de glioblastome avec MGMTp-méthylation auront une survie significativement meilleure. Le test largement utilisé pour établir le statut de la méthylation MGMTp, PCR quantitative spécifique à la méthylation (qMSP), ne permet pas une simple dichotomisation entre les tumeurs «méthylées» et «non méthylées», mais montre une «zone grise» d'incertitude diagnostique. Les patients dont les résultats des tests se situent dans cette zone grise, mais présentant une méthylation «faible» du tissu du glioblastome, auront toujours une meilleure survie que les patients avec des tumeurs totalement non méthylées, comme décrit dans cette thèse. Un consensus international doit être établi concernant à la fois les valeurs seuils pour les tests de qMSP dans le glioblastome ainsi qu'un algorithme de test internationalement accepté. De cette façon, les futures études de survie du glioblastome seront standardisées concernant les tests MGMTp et les biais seront évités. De plus, du point de vue clinique, ce consensus et cette standardisation éviteront que les patients dont les résultats des tests se situent dans la zone grise risquent de rater des opportunités thérapeutiques.

Bien que considéré comme impossible pendant des décennies, il a été prouvé au début du 21ème siècle que le cerveau humain adulte contient des cellules souches neurales (NSC) à la fois dans le gyrus denté de l'hippocampe et dans la zone sous-ventriculaire (SVZ), qui est le plus grand réservoir des NSC. Le rôle de la SVZ dans le glioblastome est mal compris. Comme cette thèse le montre également, les patients souffrant de glioblastome en contact avec SVZ ont une survie significativement pire bien que l'explication de cette observation reste pour l'instant insaisissable. La corrélation avec la présence de NSC semble évidente et il existe de plus en plus de preuves de la présence de cellules de propagation de tumeurs cérébrales
(BTPC) dans la SVZ en cas de glioblastome. L'absence d'éradication de ces cellules entraînera inévitablement une récidive tumorale, ce qui est en effet le sort de l'écrasante majorité des patients atteints de glioblastome. Par conséquent, il semble logique d'irradier le plus grand réservoir de ces BTPC, à savoir le SVZ, car la radiothérapie est bien entendu déjà un élément essentiel du traitement des patients atteints de glioblastome. Cependant, les rapports sur l'impact sur la survie de l'irradiation SVZ dans le glioblastome offrent des résultats

contradictoires. Certaines publications, comme c'est le cas avec le rapport discuté dans cette thèse, ne montrent aucun effet ou même des effets néfastes de doses de rayonnement SVZ plus élevées sur la survie des patients atteints de glioblastome. Des études prospectives soigneusement conçues sont nécessaires pour surmonter les limites des études rétrospectives concernant l'irradiation SVZ. La présence de NSC normaux dans la SVZ et leur vulnérabilité aux radiations restent un point d'attention inquiétant.

Il a été démontré que la résection totale brute (GTR) entraîne une survie significative des patients atteints de glioblastome, bien que ces études soient sujettes à des problèmes méthodologiques, en particulier concernant la mesure de la tumeur. Cependant, une intervention neurochirurgicale qui crée un nouveau déficit neurologique, annulera malheureusement les effets bénéfiques de la GTR. La résection partielle du glioblastome, non seulement comme mesure palliative pour soulager les symptômes dus à l'effet de masse, mais aussi pour prolonger la survie, reste très controversée, en particulier à «l'âge moléculaire» du glioblastome. Un rapport inclus dans cette thèse montre que dans le cas de patients atteints de glioblastome non méthylé de type sauvage IDH, la résection partielle se traduit par une survie globale significative, mais modeste, meilleure, par rapport aux patients traités par biopsie uniquement. Cela peut offrir un certain espoir aux patients présentant des marqueurs moléculaires défavorables au début de leur maladie.

Outre l'âge au moment du diagnostic, l'état du patient et la mutation IDH, ainsi qu'une faible méthylation MGMTp et une résection partielle de la tumeur sont des facteurs pronostiques favorables pour la survie des patients atteints de glioblastome. Cependant, le contact SVZ du glioblastome au moment du diagnostic s'applique comme un facteur pronostique défavorable; tandis que l'impact du rayonnement SVZ sur la survie des patients atteints de glioblastome reste très controversé.

SOMMARIO

Nonostante il trattamento aggressivo con chemioradioterapia a base di temozolomide dopo l'intervento chirurgico, la prognosi dei pazienti con glioblastoma rimane infausta con una sopravvivenza globale mediana di soli 15 mesi. In questa tesi vengono discussi diversi fattori prognostici, oltre all'età alla diagnosi e alle condizioni generali del paziente.

Due fattori biologici molecolari hanno un importante effetto prognostico nei pazienti con glioblastoma. In primo luogo, la mutazione dell'isocitrato deidrogenasi (IDH) gioca un ruolo fondamentale nell'attuale classificazione di glioma e glioblastoma. I pazienti affetti da glioblastoma con IDH mutata hanno una prognosi significativamente migliore rispetto ai pazienti con glioblastoma con IDH *wild type* (non mutata). Sfortunatamente, solo il 10% o meno dei pazienti con glioblastoma di nuova diagnosi appartiene al gruppo IDH mutata. In secondo luogo, il fenomeno epigenetico della metilazione del promotore del gene O6-metilguanina-DNA-metiltransferasi (MGMTp), rende il tessuto tumorale più vulnerabile agli effetti alchilanti della temozolomide perché la metilazione MGMTp silenzia il gene e la riparazione del DNA da parte di MGMT è ridotta o del tutto inattivata. Di conseguenza i pazienti con glioblastoma con metilazione MGMTp avranno una sopravvivenza significativamente migliore. Il test ampiamente utilizzato per stabilire lo stato di metilazione MGMTp, PCR quantitativa specifica per la metilazione (qMSP), non consente una semplice dicotomizzazione tra tumori "metilati" e "non metilati", ma mostra una "zona grigia" di incertezza diagnostica. I pazienti con risultati dei test all'interno di questa zona grigia, ma che mostrano una metilazione "debole" del glioblastoma, avranno comunque una sopravvivenza significativamente migliore rispetto ai pazienti con tumori completamente non metilati, come descritto in questa tesi. Dovrebbe essere stabilito un consenso internazionale riguardo sia i valori di cut-off per il test qMSP nel glioblastoma sia un algoritmo per le analisi della metilazione accettato a livello internazionale. In questo modo, i futuri studi sulla sopravvivenza del glioblastoma saranno standardizzati per quanto riguarda il test MGMTp e saranno evitati i bias. Inoltre, dal punto di vista clinico, questo consenso e standardizzazione impediranno che i pazienti con risultati dei test all'interno della zona grigia possano correre il rischio di perdere opportunità terapeutiche.

Sebbene considerato impossibile per decenni, all'inizio del XXI secolo è stato dimostrato che il cervello umano adulto contiene cellule staminali neurali (NSC) sia nel giro dentato dell'ippocampo sia nella zona subventricolare (SVZ); quest'ultima inoltre è risultata essere la più grande riserva di NSC. Il ruolo della SVZ nel glioblastoma è scarsamente compreso. Come mostra anche questa tesi, i pazienti affetti da glioblastoma a contatto con la SVZ hanno una sopravvivenza significativamente peggiore sebbene la spiegazione per questa osservazione rimanga al momento sfuggente. La correlazione con la presenza di NSC sembra ovvia e vi sono prove crescenti della presenza di cellule di propagazione del tumore cerebrale (BTPC) nella SVZ in caso di glioblastoma. La mancata eliminazione di queste cellule porterà inevitabilmente alla recidiva del tumore, che in effetti è il destino della stragrande maggioranza dei pazienti con glioblastoma. Pertanto, sembra logico dover irradiare la più grande riserva di questi BTPC, vale a dire la SVZ, poiché la radioterapia è ovviamente già una parte essenziale del trattamento dei pazienti con glioblastoma. Tuttavia, i rapporti sull'impatto dell'irradiazione della SVZ nel glioblastoma sulla sopravvivenza offrono risultati

contrastanti. Alcune pubblicazioni, come nel caso dello studio discusso in questa tesi, non mostrano alcun effetto o addirittura effetti dannosi della radioterapia mirata della SVZ sulla sopravvivenza dei pazienti con glioblastoma. Sono necessari studi prospettici progettati con cura per superare i limiti degli studi retrospettivi riguardanti l'irradiazione della SVZ. La presenza di NSC normali nella SVZ e la loro vulnerabilità alle radiazioni rimane un tema che merita particolare attenzione.

È stato dimostrato che la resezione totale (GTR) si traduce in una sopravvivenza del paziente con glioblastoma significativamente più lunga, sebbene questi studi siano soggetti a problemi metodologici, in particolare riguardo alla misurazione del tumore. Tuttavia, l'intervento neurochirurgico che crea un nuovo deficit neurologico, purtroppo annullerà gli effetti benefici della GTR. La resezione parziale del glioblastoma, non solo come misura palliativa per alleviare i sintomi dovuti all'effetto massa, ma anche per prolungare la sopravvivenza, rimane molto controversa soprattutto alla luce delle caratteristiche molecolari del glioblastoma appena discusse. Un rapporto incluso in questa tesi mostra che in caso di pazienti con glioblastoma non metilato con IDH *wild type*, la resezione parziale si traduce in una sopravvivenza globale significativamente migliore, seppur modesta, rispetto ai pazienti che sono stati trattati solo con biopsia. Questo può quindi offrire qualche speranza a quei pazienti con marcatori molecolari sfavorevoli all'inizio della loro malattia.

Oltre all'età alla diagnosi, le condizioni generali del paziente e la mutazione dell' IDH, anche la debole metilazione MGMTp e la resezione parziale del tumore sono fattori prognostici favorevoli per la sopravvivenza del paziente affetto da glioblastoma. L'impatto della radiazione della SVZ sulla sopravvivenza dei pazienti con glioblastoma, invece, rimane molto controverso. Infine il contatto del glioblastoma con la SVZ al momento della diagnosi viene considerato come fattore prognostico sfavorevole.

Περίληψη

Παρά την χρήση επιθετικής χημειοθεραπείας ταυτόχρονα με ακτινοθεραπεία μετά από χειρουργείο, οι ασθενείς με γλοιοβλάστωμα έχουν δυσμενή πρόγνωση τους με συνολική επιβίωση μόνο 15 μηνών.
Στη μελέτη μας, εκτός από την ηλικία κατά τη διάγνωση και την γενική κατάσταση του ασθενούς, εξετάσαμε και άλλους προγνωστικούς παράγοντες. Δύο μοριακοί δείκτες εξετάστηκαν για να εκτιμηθεί εάν μπορούν να συμβάλουν σημαντικά στην πρόγνωση των ασθενών με γλοιοβλάστωμα.
Πρώτον, η μετάλλαξη του γονιδίου IDH1 που παίζει κεντρικό ρόλο στην τρέχουσα ταξινόμηση των γλοιωμάτων. Οι ασθενείς με γλοιοβλάστωμα με μετάλλαξη στο IDH γονίδιο έχουν καλύτερη πρόγνωση συγκριτικά με τους ασθενείς με κακοήθεια χωρίς την συγκεκριμένη μετάλλαξη (wild type IDH). Δυστυχώς, μόνο 10% των ασθενών με πρόσφατα διαγνωσμένο γλοιοβλάστωμα, παρουσιάζουν την μετάλλαξη στο IDH γονίδιο.
Δεύτερον, η υπερμεθυλιωση του υποκινητή του MGMT γονιδίου. Η παρουσία του αντανακλά την ανικανότητα του όγκου να επιδιόρθωση τις βλάβες που προκαλούν οι αλκυλιουντες παράγοντες, και πιο συγκεκριμένα η τεμοζολομίδη. Οι ασθενείς με υπερμεθυλιωση του υποκινητή του MGMT δείχνουν καλύτερη επιβίωση.
Η μέθοδος που χρησιμοποιείται γενικά για την ανάλυση της υπερμεθυλιωση του υποκινητή του MGMT γονιδίου είναι ποσοτική PCR ειδική για μεθυλίωση (QMSP), δεν επιτρέπει απλό διαχωρισμό μεταξύ των όγκων που παρουσιάζουν η όχι υπερμεθυλιωση. Διαμορφώνεται μια "γκρίζα ζώνη" διαγνωστικής αβεβαιότητας, όπου οι ασθενείς με γλοιοβλάστωμα με ελαφροί μεθυλίωση θα έχουν πάντα καλύτερη επιβίωση από ασθενείς άνευ μεθυλίωση.

Πρέπει να καθοριστεί μια διεθνή ομοφωνία σχετικά με τα όρια των τεστ qMSP για το γλοιοβλάστωμα καθώς και ένα διεθνές αποδεκτό αλγόριθμο των τεστ.
Με αυτόν τον τρόπο, οι μελλοντικές μελέτες που θα εξετάζουν την επιβίωση των ασθενών με γλοιοβλάστωμα θα γίνονται με πιο τυποποιημένα τεστ MGMTP και θα αποφευχθούν τυχόν προκατειλημμένες μετρήσεις.
Και από κλινική άποψη, χάρη αυτής της ομοφωνίας και της τυποποίησης, οι ασθενείς με τεστ qMSP που βρίσκονται στην «γκρίζα ζώνη » δεν θα χάνουν πια πιθανή δυνατότητα θεραπείας.
Αν και είχε θεωρηθεί αδύνατο για δεκαετίες, έχει αποδειχθεί στις αρχές του 21ου αιώνα πως ο ενήλικος ανθρώπινος εγκέφαλος περιέχει νευρικά βλαστοκύτταρα (NSC), στην οδοντωτή έλικα του ιππόκαμπου και στην υπόκοιλιακή ζώνη (SVZ), η οποία αποτελεί και την μεγαλύτερη δεξαμενή των NSCs.
Ο ρόλος του SVZ στο γλοιοβλάστωμα δεν είναι ώμος καλά κατανοητός.
Όπως το αποδεικνύει επίσης και η παρούσα μελέτη, οι ασθενείς που παρουσιάζουν γλοιοβλάστωμα που έρχεται σε επαφή με το SVZ έχουν σημαντικά κατώτερη επιβίωση, αν και αυτό το φαινόμενο παραμένει ανεξήγητο. Η συσχέτιση με την παρουσία της NSC φαίνεται προφανή και υπάρχουν αυξανόμενες ενδείξεις για την παρουσία

κυττάρων πολλαπλασιασμού όγκου του εγκεφάλου (BTPCs) στο SVZ σε περίπτωση γλοιοβλάστωμα.
Η μη εκρίζωση αυτών των κυττάρων οδηγεί αναπόφευκτα σε υποτροπή του όγκου, όπως πράγματι παρατηρείται στην πλειοψηφία των ασθενών με γλοιοβλάστωμα.
Ως εκ τούτου, φαίνεται λογικό να δοθεί ακτινοθεραπεία στην μεγαλύτερη δεξαμενή των BTPC, δηλαδή το SVZ, όπως Ίδη αποτελεί βασικό στοιχείο της θεραπείας του γλοιοβλάστωματος.
Ωστόσο, οι δημοσιεύσεις σχετικά με την επιρροή της ακτινοθεραπείας του SVZ στα πλαίσια του γλοιοβλαστωμάτος δείχνουν αντιφατικά αποτελέσματα.
Ορισμένες δημοσιεύσεις, όπως και η παρούσα μελέτη, δεν δείχνουν καμία θετική επίδραση στην επιβίωση των ασθενών με γλοιοβλάστωμα παρά μόνο αρνητικές επιδράσεις στα πλαίσια των υψηλότερων δόσεων ακτινοθεραπείας του SVZ .
Στο μέλλον χρειάζεται να γίνουν κατάλληλα διαμορφωμένες μελέτες για να ξεπεραστούν οι περιορισμοί των αναδρομικών μελετών σχετικά με την ακτινοθεραπεία του SVZ.
Η παρουσία κανονικών NSCS στο SVZ και η ευθραυστότητά τους προς της ακτινοθεραπεία παραμένουν ένα σημαντικό θέμα ανησυχίας.
Είναι αποδεδειγμένο ότι η ευρεία χειρουργική εκτομία (GTR) έχει ως αποτέλεσμα μεγαλύτερη επιβίωση σε ασθενείς με γλοιοβλάστωμα, αν και οι μελέτες παρουσιάζουν μεθοδολογικά ελλείματα ως προς αφορά τη λήψη μετρήσεων του όγκου.
Εάν η χειρουργική αφαίρεση του όγκου δημιουργεί νέα νευρολογικά ελλείμματα, επηρεάζει δυσμενώς την νευρολογική λειτουργία του ασθενούς. Μια όσο το δυνατόν ασφαλέστερη ογκολογικά εξαίρεση εξασφαλίζει την ανακούφιση των συμπτωμάτων που οφείλονται στη πίεση του όγκου και παρατείνει την επιβίωση., παραμένει πολύ αμφιλεγόμενη, ειδικά στην "μοριακή εποχή" του γλιοβλαστώματος.
Ένα άρθρο αναφοράς που περιλαμβάνεται στη μελέτη μάς, δείχνει πως για ασθενείς με μη μεθυλιωμένο γλοιοβλάστωμα IDH wild type, η περιορισμένη εκτομή έχει σαν αποτέλεσμα μεγαλύτερη επιβίωσης σε σύγκριση με απλή βιοψία.
Αυτό μπορεί να προσφέρει κάποια ελπίδα σε ασθενείς με δυσμενείς μοριακούς δείκτες στην αρχή της ασθένειάς τους.
Εκτός από την ηλικία και την γενική κατάσταση του ασθενούς κατά τη διάγνωση, η μετάλλαξη του γονιδίου IDH, καθώς και υπερμεθυλιωση του υποκινητή MgMT και η ασφαλές εκτομή του όγκου είναι ευνοϊκοί προγνωστικοί παράγοντες για την επιβίωση των ασθενών με γλοιοβλάστωμα.
Εάν το γλοιοβλάστωμα έρχεται σε επαφή με το SVZ κατά τη διάγνωση αποδεικνύεται δυσμενής προγνωστικός παράγοντας. Η πιθανή επιρροή της ακτινοθεραπείας του SVZ στην επιβίωση των ασθενών με γλοιοβλάστωμα παραμένει όμως συζητήσιμη.

RESUMO

Apesar do tratamento agressivo com quimiorradioterapia à base de temozolomida após a cirurgia, o prognóstico dos pacientes com glioblastoma permanece sombrio, com uma sobrevida global mediana de apenas 15 meses. Nesta tese, vários fatores prognósticos são discutidos, próximos à idade no diagnóstico e condição do paciente.

Dois fatores biológicos moleculares têm um importante efeito prognóstico em pacientes com glioblastoma. Em primeiro lugar, a mutação da isocitrato desidrogenase (IDH) desempenha um papel fundamental na classificação atual de glioma e glioblastoma. Os pacientes que sofrem de glioblastoma mutado com IDH têm um prognóstico significativamente melhor do que os pacientes com glioblastoma de tipo selvagem com IDH. Infelizmente, apenas 10% ou menos dos pacientes com glioblastoma recém-diagnosticados pertencerão ao grupo com mutação IDH. Em segundo lugar, o fenômeno epigenético de metilação do promotor do gene O6-metilguanina-DNA-metiltransferase (MGMTp) torna o tecido tumoral mais vulnerável aos efeitos alquilantes da temozolomida porque MGMTp-metilação silencia o gene e o reparo do DNA por MGMT é reduzido ou até parou. Assim, pacientes com glioblastoma com metilação MGMTp terão uma sobrevida significativamente melhor. O teste amplamente utilizado para estabelecer o status de metilação MGMTp, PCR específico de metilação quantitativa (qMSP), não permite uma dicotomização simples entre tumores "metilados" e "não metilados", mas mostra uma "zona cinza" de incerteza diagnóstica. Pacientes com resultados de teste dentro desta zona cinza, mas mostrando metilação "fraca" do tecido de glioblastoma, ainda terão uma sobrevida significativamente melhor do que pacientes com tumores totalmente não metilados, como é descrito nesta tese. Um consenso internacional deve ser estabelecido em relação aos valores de corte para o teste de qMSP em glioblastoma, bem como um algoritmo de teste aceito internacionalmente. Dessa forma, futuros estudos de sobrevida do glioblastoma serão padronizados em relação ao teste MGMTp e o viés será evitado. Além disso, do ponto de vista clínico, este consenso e padronização evitarão que pacientes com resultados de testes dentro da zona cinza corram o risco de perder oportunidades terapêuticas.

Embora considerado impossível por décadas, foi comprovado no início do século 21 que o cérebro humano adulto contém células-tronco neurais (NSCs) tanto no giro denteado do hipocampo quanto na zona subventricular (SVZ), que é o maior reservatório dos NSCs. O papel do SVZ no glioblastoma é mal compreendido. Como esta tese também mostra, os pacientes que sofrem de glioblastoma em contato com SVZ têm uma sobrevida significativamente pior, embora a explicação para essa observação permaneça indefinida por enquanto. A correlação com a presença de NSCs parece óbvia e há evidências crescentes para a presença de células de propagação de tumor cerebral (BTPCs) na SVZ no caso de glioblastoma. A falha em erradicar essas células resultará inevitavelmente na recorrência do tumor, que de fato é o destino da esmagadora maioria dos pacientes com glioblastoma. Portanto, parece lógico irradiar o maior reservatório desses BTPCs, ou seja, o SVZ, uma vez que a radioterapia já é uma parte essencial do tratamento de pacientes com glioblastoma. No entanto, os relatórios sobre o impacto da irradiação de SVZ na sobrevida no glioblastoma oferecem resultados conflitantes. Algumas publicações, como é o caso do relatório discutido nesta tese, não mostram nenhum efeito ou mesmo efeitos prejudiciais

de altas doses de radiação de SVZ na sobrevida de pacientes com glioblastoma. Estudos prospectivos cuidadosamente desenhados são necessários para superar as limitações dos estudos retrospectivos sobre irradiação de SVZ. A presença de NSCs normais no SVZ e sua vulnerabilidade à radiação continuam a ser um ponto de atenção preocupante.

Foi demonstrado que a ressecção total macroscópica (GTR) resulta em uma sobrevida significativamente maior do paciente com glioblastoma, embora esses estudos sejam propensos a questões metodológicas, especialmente em relação à medição do tumor. No entanto, a intervenção neurocirúrgica que cria um novo déficit neurológico, infelizmente cancelará os efeitos benéficos da GTR. A ressecção parcial do glioblastoma, não apenas como uma medida paliativa para aliviar os sintomas devido ao efeito de massa, mas também para prolongar a sobrevida, permanece altamente controversa, especialmente na "idade molecular" do glioblastoma. Um relatório incluído nesta tese mostra que, no caso de pacientes com glioblastoma não metilado de tipo selvagem de IDH, a ressecção parcial resulta em uma sobrevida global significativa, mas modesta, em comparação com os pacientes que foram tratados apenas com biópsia. Isso pode oferecer alguma esperança aos pacientes com marcadores moleculares desfavoráveis no início de sua doença.

Próximo à idade no diagnóstico, a condição do paciente e a mutação IDH, também a metilação do MGMTp fraca e a ressecção parcial do tumor são fatores prognósticos favoráveis para a sobrevida do paciente com glioblastoma. embora o impacto da radiação SVZ na sobrevida do paciente com glioblastoma permaneça altamente controverso, o contato SVZ do glioblastoma no diagnóstico se aplica como um fator prognóstico desfavorável.

RESUMEN

El tratamiento de los pacientes con gliobastoma sigue siendo la cirugía seguida de radioterapia y quimioterapia, sobre todo temozolomida. A pesar de la agresividad de este tratamiento, el pronóstico de los pacientes con gliobastoma es bastante sombrío. La mediana de supervivencia es de tan solo unos 15 meses. En esta tesis se discuten varios factores pronósticos, además de la edad al momento del diagnóstico y la condición general del paciente.

En la actualidad se describen dos factores biológicos moleculares que influyen considerablemente en el pronóstico de los pacientes con gliobastoma. El primero es la mutación de la isocitrato deshidrogenasa (IDH). Esta mutación es tan importante que desde 2016 la clasificación del glioma está basada parcialmente en ella. Los pacientes con un gliobastoma IDH-mutado tendrían significativamente un pronóstico más favorable. Desafortunadamente, tan solo un 10% de los pacientes diagnosticados con gliobastoma pertenecen a este grupo con IDH-mutado.
El segundo es un factor epigenético, la metilación del promotor del gen O6-metilguanina-ADN-metiltransferasa (MGMT). Cuando esta metilación está presente en el gliobastoma, el tejido tumoral se vuelve más vulnerable a los efectos alquilantes de la temozolomida porque la metilación de MGMT silencia el gen y la reparación de ADN por MGMT disminuye o incluso se detiene. De ahí que los pacientes con glioblastoma con metilación de MGMTp tengan una supervivencia significativamente mejor. Sin embargo, la prueba ampliamente utilizada para establecer el estado de metilación de MGMTp, la PCR cuantitativa específica de metilación (qMSP), no permite una dicotomización simple entre tumores "metilados" y "no metilados", muestra una "zona gris" de incertidumbre diagnóstica. En este sentido cabe destacar, como se estudia en esta tesis, que incluso los pacientes, en los cuales el glioblastoma parece mostrar una metilación “débil”, tendrían una supervivencia significativamente mejor que los pacientes con tumores en los que no hay metilación génica.
Debe establecerse un consenso internacional tanto en los valores de corte para las pruebas de qMSP en el glioblastoma como en un algoritmo de prueba a seguir con respecto a los diferentes análisis técnicos para determinar la metilación del promotor MGMT. De esta manera, los futuros estudios de supervivencia del glioblastoma se estandarizarán con respecto a las pruebas de MGMTp y se evitarán sesgos. Además, desde el punto de vista clínico, este consenso y estandarización evitarán que los pacientes cuyos resultados se encuentren de la zona gris puedan correr el riesgo de perder oportunidades terapéuticas.

Aunque se consideró imposible durante décadas, a principios del siglo XXI se demostró que el cerebro humano adulto contiene células madre neurales (NSC) tanto en el giro dentado del hipocampo como en la zona subventricular (SVZ), que es el reservorio más grande de NSC. El papel de SVZ en el glioblastoma es importante pero poco conocido. Como también muestra esta tesis, los pacientes que padecen glioblastoma en contacto con SVZ tienen una supervivencia significativamente peor. Por ahora, es difícil encontrar una explicación a esto. La correlación con la presencia de NSC parece obvia y existe una creciente evidencia de la presencia de células de propagación de tumores cerebrales (BTPC) en SVZ en caso de glioblastoma. Si no se erradican estas células, inevitablemente el tumor volverá a aparecer; de hecho, es lo que ocurre en la inmensa mayoría de los pacientes con glioblastoma. Por lo

tanto, parece lógico irradiar el mayor depósito de estas BTPC, a saber, SVZ, ya que la radioterapia es, por supuesto, una parte esencial del tratamiento del paciente con glioblastoma. Sin embargo, los informes sobre el impacto de la irradiación en SVZ ofrecen resultados contradictorios. Algunas publicaciones, como es el caso del informe discutido en esta tesis, no muestran ningún efecto en lo que a la supervivencia de los pacientes con glioblastoma se refiere o incluso efectos perjudiciales de dosis más altas de radiación en SVZ. Se necesitan estudios prospectivos cuidadosamente diseñados para superar las limitaciones de los estudios retrospectivos sobre la irradiación en SVZ. La presencia de NSC normales en SVZ y su vulnerabilidad a la radiación siguen siendo un punto de atención preocupante.

Se ha demostrado que la resección total macroscópica (RTG) da como resultado una supervivencia del paciente con glioblastoma significativamente más prolongada, aunque estos estudios son propensos a problemas metodológicos, especialmente en relación con la medición del tumor. Sin embargo, la intervención neuroquirúrgica crea un nuevo déficit neurológico y desafortunadamente anulará los efectos beneficiosos de la RTG. La resección parcial del glioblastoma, no solo como medida paliativa para aliviar los síntomas debidos al efecto de masa, sino también para prolongar la supervivencia, sigue siendo muy controvertida, especialmente en la "edad molecular" del glioblastoma. Un informe incluido en esta tesis muestra que en el caso de pacientes con glioblastoma no metilado de tipo salvaje IDH, la resección parcial da como resultado una supervivencia general significativa, modesta, pero mejor en comparación con los pacientes que fueron tratados solo con biopsia. Esto puede ofrecer algo de esperanza a los pacientes con un perfil genómico tumoral de pronóstico desfavorable al inicio de su enfermedad.

Además de la edad en el momento del diagnóstico, el estado del paciente y la mutación IDH, también la metilación débil de MGMTp y la resección parcial del tumor son factores pronósticos favorables para la supervivencia del paciente con glioblastoma. Sin embargo, el contacto de SVZ con el glioblastoma en el momento del diagnóstico se considera un factor pronóstico desfavorable, mientras que el impacto de la radiación en SVZ en estos pacientes sigue siendo muy controvertido.

ÅTERUPPTA

År 2021 kommer behandlingen av patienter med gliobastom fortfarande bestå av resektion följt av strålning och kemoterapi, särskilt temozolomid. Trots detta aggressiva behandling förblir prognosen för patienter med glioblastom mycket dyster. Medianöverlevnaden är bara 15 månader. Förutom ålder och allmäntillstånd hos patienten finns det flera prognostiska faktorer. Några av de har undersökts vidare i denna avhandling.

Två molekylärbiologiska faktorer påverkar starkt prognosen för patienter med glioblastom. Först, den mutationen av *isocitrate dehydrogenase* (IDH). Denna IDH-mutation är så viktig att hela gliomklassificeringen sedan 2016 har baserats på denna abnormitet. För gliobastom betyder en IDH-muterad tumör en betydligt bättre överlevnadschansen för patienten. Tyvärr tillhör endast 10% eller mindre av patienten som står inför den nya diagnosen glioblastom till denna grupp. Den andra faktorn är en epigenetisk faktor, nämligen metylering av promotorn för *O6-methylguanine-DNA-methyltransferase* (MGMT) genen. När denna metylering är närvarande i glioblastom fungerar DNA-reparationsproteinet MGMT mindre effektiv eller slutar till och med att fungera, vilket gör tumörvävnaden mycket känsligare för den alkylerande effekten av temozolomid. I sin tur förbättrar detta patientens överlevnad avsevärt. I den teknik som ofta används för att bestämma närvaron av denna metylering, kvantitativ metyleringsspecifik PCR (qMSP), inträffar dock en så kallad ”grå zon”, inom vilken finns diagnostisk osäkerhet. Det är viktigt att, som studerat i denna avhandling, även patienter vars glioblastom bara verkar vara ”svagt” metylerat, har en signifikant bättre överlevnad än patienter i vilka denna metylering är helt frånvarande. För patienter med resultatet inom denna grå zon är det möjligt att de förlorar terapeutiska chanser. På grund av diagnostiska osäkerhet kan vetenskaplig forskning om överlevnad hos patienter med glioblastom och MGMT grumlas. Lösningen på detta problem består i att nå en internationell consensus om referensvärden för qMSP och en algoritm som ska följas med avseende på de olika tekniska analyserna för MGMT-promotor-metyleringsspecificering.

Trots årtionden av vetenskaplig tro på det motsatta, visades det i början av detta århundrade obestridligt att neurala stamceller också finns i vuxna människors hjärnor, nämligen i den tandade gyrus i hippocampus och subventrikulär zon (SVZ). Denna zon spelar en viktig men otillräcklig förstått roll för glioblastom. Patienter vars tumör kommer i kontakt med SVZ vid diagnosen

har en avsevärd sämre prognos än gruppen patienter som inte gör det, vilket visades igen i denna avhandling. Den exakta orsaken till detta är fortfarande otillräckligt känd, även om många skribenter tror att det måste finnas en koppling till förekomsten av neutrala stamceller i SVZ. Faktum är att de senaste publikationerna visar att, troligen, stamceller som degenererar maligna är ursprunget till glioblastom. Så länge dessa stamceller inte utrotas kommer tumören oundvikligen tillbaka, vilket är ödet för nästan alla patienter som lider av glioblastom. Eftersom strålbehandling redan är en fast och validerad del av standardbehandling verkar det logiskt att också bestråla den största potentiella reservoaren av glioblastomstamceller, SVZ. Studierna om strålningens inverkan på SVZ är dock motstridiga. Vissa publikationer, inklusive studien som presenteras i denna avhandling, visar att bestrålning av SVZ inte kan ha någon eller till och med en negativ effekt på överlevnaden av glioblastompatienter. För att ge större tydlighet i detta viktiga ämne kan endast noggrant utarbetade prospektiva studier övervinna de betydande bristerna i retrospektiva studier som utfördes. Men försiktighet rekommenderas, eftersom strålning också påverkar neurala stamceller.

Neurokirurgisk resektion av glioblastom är fortfarande en av pelarna i behandlingen. Även om det finns metodiska debatter om mätning av tumörvolymer, visar många studier att total resektion av färglagt tumördelen av glioblastom förlänger patientens överlevnad signifikant. Dock förorsaka ny neurologisk skada på grund av fullständig resektion av glioblastom kan upphäva denna fördel. Effekterna av partiell resektion av glioblastom på patientöverlevnad, utöver enbart minskning av tumörmasseffekten, är kontroversiella, särskild i ljuset av de molekylärbiologiska faktorer som redan diskuteras. I denna avhandling presenteras en studie som visar att partiell tumörresektion hos glioblastompatienter med MGMT-ometylerade IDH-vildtumörer resulterar i en signifikant, men blygsam, förlängning av överlevnaden jämfört med patienter som genomgick enbart biopsi. Detta ger lite hopp för patienter som startar sin sjukdom med en ogynnsam tumörgenetisk profil.

Förutom ålder och allmäntillstånd anses närvaron av IDH-mutationen, närvaron av en ”svag” MGMT-promotormetylering och en partiell resektion av tumören också som gynnsamma prognostiska faktorer hos patienter med glioblastom. Däremot är glioblastomens kontakt med SVZ vid diagnos en ogynnsam prognostisk faktor medan rollen för SVZ-bestrålning hos dessa patienter förblir oklar.

ÖZET

2021'de, glioblastomdan muzdarip hastaların tedavisi hala rezeksiyondan sonra radyasyon ve kemoterapi, özellikle temozolomidden oluşacak. Bu agresif yaklaşıma rağmen, glioblastom hastalarının prognozu çok zayıftır. Ortalama hayatta kalma süresi sadece 15 aydır. Hastanın tanı anındaki yaşı ve genel durumunun yanı sıra, bazıları bu tezde daha ayrıntılı incelenen birkaç prognostik faktör vardır.

Şu anda glioblastomlu hastaların prognozunu güçlü bir şekilde etkileyen iki moleküler biyolojik faktör tanımlanmaktadır. Birincisi, izositrat dehidrojenazın (IDH) mutasyonudur. Bu IDH mutasyonu o kadar önemlidir ki, 2016'dan beri tüm glioma sınıflandırması kısmen bu anormalliğe dayanmaktadır. Glioblastom için IDH-mutasyona uğramış bir tümör aynı zamanda hasta için önemli ölçüde daha iyi bir hayatta kalma anlamına gelir. Ne yazık ki, yeni glioblastom tanısı ile karşılaşan hastaların sadece %10'u veya daha azı bu gruba ait olacaktır. İkinci faktör epigenetik bir faktördür, yani O6-metilguanin DNA metil transferaz (MGMT) geninin promoterinin metilasyonu. Bu metilasyon glioblastomda mevcut olduğunda, DNA onarım proteini MGMT daha az verimli çalışır veya hatta çalışmayı durdurarak tümör dokusunu temozolomidin alkilleyici etkisine çok daha duyarlı hale getirir. Bu da hastanın hayatta kalmasını önemli ölçüde artırır. Bununla birlikte, bu metilasyonun varlığını belirlemek için sıklıkla kullanılan teknikte, kantitatif metilasyona özgü PCR (qMSP), içinde tanısal belirsizliğin olduğu sözde "gri bölge" oluşur. Ek olarak, bu tezde çalışıldığı gibi, glioblastomları sadece 'zayıf' metillenmiş gibi görünen hastaların bile, bu metilasyonun tamamen bulunmadığı hastalara göre önemli ölçüde daha iyi bir sağkalıma sahip olması önemlidir. Bu gri alan içinde test sonuçları olan hastalar, tedavi fırsatlarını kaybedebilir. Glioblastom ve MGMT'li hastaların sağkalımına ilişkin bilimsel araştırmalar da bu tanısal belirsizliğin varlığıyla gölgelenebilir. Bu problemin çözümü, qMSP için referans değerleri ve MGMT promoter metilasyon tespiti için farklı teknik analizler ile ilgili izlenecek bir algoritma ile ilgili uluslararası bir fikir birliğine ulaşmaktır.

Aksine onlarca yıllık bilimsel inanca rağmen, bu yüzyılın başında nöral kök hücrelerin yetişkin insan beyninde, yani hipokampusun dentat girusunda ve subventriküler bölgede (SVZ) bulunduğu reddedilemez bir şekilde kanıtlandı. Bu bölge, glioblastomd önemli ancak yeterince anlaşılmamış bir rol oynar. Tanı anında tümörü SVZ ile temas eden hastalar, temas etmeyen hasta grubuna göre açıkça daha kötü prognoza sahiptir ve bu da bu tezde yine gösterilmiştir. Pek çok yazar SVZ'deki nöral kök hücrelerin varlığı ile bir bağlantı olması gerektiğine inanmasına rağmen, bunun kesin nedenleri hala yeterince bilinmemektedir.Dahası, son yayınlar, büyük olasılıkla, malign dejenere olan kök hücrelerin glioblastomun kökeninde olduğunu göstermektedir. Bu kök hücreler ortadan kaldırılmadığı sürece, tümör kaçınılmaz olarak nüksetecektir ki bu aslında neredeyse tüm hastaların kaderidir glioblastom muzdarip. Radyoterapi standart glioblastoma tedavisinin zaten sabit ve onaylanmış bir parçası olduğundan, glioblastom kök hücrelerinin en büyük potansiyel rezervuarı olan SVZ'yi de ışınlamak mantıklı görünmektedir. Bununla birlikte, radyasyonun SVZ üzerindeki etkisine ilişkin çalışmalar çelişkilidir. Bu tezde sunulan çalışma da dahil olmak üzere bazı yayınlar, SVZ'nin ışınlanmasının glioblastom hastalarının sağkalımı üzerinde hiçbir etkisi olmadığını veya hatta

olumsuz bir etkisi olabileceğini göstermektedir. Bu önemli konuya daha fazla açıklık sağlamak için yapılan retrospektif çalışmaların önemli eksikliklerini ancak dikkatle tasarlanmış ileriye dönük çalışmalar giderebilir. Ancak, radyasyon nöral kök hücreleri de etkileyeceğinden dikkatli olunması önerilir.

Glioblastomun nöroşirürji rezeksiyonu tedavinin temellerinden biri olmaya devam etmektedir. Tümör hacimlerinin ölçümü ile ilgili metodolojik tartışmalar olmasına rağmen, birçok çalışma glioblastomun boyanan tümör kısmının toplam rezeksiyonunun hastanın sağkalımını önemli ölçüde uzattığını göstermektedir. Tam bir rezeksiyon elde edilse bile yeni nörolojik hasara neden olmanın bu faydayı olumsuz etkilediği unutulmamalıdır. Glioblastomun kısmi rezeksiyonunun hasta sağkalımı üzerindeki etkileri, tümör kitle etkisini azaltmanın ötesinde, özellikle daha önce tartışılan moleküler biyolojik faktörler ışığında tartışmalıdır. Bu tezde, MGMT metillenmemiş IDH vahşi tip tümörlü glioblastom hastalarında kısmi tümör rezeksiyonunun, sadece biyopsi yapılan hastalara kıyasla, mütevazı olsa da önemli bir sağkalım süresine neden olduğunu gösteren bir çalışma sunulmuştur. Bu, hastalıklarına olumsuz bir tümör genetik profiliyle başlayan hastalara biraz umut vermektedir.

Hastanın yaşı ve durumuna ek olarak, IDH mutasyonunun varlığı, "zayıf" bir MGMT promoter metilasyonunun varlığı ve tümörün kısmi rezeksiyonu da glioblastom hastalarında olumlu prognostik faktörler olarak kabul edilir. Bunun tersine, glioblastomun tanı anında SVZ ile teması, olumsuz bir prognostik faktör iken, bu hastalarda SVZ ışınlamasının rolü belirsizliğini korumaktadır.

Краткое содержание

Как прежде, в 2021 году лечение пациентов, страдающих глиобластомой, заключается в резекции опухоли с последующей лучевой и химиотерапией, в частности темозоломидом. Несмотря на такой агрессивный подход, прогноз у этих пациентов остаётся очень неблагоприятным: средняя выживаемость составляет приблизительно 15 месяцев. Помимо возраста и общего состояния пациента на момент постановки диагноза, существуют другие прогностические факторы и некоторые из них более детально исследованы в этой диссертации.

В настоящее время описаны два молекулярно-биологических фактора, которые в важной степени влияют на прогноз пациентов с глиобластомой.
Первый – это мутация изоцитратдегидрогеназы (IDH). Эта мутация настолько важна, что вся классификация глиом с 2016 года отчасти основана на этой аномалии. Сверх того, наличие в глиобластоме мутации IDH значительно улучшает выживаемость пациентов. К сожалению, только 10% и даже меньше пациентов с новым диагнозом глиобластомы принадлежат к этой группе.
Второй фактор - это эпигенетический фактор, а именно, метилирование промотора гена O^6-метилгуанин-ДНК-метилтрансферазы (MGMT). Когда это метилирование присутствует в глиобластоме, белок репарации ДНК MGMT работает менее эффективно или даже перестает работать, делая ткань опухоли более чувствительной к алкилирующему эффекту темозоломида. Это, в свою очередь, существенно продлевает жизнь пациентов.
Однако в методике количественной метил-специфичной ПЦР (qMSP), часто используемой для определения наличия метилирования, существует так называемая «серая зона», в пределах которой есть диагностическая неопределенность. Важно, как показано в этой диссертации, что пациенты, глиобластома которых оказалась только «слабо» метилированной, имели гораздо лучшую выживаемость, чем пациенты, у которых это метилирование полностью отсутствует. При этом пациенты с результатами в «серой зое» могут лишиться терапевтических возможностей. В дополнение, научное исследование выживаемости пациентов с глиобластомой и MGMT также может быть затруднено из-за существованиия этой диагностической неясности. Решение этой проблемы состоит в достижении международного консенсуса относительно рекомендованных значений qMSP и алгоритма использования различных технических тестов для определении метилирования промотора MGMT.

Несмотря на десятилетия научных убеждений в обратном, в начале этого столетия было неопровержимо доказано, что нервные стволовые клетки также присутствуют в мозге взрослого человека, а именно в зубчатой извилине гиппокампа и субвентрикулярной зоне (SVZ). Эта зона играет важную, но недостаточно изученную роль при глиобластоме. Пациенты, у которых при постановке диагноза опухоль контактирует с SVZ, имеют явно худший прогноз, чем пациенты, у которых этого не происходит, что также продемонстрировано в этой диссертации.

Точная причина(ы) этого все ещё недостаточно известна, хотя многие авторы полагают, что это связано с присутствием нервных стволовых клеток в SVZ. Недавние публикации показывают, что, скорее всего, происхождение глиобластомы следует искать в стволовых клетках, которые перерождаются в злокачественные. Пока «стволовые клетки глиобластомы» не уничтожены, опухоль неизбежно будет рецидивировать и это является судьбой почти всех пациентов, страдающих глиобластомой.

Поскольку лучевая терапия является уже проверенной и действенной частью стандартного лечения глиобластомы, становится логичным также облучение субвентрикулярной зоне (SVZ), как самого большого потенциального резервуара стволовых клеток глиобластомы. Однако исследования о влиянии излучения на SVZ противоречивы. Некоторые публикации, в том числе исследование, представленное в этой диссертации, показывают, что облучение SVZ может вообще не иметь эффекта или даже негативно влиять на продолжительность жизни пациентов с глиобластомой. Только тщательно спланированные проспективные исследования могут преодолеть значительные недостатки уже проведенных ретроспективных исследований и внести большую ясность в таком важном вопросе. Но необходима осторожность, поскольку облучение также будет оказывать воздействие на здоровые нервные стволовые клетки.

Нейрохирургическая резекция глиобластомы остаётся одним из столпов лечения. Несмотря на то, что существуют методологические дебаты относительно определения размеров опухоли, многие исследования показывают, что полная резекция окрашенной опухолевой части глиобластомы значительно продлевает жизнь пациентов. Следует отметить, что возникновение нового неврологического повреждения сводит на нет это преимущество, даже если выполнена полная резекция.

Эффект частичной резекции глиобластомы на продолжительность жизни пациентов, и не только посредством уменьшения размера опухоли, является спорным, особенно в свете уже обсужденных молекулярно-биологических факторов. В этой диссертации представлено исследование, показывающее, что частичная резекция опухоли у пациентов с MGMT-неметилированной IDH-wildtype глиобластомой приводит к значительному, хотя и умеренному, увеличению выживаемости пациентов по сравнению с пациентами, перенесшими только биопсию. Это даёт некоторую надежду пациентам, у которых болезнь начинается с неблагоприятного генетического профиля опухоли.

Кроме возраста, состояния пациента и наличия мутации IDH, наличие «слабого» метилирования промотора MGMT и частичная резекция опухоли также считаются благоприятными прогностическими факторами у пациентов с глиобластомой. С другой стороны, контакт глиобластомы с SVZ при постановке диагноза является неблагоприятным прогностическим фактором, в то время как роль облучения SVZ остаётся неясной.

概要

儘管手術後採用基於替莫唑胺的放化療進行了積極治療，但膠質母細胞瘤患者的預後仍然不佳，中位總生存期僅為15個月。 本文討論了幾個預後因素，除了診斷時的年齡和病情。

兩種分子生物學因素在膠質母細胞瘤患者中具有重要的預後作用。首先，異檸檬酸脫氫酶（IDH）突變在目前的神經膠質瘤和膠質母細胞瘤分類中起著關鍵作用。 IDH突變的膠質母細胞瘤患者的預後明顯優於IDH野生型膠質母細胞瘤患者。不幸的是，新診斷的膠質母細胞瘤患者中只有10%或更少會屬於IDH突變組。其次，O6-甲基鳥嘌呤-DNA-甲基轉移酶基因啟動子（MGMTp）的表觀遺傳現象使腫瘤組織更易受替莫唑胺的烷基化作用，因為MGMTp-甲基化使基因沉默並且MGMT的DNA修復降低或甚至停了下來因此，患有MGMTp-甲基化的膠質母細胞瘤患者將具有明顯更好的存活率。廣泛用於確定MGMTp甲基化狀態的測試，即定量甲基化特異性PCR（qMSP），不能在“甲基化”和“未甲基化”腫瘤之間進行簡單的二分法，但顯示出診斷不確定性的“灰色區域”。如本論文所述，在灰色區域內具有測試結果但顯示膠質母細胞瘤組織“弱”甲基化的患者仍將比具有完全未甲基化腫瘤的患者俱有明顯更好的存活率。對於膠質母細胞瘤qMSP測試的臨界值以及國際公認的測試算法，應該建立國際共識。這樣，將來的膠質母細胞瘤生存研究將在MGMTp測試方面進行標準化，並避免偏倚。而且，從臨床角度來看，這種共識和標準化將防止在灰色區域內獲得測試結果的患者可能失去治療機會的風險。

儘管幾十年來被認為是不可能的，但在21世紀初已經證明，成年人類大腦在海馬齒狀回和腦室下區域（SVZ）中都含有神經乾細胞（NSC），這是最大的水庫NSC。尚不清楚SVZ在膠質母細胞瘤中的作用。正如該論文還表明的那樣，儘管目前尚不清楚該觀察結果，但患有SVZ接觸性膠質母細胞瘤的患者生存期明顯較差。與神經乾細胞的存在的相關性似乎很明顯，並且有越來越多的證據表明，在膠質母細胞瘤的情況下，SVZ中存在腦腫瘤繁殖細胞（BTPC）。未能根除這些細胞將不可避免地導致腫瘤復發，這確實是絕大多數膠質母細胞瘤患者的命運。因此，輻照這些BTPC的最大儲庫，即SVZ，似乎是合乎邏輯的，因為放射治療當然已經是膠質母細胞瘤患者治療的重要組成部分。但是，有關SVZ照射在膠質母細胞瘤中對生存的影響的報導提供了相互矛盾的結果。某些論文，如本論文所討論的報告一樣，顯示較高的SVZ輻射劑量對膠質母細胞瘤患者的生存沒有影響甚至是有害的影響。需要精心設計的前瞻性研究來克服有關SVZ輻射的回顧性研究的局限性。 SVZ中正常NSC的存在及其對輻射的脆弱性仍然是令人擔憂的關注點。

總體全切除術（GTR）已顯示可顯著延長膠質母細胞瘤患者的生存期，儘管這些研究容易出現方法學問題，尤其是有關腫瘤測量的問題。 但是，不幸的是，造成新的神經功能缺損的神經外科手術干預將抵消GTR的有益作用。 膠質母細胞瘤的部分切除，不僅是緩解因質量效應引起的症狀的姑息手段，而且還可以延長生存期，在膠質母細胞瘤的“分子時代”，尤其是在爭論中，它一直是引起爭議的。 本論文中包含的一份報告

顯示，與僅接受活檢的患者相比，IDH野生型未甲基化的膠質母細胞瘤患者進行部分切除可顯著提高總體生存率，但又有一定的優勢。 這可能給那些發病時分子標記不利的患者帶來希望。

除診斷時的年齡外，患者的病情和IDH突變，MGMTp甲基化弱以及部分腫瘤切除是膠質母細胞瘤患者生存的有利預後因素。 儘管SVZ輻射對膠質母細胞瘤患者生存的影響仍然存在很大爭議，但診斷時將SVZ接觸膠質母細胞瘤是不利的預後因素。

SAMANTEKT

Þrátt fyrir árásargjarna krabbameinslyfjameðferð sem byggir á temózólómíði eftir aðgerð, eru horfur glioblastoma sjúklinga dapurlegar og miðgildi heildarlifunar er aðeins 15 mánuðir. Í þessari ritgerð er fjallað um nokkra forspárþætti, fyrir utan aldur við greiningu og ástand sjúklings.

Tveir sameindalíffræðilegir þættir hafa mikilvæg forspárgildi hjá glioblastoma sjúklingum. Í fyrsta lagi gegnir stökkbreytingin ísósítrat dehýdrógenasa (IDH) lykilhlutverki í núverandi glioma og glioblastoma flokkun. Sjúklingar sem eru með IDH-stökkbreytt glioblastoma hafa marktækt betri horfur en sjúklingar með glioblastoma án þessara stökkbreytingar (wild type). Því miður munu aðeins innan við 10% nýgreindra glioblastoma sjúklinga tilheyra IDH stökkbreyttum hópi. Í öðru lagi gerir epigenetískt fyrirbæri metýleringu O6-metýlguaníns-DNA-metýltransferasa erfðaefnisins (MGMTp), æxlisvefinn viðkvæmari fyrir alkýlerandi áhrifum temózólómíðs vegna þess að MGMTp-metýlering þaggar niður genið og DNA-viðgerð með MGMT er minni eða jafnvel hætt. Semsagt, glioblastoma sjúklingar með MGMTp-metýleringu hafa marktækt betri lífslíkur.

Prófið sem mikið er notað til að staðfesta MGMTp-metýlerunarstöðu, magnbundna metýlerunarsértæka PCR (qMSP), gerir ekki ráð fyrir einfaldri tvískiptingu milli „metýleraðra“ og „ómetýleraðra“ æxla en sýnir „grátt svæði“ greiningaróvissu. Sjúklingar með niðurstöður rannsókna innan þessa gráa svæðis, en sýna „veika“ metýleringu glioblastoma vefja, munu samt hafa marktækt betri lifun en sjúklingar með fullkomlega ómetýlerað æxli, eins og lýst er í þessari ritgerð. Koma skal á alþjóðlegri samstöðu varðandi bæði skert gildi fyrir qMSP próf í glioblastoma sem og alþjóðlega viðurkenndan prófunaralgoritma. Þannig verða rannsóknir á lífslíkur sjúklinga með glioblastoma stöðluð varðandi MGMTp próf og minni líkur á hlutdrægni (bias). Ennfremur, frá klínísku sjónarmiði, mun þessi samstaða og stöðlun koma í veg fyrir að sjúklingar með niðurstöður innan gráa svæðisins eigi á hættu að missa af mögulegri meðferð.

Þótt það væri talið ómögulegt áratugum saman var það sannað í byrjun 21. aldar að heilinn hjá fullorðnum inniheldur taugastofnfrumur (NSC), bæði í tanngírus í hippocampus og í svæðunum undir heilaholunum (SVZ) þar sem mesta magnið af þessum stofnfrumum er til staðar. Hlutverk SVZ í glioblastoma er lítið þekkt. Eins og fram kemur í þessari ritgerð hafa sjúklingar sem eru með glioblastoma í nánd við þessi svæði marktækt verri lifun þó að skýringin á þessari athugun sé ennþá vandfundin í bili. Það virðast vera augljós tengls við NSC og vaxandi vísbendingar um tilvist æxlis hvetjandi frumna (BTPCs) í SVZ ef um glioblastoma er að ræða. Takist ekki að uppræta þessar frumur er endurkoma æxla óhjákvæmileg, sem sannarlega eru örlög yfirgnæfandi meirihluta glioblastoma sjúklinga. Þess vegna virðist rökrétt að geisla stærsta lón þessara BTPC í SVZ og því er geislameðferð nú þegar nauðsynlegur hluti af meðferð hjá sjúklingum með glioblastoma. Skýrslur um lifunaráhrif SVZ geislunar í glioblastoma sýna þó misvísandi niðurstöður. Sum rit, eins og raunin er með skýrsluna sem fjallað er um í þessari ritgerð, sýna engin áhrif eða jafnvel skaðleg áhrif hærri SVZ geislunarskammta á lifun glioblastoma. Vandlega hannaðar væntanlegar rannsóknir er nauðsynlegar til að vinna bug á takmörkunum

sem fylgja afturskyggnum rannsóknum varðandi SVZ geislun. Tilvist eðlilegra NSC í SVZ og viðkvæmni þeirra fyrir geislun er enn áhyggjuefni.

Sýnt hefur verið fram á að heildarskurður (GTR) hefur í för með sér verulega lengri lifun sjúklinga með glioblastoma, þó að þesskonar rannsóknir eru næmar fyrir aðferðafræðilegum villum, sérstaklega varðandi æxlamælingar. En taugaskurðlækningaaðgerðir sem skapa ný vandamál munu því miður eyða jákvæðum áhrifum GTR. Hluta aðgerð (partial) við glioblastoma, ekki aðeins sem líknandi aðgerð til að draga úr einkennum vegna massaáhrifa, heldur einnig til að lengja lifun, er enn mjög umdeild sérstaklega á „sameindaöld“ glioblastoma. Skýrsla, sem fylgir þessari ritgerð, sýnir að ef um er að ræða sjúklinga með IDH-villtýpu ómetýlerað glioblastoma, þá leiðir hluta aðgerð til umtalsverða, en hóflega, betri heildarlifun, samanborið við sjúklinga þar sem einungis var tekid sýni. Þetta gæti veitt þeim sjúklingum með óhagstæð sameindamerki nokkra von.

Fyrir utan aldur við greiningu er ástand sjúklings, IDH-stökkbreyting, veik MGMTp-metýlering og hluta æxlisskurður hagstæðir spáþættir fyrir lifun sjúklinga með glioblastoma. Áhrif SVZ geislunar á lifun glioblastoma eru áfram mjög umdeild og líta má á nálægð glioblastoma við SVZ svæðin sem óhagstæðan þátt.

FINANCING

I sincerely thank the following non-profit organizations for their financial support of this research project:

- Center for Oncology, Ghent University Hospital
- Lux Luka Foundation
- Fonds Arne Lannoy AKA Zorro

Both the *Lux Luka Foundation* as well as the *Fonds Arne Lannoy AKA Zorro* were founded by families who lost a beloved relative to high grade glioma. Their loved ones will never be forgotten and through these foundations, they bring hope to other patients.

DANKBETUIGING

Zeer veel mensen hebben grote of kleine, maar altijd waardevolle, bijdragen geleverd aan dit project. Aan hen allen welgemeende dank. Het is gepast om een aantal personen, diensten en instellingen apart te vermelden.

Veel mensen hebben geholpen met de vertalingen, waarvoor hartelijke dank.

Ik bedank oprecht alle instellingen - maar vooral alle leerkrachten, tutoren en professoren - die voor mijn vorming en langdurige opleiding hebben ingestaan: mijn alma mater, de Universiteit Gent en het Universitair Ziekenhuis Gent, maar ook het St. Jozefscollege te Tielt, mijn middelbare school. Ik heb het grote geluk gehad dat deze instellingen niet alleen investeerden in loutere kennisoverdracht maar ook in opvoeding, vorming en reflectie.

Ik wens de collegae van de diensten anatoompathologie, neurochirurgie en radiotherapie van het Universitair Ziekenhuis Gent en AZ Delta (voorheen H. Hart Ziekenhuis) Roeselare te bedanken voor de goede samenwerking. Een specifiek woord van dank is gericht aan de radiofysici van beide instellingen, zonder wiens inzet dit project nooit had kunnen slagen.

De collegae van het Universitair Ziekenhuis Gent hebben mij sinds 2011 telkens opnieuw verkozen in de Medische Raad: een groot privilege en ik probeer hun stem waardig te zijn. Deze vergadering heeft mijn horizon verruimd en mij geleerd dat de individuele arts-patiënt relatie, de grondslag van alle geneeskundig handelen, onderhevig is aan tal van invloeden dewelke artsen mee moeten aansturen. Ik bedank oprecht alle leden en voorzitters van de Medische Raden van de voorbije jaren.

Ik wens uitdrukkelijk de verpleegkundigen van UZ Gent te bedanken met wie ik al vele jaren mag samenwerken. Specifiek de verpleegkundig teams van de neurochirurgische hospitalisatieafdeling 6b, onder leiding van Dhr. W. Coppens, en van het operatiekwartier, cluster 2, onder leiding van Dhr. W. Verleyen. De verpleegkundigen ben ik veel dank verschuldigd voor hun geduld en morele steun: zij weten wat het betekent patiënten echt te helpen maar ook soms te moeten verliezen; en ze verduren de grillen van chirurgen.
Ook alle medewerkers van de polikliniek neurochirurgie-neurologie, bedank ik voor de samenwerking. Een specifiek woord van dank aan Mevr. I. Noterman, Mevr. B. Van Gerven en Dhr. J. Immegeers, die eveneens een steun en een toeverlaat zijn. Maar ik denk ook aan mensen die vroeger op het secretariaat gewerkt hebben: Mevr. B. Janssens, Dhr. R. Van Severen, Dhr. P. Standaert en Mevr. A. Van de Sype. Graag roep ik een warme herinnering op aan Mevr. A. Colle en Mevr. L. Van Langenhove die ons ontvallen zijn.

Ik druk mijn grote appreciatie uit voor de collegae neurologen van ZorgSaam Ziekenhuis Terneuzen, zowel voor het vertrouwen dat ik geniet om hun patiënten ondertussen al tien jaar te mogen behandelen maar ook voor de wekelijkse discussies & reflecties. Dankzij deze dialectiek groeien we in kennis, ervaring en empathie, waardoor we betere artsen worden.
Ik wens zeker ook een woord van dank aan de secretariaatsmedewerkers van de polikliniek neurologie aldaar te richten. Heel in het bijzonder aan Mevr. Elian Verstraeten, voor haar uitgesproken en volgehouden inzet voor de poli neurochirurgie ZorgSaam waarbij ze nooit de patiënt uit het oog verliest.

De leden van de examencommissie verdienen een speciaal woord van dank: Dr. Rutten, Prof. Dr. De Vleesschouwer, Prof. Dr. Van Eijkeren, Prof. Dr. Van Dorpe, Prof. Dr. Poppe alsook de voorzitter, Prof.

De Wagter. Hun kritiek en dialectiek tilde dit proefschrift tot een hoger niveau. De discussie tijdens de interne verdediging was een verrijkende ervaring.

Ik bedank graag mijn promotoren, Prof. Dr. T. Boterberg, Prof. Dr. J.P. Kalala en Prof. em. Dr. D. Van Roost, die mij met raad en daad bijstonden én bijstuurden waar nodig. Een gemeend woord van dank ook aan Prof. em. Dr. M. Mareel, die altijd bereid was tot kritische academische reflectie. Eveneens bedank ik Prof. em. Dr. J. Caemaert, die bij mij de interesse in neuro-oncologie, en in glioblastoma in het bijzonder, al heel vroeg had gewekt.

Bij deze gelegenheid is het ook meer dan gepast om enkele artsen, die tijdens mijn opleiding een belangrijke rol gespeeld hebben, uitdrukkelijk te bedanken. Vanuit mijn opleidingsjaar Neurologie in Wilrijk: Dr. T. Strauven, Dr. W. Vanlandeghem en Dr. R. Madou. Zij gaven mij cruciale begeleiding tijdens mijn eerste stappen in de echte geneeskunde. Mijn jaar Hoofd-Hals chirurgie in UZ Gent: Prof. em. Dr. H. Vermeersch en Dr. K. Bonte, die mij vertrouwen schonken en op die manier mij vleugels gaven. Natuurlijk de neurochirurgen die mij hebben opgeleid: Prof. em. Dr. J. Caemaert, Prof. em. Dr. D. Van Roost, Prof. Dr. J.P. Kalala, Dr. E. Baert en Dr. F. Dewaele.

Dr. H. Pinson bedank ik oprecht voor de goede samenwerking en zijn inzet voor dit project. Onze kritische discussies zorgden voor intellectuele diepgang; de humor voor ambiance. Het werk gaat verder. Een speciaal woord van dank aan Dr. C. Van den Broecke, die als een stille bron dit project steeds nieuwe kracht gaf, en zonder wie dit onderzoek niet zou gelukt zijn.

Mijn schoonouders en schoonbroers verdienen mijn dankbaarheid voor de spontaneïteit en de warmte waarmee ik jaren geleden in hun familie ben opgenomen én voor hun gedurige steun aan ons gezin, in goede maar ook in moeilijker dagen.

Mijn familie, en vooral mijn ouders, hebben het voor mij mogelijk gemaakt te mogen en te kunnen studeren. Ze moesten hiervoor soms hard tegen de stroom in roeien en zichzelf sommige zaken ontzeggen. Voor deze volgehouden inspanning en dit enorme geschenk kan ik mijn ouders, broers en zus nooit genoeg bedanken. Zij allen delen dan ook in de verdienste dat dit project geslaagd is, heel in het bijzonder mijn vader, Dhr. Wilfried Hallaert, helaas reeds overleden, en mijn lieve moeder, Mevr. Edith De Witte.

Ons gezin verdient het belangrijkste dankwoord. Zij hebben wellicht het meest moeten verduren tijdens dit project, zowel door de tijd die dit alles aan ons gezinsleven onttrok als door de last die mijn frequente preoccupatie met studie, operaties en patiëntenzorg voor hen al te vaak betekent. Hoe begripsvol en respectvol zij hier ook mee omgaan, toch is dit vaak een opgave waar zij niet zelf voor gekozen hebben.

Het laatste dankwoord is vanzelfsprekend voor Sandra Vercammen, mijn vrouw. Voor haar geduld, inzet, begrip, aanwezigheid maar bovenal voor haar liefde en zorg voor onze kinderen en voor mij.

Te St. Amandsberg, Gent, 3 augustus 2021

About the author

Giorgio Godfried Gilbert Hallaert was born in the town of Tielt (Western-Flanders, Belgium) on September 25, 1978. He grew up in a warm working-class family with 3 siblings. His high-school curriculum involved classical studies (Greek-Latin) at the local catholic secondary school of Saint Joseph. He graduated top of his class. Thanks to the continuing efforts of his family and government scholarships, he was able to start the study of medicine at Ghent University in 1996 and obtained the medical degree in 2003 summa cum laude. He was subsequentially selected for the neurosurgical training program of Ghent University Hospital. He obtained the license to practice neurosurgery in 2009. After a short working experience at Tilburg (the Netherlands), he joined the department of neurosurgery of Ghent University Hospital, where he practices both academic and general neurosurgery. He also has an outpatient consultation at ZorgSaam Hospital, Terneuzen (the Netherlands). He is married to Sandra A. W. Vercammen, and they have two children, Soraya, and Dante. They live in St. Amandsberg (Gent).

Bibliography

Stellingen

Bij het proefschrift

De overleving van patiënten met een glioblastoma

Chirurgische, radiologische, radiotherapeutische en moleculairbiologische prognostische factoren

1. De subventriculaire zone speelt een belangrijke rol bij het glioblastoma waarbij contact van de tumor met deze zone bij diagnose een ongunstige prognostische factor is maar de invloed van bestraling op de subventriculaire zone controversieel blijft. *(dit proefschrift)*

2. Een ruime, ongecompliceerde, resectie van een IDH-wildtype MGMT-ongemethyleerd glioblastoma zal, vergeleken met enkel biopsie-name, tot een verlenging met ongeveer 4 maanden van de overleving van de patiënt leiden, zelfs indien er geen totale resectie plaatsvond. *(dit proefschrift)*

3. Bij het gebruik van kwantitatieve methylatie-specifieke PCR ter bepaling van de methylatie-status van de promotor van het MGMT gen bij glioblastoma, zullen patiënten met zwak gemethyleerde tumoren een significant betere overleving hebben dan patiënten met ongemethyleerd glioblastoma. *(dit proefschrift)*

4. To demand or preach mechanical precision, even in principle, in a field incapable of it, is to be blind and to mislead others. *Isaiah Berlin*

5. Ce qui est simple est toujours faux. Ce qui ne l'est pas est inutisable. *Paul Valéry*

6. Facts are stubborn things; and whatever may be our wishes, our inclinations, or the dictates of our passions, they cannot alter the state of facts and evidence. *John Adams*

7. Der ideale Untertan eines totalitären Regimes ist nicht der überzeugte Nazi oder der überzeugte Kommunist, sondern das Individuum, für das es keinen Unterschied mehr zwischen Realität und Fiktion, zwischen wahr und falsch mehr gibt. *Hannah Arendt*

8. The reasonable man adapts himself to the world. The unreasonable one persists in trying to adapt the world to himself. Therefore, all progress depends on the unreasonable man. *George B. Shaw*

9. Spacetime tells matter how to move; matter tells spacetime how to curve. *John A. Wheeler*

10. Entre le fort et le faible, entre le riche et le pauvre, entre le maître et le serviteur, c'est la liberté qui opprime et la loi qui affranchit. *Henri Lacordaire*

11. Alles van waarde is weerloos. *Lucebert*

12. Injustice anywhere is a threat to justice everywhere. *Dr. Martin L. King Jr.*

13. The temptation to tell a chief in a great position the things he most likes to hear, is one of the commonest explanations of mistaken policy. *Winston S. Churchill*

14. Τὰ ἐξῆς ἀεὶ τοῖς προηγησαμένοις οἰκείως ἐπιγίνεται. *Marcus Aurelius*

Giorgio Hallaert
St. Amandsberg, juli 2021

[O]ί μὲν ἰππήων στρότον οἱ δὲ πέσδων
οἱ δὲ νάων φαῖσ᾽ ἐπὶ γᾶν μέλαιναν
[ἔ]μμεναι κάλλιστον ἔγω δὲ κῆν᾽
ὄττω τὶς ἔραται.

Σαπφώ

ἐνίκησᾶ μέαυτόν