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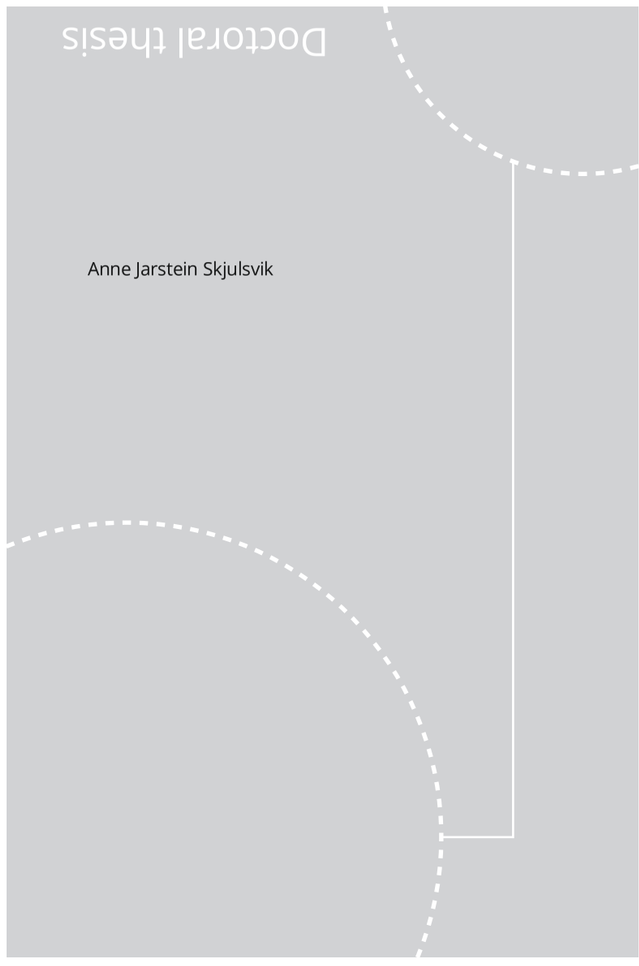
Anne Jarstein Skjulsvik

# On the prognostic factors of low-grade gliomas

Doctoral thesis



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Thesis for the Degree of Philosophiae Doctor

Trondheim, May 2020

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# Prognostiske faktorer i lavgradige hjernesvulster

### Hjernesvulsters egenskaper i forløpet ved hjernekreft

Gliomer er den vanligste typen av primære maligne hjernesvulster og disse rammer ca. 6 personer per 100 000 per år. Gliomer graderes ved mikroskopisk cellebilde og vekstmønster etter en skala på 1–4. Grad 1 regnes som godartet og ofte kurerbar ved kirurgi mens grad 4 er den mest ondartede/maligne typen hjernesvulst med en overlevelse på ca. 15 måneder fra diagnosetidspunkt. Gliomer av grad 2 kalles lavgradige gliomer. Disse svulstene er mer sjeldne enn høygradige, men rammer ofte yngre personer. Lavgradige gliomer er saktevoksende, og infiltrerer hjernevevet rundt. Behandlingen er kirurgi, hvor hensikten er å fjerne så mye som mulig av svulsten uten å påføre hjerneskade, samt å fastsette diagnosen. Fullstendig kirurgisk fjerning er umulig da svulsten vokser som enkeltceller i hjernevevet. Pasientene kan derfor få tilbakefall etter flere år.

Pasienter med lavgradige gliomer har et varierende forløp. Noen kan ha svært saktevoksende svulster og leve i årevis, mens andre raskt kan få tilbakefall. Ved tilbakefall kan enkelte av svulstene få økt malignitetsgrad. I de senere årene har man kartlagt flere mutasjoner og egenskaper ved gliomer som har vist seg å være av stor betydning for forløpet av denne sykdommen. Flere av disse mutasjonene har redefinert klassifikasjonen av hjernesvulster. I denne avhandlingen ønsket vi å se nærmere på flere av disse kjente mutasjonene og egenskapene:

I den første artikkelen undersøkte vi en markør for celledeling (Ki67/MIB1) og hvordan denne markøren fordelte seg på de ulike gradene (1-4) av hjernesvulster.

Den andre artikkelen er en såkalt populasjonsbasert studie. Her har man sett på to pasientgrupper som ble håndtert kirurgisk ulikt. Den ene gruppen fikk tidlig kirurgisk behandling, mens i den andre gruppen tok man en liten vevsprøve og ventet til svulsten begynte å vokse før man opererte. En tidligere studie på det samme materialet viste at en tilnærming med tidlig kirurgi var å foretrekke, og den andre artikkelen er en oppfølgingsstudie hvor man undersøkte den samme populasjonen for ulike mutasjoner.

I den tredje artikkelen har vi sett på hvordan de ulike subgruppene av gliomer fordeler seg i hjernen. Vi har sett på hvordan hjernesvulster med ulike mutasjoner fordeler seg spesielt i forhold til såkalte nevrogene nisjer, som er områder i hjernen hvor man vet det foregår celledeling. Det har det vært spekulert i om disse nevrogene nisjene kan være utgangspunktet for hjernesvulster.

Hovedfunnene i denne avhandlingen er:

- ✓ Det er økende grad av celledeling (målt med Ki67/MIB1) fra lavgradige til høygradige hjernesvulster, men det er også betydelig overlapp mellom de ulike malignitetsgradene
- ✓ Tidlig kirurgi gir bedre overlevelse, også hos gliomer med ulike mutasjoner
- ✓ Vi fant to potensielle nye mutasjoner i gliomer, såkalte IDH-mutasjoner
- ✓ Gliomer med ulik IDH-mutasjon fordeler seg på ulike områder i hjernen
- ✓ Gliomer med IDH-mutasjon kan potensielt utgå fra et annet område i hjernen enn gliomer uten IDH-mutasjon

Ovennevnte avhandling er funnet verdig til å forsvares offentlig

for graden PhD i molekylærmedisin.

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To Ane and Åsa for keeping me grounded.

Trondheim, April 2020

Anne Jarstein Skjulsvik

# List of papers

**PAPER I:**

**Ki-67/MIB-1 immunostaining in a cohort of human gliomas.**

Skjulsvik, A. J., Mørk, J. N., Torp, M. O., & Torp, S. H. (2014). International journal of clinical and experimental pathology, 7(12), 8905–8910.

**PAPER II:**

**Surgical resection versus watchful waiting in low-grade gliomas.**

Jakola, A. S., Skjulsvik, A. J., Myrmel, K. S., Sjåvik, K., Unsgård, G., Torp, S. H., Aaberg, K., Berg, T., Dai, H. Y., Johnsen, K., Kloster, R., & Solheim, O. (2017). Annals of oncology: official journal of the European Society for Medical Oncology, 28(8), 1942–1948. https://doi.org/10.1093/annonc/mdx230

**PAPER III:**

**Is the anatomical distribution of low-grade gliomas linked to regions of gliogenesis?**

Skjulsvik, A. J., Bø, H. K., Jakola, A. S., Berntsen, E. M., Bø, L. E., Reinertsen, I., Myrmel, K. S., Sjåvik, K., Åberg, K., Berg, T., Dai, H. Y., Kloster, R., Torp, S. H., & Solheim, O. (2020). Journal of neuro-oncology, 147(1), 147–157. https://doi.org/10.1007/s11060-020-03409-8

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# 1 INTRODUCTION

## 1.1 EPIDEMIOLOGY AND RISK FACTORS

Gliomas are the most commonly occurring malignant primary brain tumour with an incidence rate of 6/100 000 persons per year (1). Diffuse low-grade glioma has an incidence of approximately 1/100 000 per year (2). Ionizing radiation is the only known environmental risk factor for development of gliomas, but there is an unexplained and inverse association between allergies and other atopic conditions and risk of glioma (3). Men have a reported increased risk of glioma of 1.4 (4), but when adjusted for intracranial volume there is an observed lower risk of high-grade gliomas for males compared to females (5). There is a reported twofold familial risk of glioma (6) and genome-wide association studies have identified several single-nucleotide polymorphisms (SNPs) influencing risk of gliomas (7). Reported epidemiological risk factors include tall stature (8) and high socioeconomic status (9). However, a large publication of the aetiology of different types of cancer, including brain cancer, suggests that the majority of mutations are attributed to random mutations and not hereditary or environmental mutations, advocating an important effect of chance (10).

## 1.2 CLINICAL FEATURES

Patients with gliomas presents with focal or generalized symptoms, depending on the tumour growth rate, tumour size and location. Focal symptoms are related to a specific location in the brain and may include motor deficits, sensory deficits, language deficits or visual deficits. Other symptoms might be subtler cognitive dysfunctions such as personality changes or memory deficits. Generalized symptoms are not related to a specific anatomic location, and might involve generalized seizures, headaches and symptoms related to increased intracranial pressure (progressive headaches, vomiting, blurred vision and reduced consciousness) (1). Patients with low grade gliomas are frequently

diagnosed because of seizures and due to the widespread use of MRI many are diagnosed as incidental findings.

## 1.3 CLASSIFICATION OF GLIOMAS

### 1.3.1 Histopathology

Traditionally, the World Health Organization (WHO) classification of brain tumours have been based on microscopic appearance, assessed by haematoxylin and eosin (HE) stained sections in light microscope, immunohistochemically expression of proteins and ultrastructural features assessed in electron microscopy (11). Because of microscopic similarities with assumed cells of origin and their developmental differentiation states, gliomas were thought to arise from glial supportive tissue of the brain; astrocytes, oligodendrocytes or ependymal cells, which has given name to the different tumours. The tumours were classified based on histological criteria and assigned a malignancy grade (see below).

Microscopically, the WHO Classification of 2016 describes the low-grade gliomas with moderately increased cellularity compared to the normal brain. In astrocytomas the typical features are neoplastic astrocytes on a fibrillary background. The neoplastic cells may vary considerably, both with size and cytoplasmic processes. In oligodendrogliomas the typical features are monomorphic round cells with variable perinuclear halos on formalin fixated paraffin embedded (FFPE) sections. The background is usually with a dense network of capillaries. The presence of necrosis and microvascular proliferation suggests a high-grade glioma (12).

### 1.3.2 WHO grade

The WHO classification includes a malignancy grade applicable across tumour entities and is based on microscopic features such as mitoses, necrosis, and

microvascular proliferation. Table 1 illustrates these histopathological findings and their relation to the diagnostic entities.

*Table 1: Histopathological features of the different WHO grades with corresponding diagnostic entities.*

| **WHO grade** | | **Diagnosis** |
|---|---|---|
| **I** | Low proliferative potential and the possibility of cure after surgical resection | Pilocytic astrocytoma |
| **II** (low-grade) | Infiltrative in nature and often recur, despite having low levels of proliferative activity | Astrocytoma<br>Oligodendroglioma |
| **III** (high-grade) | Clear histological evidence of malignancy, including nuclear atypia and increased mitotic activity | Anaplastic astrocytoma<br>Anaplastic oligodendroglioma |
| **IV** (high-grade) | Cytologically malignant, mitotically active neoplasms with necrosis and/or microvascular proliferation | Glioblastoma |

The diffuse gliomas include grade II and III astrocytomas, grade II and III oligodendrogliomas, the former grade II and III oligoastrocytomas, and grade IV glioblastomas. Grade I tumours (pilocytic astrocytomas) are considered a separate entity due to their circumscribed growth, benign clinical course and possibility of cure after surgical resection alone. A WHO grade II is considered a low-grade glioma while WHO grade III and IV are considered high-grade gliomas. Grade II lesions are infiltrative growing lesions that tend to recur, and they may progress to higher grades of malignancy over time. For example, a grade II astrocytoma could transform into a grade III anaplastic astrocytoma and may eventually end up as a grade IV glioblastoma.

### 1.3.3 WHO Classification 2016 – a paradigm shift

In 2016 came the revised WHO classification from 2007, which incorporates molecular parameters such as isocitrate dehydrogenase (IDH) and 1p19q co-deletion (11, 12), see table 2 and figure 1.

*Table 2 Comparing WHO 2007 and WHO 2016*

| WHO grade | WHO 2007 | WHO 2016 (revised 2007) |
|---|---|---|
| Grade II | Diffuse astrocytoma | Diffuse astrocytoma; IDH mutated |
| | | Diffuse astrocytoma; IDH wild type |
| | Oligodendroglioma | Oligodendroglioma; IDH mutated, 1p19q co-deleted |
| | Oligoastrocytoma | n/a |
| | | |
| Grade III | Anaplastic astrocytoma | Anaplastic astrocytoma; IDH mutated |
| | | Anaplastic astrocytoma; IDH wild type |
| | Anaplastic oligodendroglioma | Anaplastic oligodendroglioma; IDH mutated, 1p19q co-deleted |
| | Anaplastic oligoastrocytoma | n/a |
| | | |
| Grade IV | Glioblastoma | Glioblastoma; IDH mutant (secondary) |
| | | Glioblastoma; IDH wild type (primary) |

This classification now groups all diffuse gliomas together and they are classified based on both phenotype and genotype. The diffuse gliomas include astrocytic tumours (WHO grade II and III), oligodendroglial tumours (WHO grade II and III), the oligoastrocytomas (WHO grade II and III) and glioblastomas (WHO grade IV). In this classification the glioblastomas without IDH mutation also carry the synonymous term “IDH wild type primary glioblastoma” as these glioblastomas typically arises de novo. The

glioblastomas with IDH mutation are also called “secondary glioblastoma, IDH-mutant” as they develop though malignant progression of a diffuse astrocytoma or anaplastic astrocytoma (12).

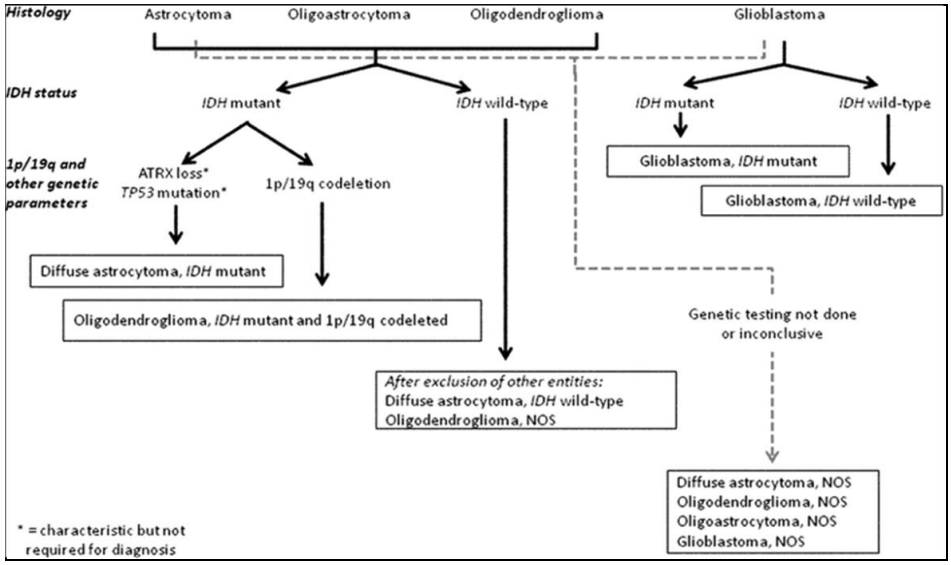


*Figure 1: Simplified algorithm for classification of the diffuse gliomas based on histological and genetic features. From Cavenee et al. with permission from Dr. David N. Louis.*

### 1.3.4 IDH mutation

Mutations in the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent isocitrate dehydrogenase (IDH) genes of the Krebs cycle, IDH1 and IDH2, are considered early events in gliomagenesis (13, 14). The IDH-mutation family consists of heterozygous point mutations at residue R132 in IDH1, R172 and R140 in IDH2. Initially reported in an exome sequencing study on secondary glioblastomas with a history of a lower grade precursor tumour (15), the IDH1 mutation was subsequently reported in both astrocytomas and oligodendrogliomas (16). Although IDH1 is by far the most prevalent, an IDH2 mutation has also been found in the same set of gliomas (17). Approximately 70% of these tumour entities carry point mutations in codon 132, and of these,

more than 90% are mutations of the R132H type (17), for which there is a commercially available monoclonal antibody (18). The cancer-associated IDH mutations are unusual by the fact that they do not simply lead to a loss of function but result in a new metabolic enzymatic activity that produces a potential oncometabolite. The point mutations leads to a gain-of function ability of the enzyme to catalyse the NADPH-dependent reduction of alpha-ketoglutarate to R(-)-2-hydroxyglutarate (2HG). This may in turn inhibit both histone and deoxyribonucleic acid (DNA) demethylation and alter epigenetic regulation of stem and progenitor cell differentiation (19, 20). In addition, these mutations may also give tumour cells a growth advantage through activation of pathways mediated by HIF1α, a key factor in the cellular response to hypoxia (21).

### 1.3.5 1p19q co-deletion

First reported in 1994, oligodendroglial tumours show loss of heterozygosity (LOH) for chromosome arms 1p and 19q (22). It is now known to be an unbalanced translocation and subsequent combined whole-arm loss of chromosome 1p and 19q (23, 24) and is invariably associated with IDH-mutation (17). The translocation between chromosomes 1 and 19 [t(1;19)(q10;p10)] creates two derivative chromosomes, der(1;19)(p10;q10) [+] and der(1;19)(q10;p10) [C], and is followed by loss of the derivative chromosome containing 1p and 19q [+] (25).The presence of both 1p and 19q deletion, called 1p19q co-deletion, is the genetic hallmark of oligodendrogliomas. 1p19q co-deletion is associated with a better chemotherapeutic response and overall survival (26-28), but the partial loss of 1p results in a much poorer prognosis (29).

### 1.3.6 Telomerase reverse transcriptase (TERT) and α-thalassemia/ mental retardation syndrome X-linked (ATRX)

These two mutually exclusive mutations play a role in telomere maintenance in gliomas. Telomeres are repetitive elements at the end of chromosomes. They protect chromosomes from DNA-degradation but telomere shortening occurs with cell division (30). Tumour cells maintain their telomere length and escape senescence either via re-activation of telomerase or through telomerase-independent mechanisms collectively called alternative lengthening of telomeres.

TERT

Point mutations in the promoter region of the telomerase reverse transcriptase leads to increased activity of the catalytic subunit of telomerase. The enzyme adds nucleotides to telomeres and the activity of telomerase is relatively low in normal tissue, which allows cell senescence and apoptosis. Somatic point mutations that substitute a cytosine for a thymidine at position 228 (C228T) and 250 (C250T) of the TERT gene promoter (pTERT) results in an abnormal reactivation of telomerase complex. This alters telomere lengthening and lead to prolonged longevity of tumour cells by escaping from the tumour cell senescence (31). Though this is the most important effect of TERT promoter mutations, a range of other telomere lengthening-independent functions of TERT significantly contribute to cancer initiation and progression, which include its effects on mitochondria, ubiquitin-proteasomal system, gene transcription, micro- ribonucleic acid (RNA) expression, DNA damage repair and RNA-polymerase activity (32). TERT promoter mutations are found in >95% of oligodendrogliomas (33, 34) where they are associated with better survival (35), but also in a large proportion of IDH wild type gliomas with poor survival (34).

ATRX

The ATRX protein was first discovered through a study assessing patients with the x-linked mental retardation syndrome presenting with α-thalassemia. ATRX function as a regulator of chromatin remodelling and transcription whose main function is the deposition of the histone variant H3.3 into telomeres (36). In low-grade gliomas inactivating alterations of ATRX are frequent (86%) and this includes mutations, deletions, gene fusion, or a combination of these events (37) which induce alternative lengthening of telomeres. Loss of protein expression can be detected by immunohistochemistry and is a surrogate marker for ATRX mutations (34). ATRX is invariably present with IDH mutation but is almost mutually exclusive with 1p19q co-deletion (38) and activating mutations in the TERT gene, making it both a prognostic and predictive biomarker.

### 1.3.7 TP53 mutation

The TP53 gene and its product the p53 protein, affectionately known as “the guardian of the genome”, is important maintaining genome integrity and preventing the proliferation of cells with damaged DNA (39). The gene is mutated in about half of human cancers and in gliomas the mutation is associated with IDH mutation and ATRX inactivation (40). The p53 immunostaining may act as a surrogate marker for TP53 mutation (41) but p53 immunohistochemistry detects both wild type and mutant protein, and will also stain positive in any condition causing wild type accumulation (42).

### 1.3.8 O6-methylguanine–DNA methyltransferase (MGMT)

The MGMT gene encodes a DNA-repair protein. Chemotherapy-induced effects, especially O6-methylguanine, trigger cytotoxicity and apoptosis and high levels of MGMT activity in cancer cells counteract the therapeutic effect of alkylating agents in chemotherapy. Promoter methylation of the MGMT gene is

associated with a loss of MGMT expression and diminished DNA-repair activity, which translates clinically into response to chemotherapy and improved survival in glioblastomas (43) and is therefore considered a predictive biomarker of treatment response. The MGMT promoter methylation is associated with both IDH mutation and TP53 mutation (44, 45).

### 1.3.8 Proliferation marker Ki-67/MIB-1 (MIB-1)

During the eukaryotic cell division, the cell goes through different stages of which allows for one cell to produce two daughter cells. The MIB-1 antigen is expressed during all phases of cell division except G0 and the initial part of G1. The marker is therefore a surrogate marker for mitotic cells and a proliferation index can be determined. Diffuse astrocytomas have a proliferation index around 4% while anaplastic astrocytomas are in the range of 5-10% (11). Studies have shown that an increase in MIB-1 proliferation index is associated with an increase in malignancy grade (46, 47). In addition, the index may vary both within a single tumour and with increasing malignancy grade (11, 48, 49).

### 1.3.9 H3 K27M mutation

A diffusely infiltrative glioma called diffuse midline glioma, H3 K27 M mutant is a rare and relatively new entity in children and young adults (11). Diffuse midline gliomas of the brain stem, spinal cord, and diencephalon often harbour a K27M mutation and most patients die within 2 years. The H3 K27M mutation can be detected with a mutation- specific antibody (42).

## 1.4 SURVIVAL

Diffuse low-grade gliomas remain incurable, and eventually a fatal disease, due to several factors. Because of their infiltrative growth, they advance well beyond the radiological margins making surgical resection impossible even with

improved neurosurgical techniques. Diffuse low-grade gliomas tend to recur within years of resection, and when they do recur it may be with a higher malignancy grade and increased growth rate. The mean age at time of diagnosis is around 40 years of age. The median survival rates for diffuse low-grade gliomas were previously reported from 5-10 years. The molecular markers of gliomas are strongly associated with survival (40, 50), and it is clear that median survival estimates range from approximately 2 to 12 years or more depending on the molecular diagnosis (11).

## 1.5 THEORY OF ORIGIN

For many decades, the classification system of gliomas remained unchanged, and gliomas were assumed to arise by de-differentiation and malignant transformation of fully matured glial cells. It was also believed that mature glia was the only dividing cell in the adult brain and that neural stem cells was only present during development of the brain. Then animal models indicated that gliomas can arise from a several different cell types; including neural stem cells, astrocytes, or oligodendrocyte precursor cells (OPC) (51):

- Neural stem cells are found in neurogenic niches and give rise to neurons, astrocytes and oligodendrocyte precursor cells (OPC).
- Mature astrocytes may undergo proliferation and generate a significant cell population, which may support the hypothesis that mature astrocytes could serve as cells of origin for cancer.
- The oligodendrocyte precursor cell (OPC), also called NG2 cells or polydendrocytes, are the largest dividing population in the human brain. This fact coupled with both their shared immunohistochemical and molecular markers with gliomas and their ability to readily form gliomas in murine models make them a likely candidate for glioma formation (51-53).

## 1.6 NEUROGENIC NICHES

With the discovery of adult neurogenesis (54) and the neurogenic niches in the mammalian brain (55-57), came a renewed interest for the cancer stem cell theory. This theory states that all tumours contain a fraction of stem cells (cancer stem cells, CSC) with indefinite potential for self-renewal that drive oncogenesis (58). The subventricular zone (SVZ) and the subgranular zone (SGZ) are the two neurogenic niches in the human brain: The SVZ is in the lateral walls of the lateral ventricles and the SGZ is located in the dentate gyrus of the hippocampus (59).

The SVZ is the largest neurogenic niche and gliomas may develop from neural stem cells originating within this niche (60). In support of this, multipotent cells expressing Nestin, a stem cell/ progenitor marker, were isolated from human gliomas and shown to initiate glioma formation in mouse models (61). But such xenograft studies do not fully capture the glioma formation, and several other murine models have been developed to show oncogenes involved in glioma formation (62-64). In a study using patient brain tissue where astrocyte-like neural stem cells with driver mutations migrate from the SVZ and initiate glioma development in distant brain regions (65). The presence of cancer stem cells does have therapeutic implications (66), and it has been suggested that existing tumours may also take advantage of the neurogenic niches, such as the SVZ, in tumour maintenance and recurrence (67).

In clinical research, radiographic studies do show a distinct pattern of gliomas, and gliomas tend to occur near the periventricular area of the brain. Involvement of the SVZ indicates poor clinical outcome at least in glioblastomas (68, 69) and targeted radiation therapy of the subventricular zone might be useful in some patients (70, 71). There is also some evidence of reduced survival in low-grade gliomas in contact with the SVZ (72, 73).

## 1.7 ANATOMICAL LOCATION

There are anatomical differences in the distribution of gliomas. Low-grade gliomas are often found in eloquent regions of the brain (74). There are several studies on anatomical distribution of low grade gliomas: Qi et al. published a retrospective study of 193 gliomas of grade II and III showing and reported that IDH-mutated gliomas were mainly located into a single lobe, such as the frontal lobe, temporal lobe, or cerebellum whereas IDH wild type tumours more often were located in diencephalon or brainstem (75). The frontal lobes were also a site of predilection for IDH-mutated gliomas in another study including 92 low-grade gliomas (76). In a study on 146 low-grade gliomas Wang et al. found that IDH-mutated gliomas were preferentially located in the frontal lobes, specifically around the rostral extension of the lateral ventricles (77), a location more often seen linked to IDH-mutated glioblastomas (78). However, interpreting these studies in light of the WHO 2016 classification is not straightforward, as only one of these studies did a complete IDH-mutation status analysis including both IDH1 and IDH2 mutations (75). 1p19q co-deleted tumours, oligodendrogliomas, follow a similar distribution as IDH-mutation and several studies have shown a preference for frontal lobe in co-deleted tumours (79, 80). Further, frontal tumours have been associated with a better prognosis (81). A more recent study by Darlix et al. reported that frontal tumours were frequently IDH-mutant (87.1% compared with 57.4%) and 1p19q co-deleted (45.2% compared with 17.0%) than temporo-insular tumours (82). The location of gliomas is therefore important and may indicate molecular status and hence prognosis.

## 1.8 IMAGING OF GLIOMAS

In patients with suspected brain tumour, imaging with both computed tomography (CT) and magnetic resonance imaging (MRI) is usually performed. MRI, including T2-weighted and fluid-attenuated inversion recovery sequences (FLAIR), and T1-weighted sequences before and after gadolinium use, is the standard method for the detection of a gliomas according to international

recommendations for tumour imaging protocols (83, 84). Also, angiography sequences can aid the surgical strategy, and amino acid PET may aid in defining metabolic hotspots suited for biopsy (85). In addition to the basic anatomic sequences on MRI (T1, T2, FLAIR and T1 after contrast), there are also several advanced sequences which focus more on physiological and biochemical properties. Examples of advanced sequences are diffusion weighted imaging, susceptibility weighted imaging, magnetic resonance spectroscopy, perfusion weighted imaging, diffusion tensor imaging and blood oxygen level dependent functional MRI. MRI tractography based on diffusion tensor imaging and/or functional MRI is sometimes used for surgical planning, while the other sequences are often used to rule out differential diagnoses such as cerebral abscesses or pseudoprogression.

Low-grade gliomas in adults generally appear with low density on nonenhanced CT, with low signal intensity on T1-weighted and hyperintense on T2-weighted and FLAIR images. There is usually no peritumoral oedema. A more heterogenous signal intensity on T1-weighted and T2-weighted images, in addition to calcifications, are often found in oligodendrogliomas (86). Contrast enhancement is usually heterogenous and occurs in up to 15–39% of supratentorial low-grade gliomas in adults (87). Preoperative contrast enhancement was previously reported to be 18% in the study population of paper II (2) and Bø et al. reported 28 % in his study (88). High-grade gliomas have heterogenous contrast enhancement or a ring-like pattern on T1-weighted images. A central core of necrosis is present in glioblastoma and they often have peritumoral oedema (89). Efforts to distinguish different genotypes of gliomas by imaging are emerging, but accuracy is still modest (86).

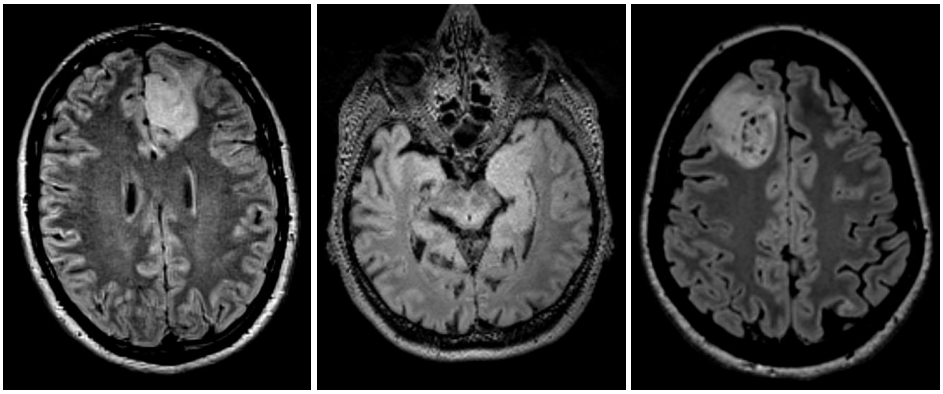

*Figure 2: Preoperative T2-weighted MRI-images of three diffuse gliomas, all WHO grade II. From the study population in paper III.*

## 1.9 TREATMENT

### 1.9.1 Surgery

Historically, diffuse low-grade gliomas were considered benign due to their slow growth. In the last decades this view has changed and the latest EANO-guidelines (85) now recommends maximum safe resection preferably in high-volume specialist centres. The guidelines also state that it is “uncertain whether the extent for resection truly matters”, a topic that was subsequently debated (90, 91). An updated Cochrane-analysis also states that although there is improved survival correlating to higher extent of resection, there is still lacking randomized control trials to make definitive clinical decisions and each case should be assessed individually (92). A study comparing surgery to no surgery is probably unlikely to happen, especially after publications where the completeness of initial resection of a diffuse glioma is an independent predictor of both progression-free survival and overall survival (93, 94).

Management of glioma is dependent on tissue diagnosis and the assessment of molecular markers, and surgery is performed with both diagnostic and therapeutic objectives. But the infiltrative growth of glioma cells is likely incompatible with achieving total resection (95, 96).

### 1.9.2 Adjuvant treatment

As surgery alone is not curative, patients with diffuse low-grade gliomas ultimately require adjuvant therapy. Both chemotherapy and radiotherapy play a role in the management of gliomas, but the question of whether to give adjuvant therapy, what to give and when to give is less certain (85). Thus, the EANO recommendations are based on factors predicting prolonged time to progression, transformation and survival: For patients regarded as low risk, i.e. age <40 years and gross total resection (GTR) follow-up is with MRI surveillance, but for patients regarded as 'high risk', i.e. age >40 or incomplete resection, adjuvant therapy is recommended.

RADIOTHERAPY

The dose and schedule are dependent on the grade of the tumour. The EANO guidelines (85) recommends that the radiation target volume includes the residual tumour volume, including the surgical bed, in addition to a 1.0-2.5 cm margin. The target volume should be modified to reduce radiotherapy to areas of high risk of radiotherapy-associated toxicity (i.e. retina, hippocampus). Methods of more focused radiotherapy such as intensity-modulated or image-guided radiotherapy are also available.

CHEMOTHERAPY

Temozolomide (TMZ) or the combination of procarbazine, lomustine and vincristine, collectively referred to as PCV, are the two main options. Previous studies on treatment regimens were conducted in the era before molecular markers and are difficult to translate into today's clinical practice (97, 98). Still, only the effectiveness of radiotherapy plus PCV has been proven in a randomized clinical trial (97).

The latest EANO guidelines has the following recommendations for therapy (85): For diffuse low-grade gliomas, maximal surgical resection is considered the best initial therapeutic measure with a watch-and-wait strategy once the diagnosis is established. However, IDH wild type tumours can have an aggressive course resembling glioblastomas and should be considered for further treatment. In anaplastic astrocytomas, WHO grade III, the standard treatment includes maximal surgical removal or biopsy followed by radiotherapy. Maintenance temozolomide has also been introduced. For glioblastomas, WHO grade IV there are no specific treatment recommendations for different subtypes. The standard of treatment is gross total resection when feasible, followed by radiotherapy with the addition of temozolomide. The effect of temozolomide is more apparent in patients with MGMT promoter- methylated glioblastoma (43) and MGMT status often guides treatment in the elderly. In oligodendrogliomas the standard treatment is radiotherapy followed by PCV if treatment beyond surgery is considered necessary.

TUMOUR TREATING FIELDS

A novel treatment modality is tumour treating fields. This involves the patient carrying a mobile electrical device for more than 18 hours per day and having 4 arrays of transducers continuously fixed to the shaved scalp (99) . The device delivers alternating electrical fields to the brain which cause mitotic arrest and apoptosis of rapidly dividing glioblastoma cells. This modality has been shown to improve both progression-free and overall survival in patients with glioblastoma (99, 100).

## 1.10 PROGNOSTIC FACTORS

Gliomas constitute a heterogenous group of diseases where some tumours remain stable for years while others rapidly progress. Prognostic factors

obviously play a role, and both tumour-, patient-, and treatment-related factors are of importance, see table 4.

*Table 4 Prognostic factors in gliomas. Modified from Stupp et al. (101). Outlined factors are addressed specifically in this thesis.*

| **Tumour** | **Patient** | **Treatment** |
|---|---|---|
| Histology | Age | Radical surgery |
| **WHO grade** | Performance status (KPS) | Radiotherapy |
| **Location** | Neurologic function | Chemotherapy |
| Tumour size | Seizure status | |
| Contrast enhancement | Corticosteroid dependency | |
| **Ki67/MIB-1** | Cognitive function | |
| **IDH-mutation status** | | |
| **1p19q co-deletion status** | | |

Tumour-related prognostic factors and the molecular markers in the latest WHO classification divide low-grade gliomas in diagnostic groups that have both prognostic and therapeutic implications. The main molecular groups being oligodendroglioma IDH mutated and 1p19q co-deleted, astrocytoma IDH mutated and astrocytoma IDH wild type.

Patient populations can be divided by different prognostic groups, and for prediction of survival there are two scores in use:

PIGNATTI

The Pignatti score is used in the prediction of survival in patients with diffuse low-grade glioma. The score incorporates patient age ≥40 years, tumour diameter ≥6 cm, tumour crossing midline, presence of neurologic deficit and astrocytoma histology, where the presence of each factor gives one point. Higher score indicates a worse prognosis, and scores of ≥3 are considered high risk with a significantly shorter median survival time (102).

UCSF

The University of California at San Francisco Low-Grade Glioma Prognostic Scoring System (UCSF) is similar but designed as a preoperative scoringsystem. Here, presumed eloquence of tumour location, a KPS (Karnofski performance status) ≤80, patient age >50 years and tumour diameter >4 cm each gives one point, and total score is inversely proportional to predicted survival (103).

# 2 OBJECTIVES

The overall aim of this thesis was to explore prognostic markers in patients with diffuse low-grade gliomas. Both WHO grade, molecular markers and tumour location are known tumour-related prognostic factors. The following aims were formulated:

**Paper I** **Ki-67/MIB-1 immunostaining in a cohort of human gliomas**

Evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas.

**Paper II** **Surgical resection versus watchful waiting in low-grade gliomas**

Assess molecular markers and long-term survival in two population-based parallel cohorts of low-grade gliomas WHO grade II with different surgical treatment strategies; upfront surgical resection compared with watchful waiting.

**Paper III** **Is the anatomical distribution of low-grade gliomas linked to regions of gliogenesis?**

Analyse the anatomical distribution of low-grade gliomas of WHO grade II and explore whether molecular subtype could be linked to distance from lesions to the two neurogenic niches in human adults, namely the subventricular zone and the subgranular zone.

# 3 MATERIALS AND METHODS

## 3.1 PATIENT POPULATION

In paper I a consecutive series on gliomas in adults (over 16 years of age) who underwent operations at St. Olavs hospital, Trondheim University Hospital, during the time period 1998-2013. The inclusion was based on histopathology alone by searching the Department of Pathology database.

For paper II we used population-based inclusion from two of the four geographical health regions in Norway, North-Norway (Helse Nord) and Mid-Norway (Helse Midt-Norge). Population based meaning the inclusion of all patients receiving a tissue diagnosis of diffuse low-grade glioma at the two university hospitals 1998 through 2009 in patients 18 years or older. The two hospitals, University Hospital of North Norway and St. Olavs hospital, Trondheim University Hospital, serve exclusively in the health regions with regional referral practice. The inclusion of the cohort was retrospective and based on histopathology alone. Follow-up ended 1 January 2016.

For paper III patients with a histopathology diagnosis of diffuse low-grade glioma at the University Hospital of North Norway from 1998 through 2009 and at St. Olavs hospital, Trondheim University Hospital, from 1998 through 2015 were included.

## 3.2 ETHICAL CONSIDERATIONS

All studies were approved by the Regional Committee for Medical and Health Research Ethics in Health Region Mid-Norway. The need for informed consent was waived by the Regional Committee for Medical Research Ethics.

## 3.3 TISSUE SAMPLES

For paper I, II and III pathology reports, formalin-fixated paraffin embedded (FFPE) tissue samples and haematoxylin -erythrosine (HE) stained sections were retrieved from the diagnostic biobank at the Department of Pathology at St. Olavs hospital, Trondheim University Hospital.

For paper II and III samples were also retrieved from the diagnostic biobank at Department of Pathology at University Hospital of North Norway (UNN). Representative tissue blocks containing tumour tissue were chosen. For paper II a blinded review was performed as previously described (2).

## 3.4 IMAGING

For paper II and III diagnostic MRIs were retrieved. In paper II preoperative MRIs were available for review in 142 of the 153 patients with diffuse low-grade glioma. For paper III 159 patients were included and their images were acquired on 18 different MRI systems on 10 different locations with field strengths between 0.5 and 3.0T (16 on 0.5T, 4 on 1.0T, 86 on 1.5T and 53 on 3.0T). Of the MRI-sequences used for pre-operative tumour segmentation 122 were Fluid- Attenuated Inversion Recovery (FLAIR) images, 30 were T2-weighted images and seven were T1-weighted contrast enhanced images.

## 3.5 SEGMENTATION

In paper III the segmentations were performed by a radiologist in the open source software 3D Slicer 4.4.0 (http://www.slicer.org), a software platform for quantitative imaging (104). Both FLAIR images, T2-weighted images and T1-weighted contrast enhanced images were used. Manual segmentation is considered the gold standard, but being time consuming this is not always feasible. Because gliomas are infiltrative tumours, their true border are not visualized by contemporary imaging techniques. Also, variability in the

perceived tumour border results in high intra- and inter-rater variability in both manual and automated segmentation (105). In paper III we used semi-automatic segmentation where computer-made segmentation was edited by a radiologist (106). However, variations in imaging appearance are known to differ among molecular subtypes. Astrocytomas are considered more well-defined than oligodendrogliomas (82, 86) making the radiological size of oligodendrogliomas more uncertain.

## 3.6 IMAGE ANALYSIS

In paper III the segmented MRI images were all registered to the standard Montreal Neurological Institute (MNI) coordinate space, which is defined by the ICBM-152 brain template (107). The registration pipeline, which was based on the Advanced Normalization Tools toolkit (108, 109). The registrations were controlled manually. The registered segmentations were then summed voxel-wise to create maps of the tumour distribution.

Surfaces representing the SVZ were created manually along the lateral walls of the lateral ventricles, based on the description given by Vescovi et al. (110) and the rendering of the lateral ventricles given in the Hammersmith atlas (111). This atlas complies with the MNI space, and the distances from the registered tumours to the SVZ could thus be calculated directly.

The location of the SGZ was defined using the high-resolution atlas of the hippocampus and subfields created by the CoBrA Lab (112) which is available at http://cobralab.ca/atlases/Hippocampus-subfields/. The atlas was first registered to the MNI space, and the location of the SGZ was then defined as the centre of mass of the dentate gyrus.

## 3.7 LABORATORY METHODS

### 3.7.1 Immunohistochemistry

Immunohistochemistry is a method were labelled antibodies bind to and detects specific antigens in tissues or cells. It is now considered an essential tool in surgical pathology labs and it is used both for diagnostic, genetic and therapeutic purposes (113). Antibodies are immunoglobulin molecules with two main units, a pair of light chains (kappa or lambda) and a pair of heavy chains (gamma, alpha, mu, delta or epsilon) linked together by disulphide bonds. The three-dimensional structure of the antigen determines the antibodies ability to recognize the antigen. Formalin fixation induces protein crosslinks, thus reducing the antigenicity by modifying the antigen's protein conformation (114). Both enzyme digestion and other methods such as heat-induced antigen retrieval methods are used to increase antigenicity.

Antigens capable of inducing antibody formation are called an antigenetic determinant or epitope. Each antigen can contain more than one epitope and epitopes may be both continuous or discontinuous. Epitopes are a cluster of amino-acid-residues able to bind specifically to an area named paratope (antigen-binding site) on the antibody. Antibodies themselves may function both as antibodies and as antigens. Labelled antibodies may bind directly to the antigen, but may also serve as an antigen itself, allowing a secondary antibody to bind to the already formed antigen-antibody complex. Antibodies may be both monoclonal, recognizing only one epitope, or polyclonal where they recognize several epitopes within one antigen. Monoclonal antibodies are often considered more specific compared to the polyclonal antibodies, but because many antigens have similar epitopes resulting in cross-reactivity.

For visualization, direct labelling of the primary antibody or indirect labelling of a secondary antibody may be used. Secondary labelling lowers the need for a high primary antibody concentration and negative control can be produced by omitting the primary antibody. In addition, an external negative and positive control should be used, as well as an internal control (115).

*Table 5: The antibodies used in this thesis*

| | Antibody | Clone | Type | Manufacturer | Dilution |
|---|---|---|---|---|---|
| Paper I | Ki67/MIB-1 | MIB1 | monoclonal | Immunotech | 1:100 |
| | Ki67/MIB-1 | MIB1 | monoclonal | Dako | 1:600 |
| Paper II | IDH1-R132H | H09 | monoclonal | Dianova | 1:100 |
| | ATRX | | polyclonal | Sigma Aldrich | 1:500 |

PAPER I

All tumour samples were fixed in buffered formalin, usually for not more than 24 hours, and then embedded in paraffin. Paraffin sections (3-µm-thick) were cut and mounted on Superfrost glass slides, deparaffinized, and dehydrated. Different antigen retrieval methods were used during the study period, including pressure cooking, microwave oven, and water bath. The Ki-67/MIB-1 antibody was supplied by Immunotech (Hamburg, Germany) and by DAKO (Glostrup, Denmark). The working dilution was 1:100 or 1:600 depending on the detection system used. The sections were incubated for 40 min at room temperature. Automatized immunohistostainers and detection systems were purveyed by DAKO (TechMate 500, Autostainer Plus, Autostainer Link 48). The staining procedures were performed according to the manufacturer's recommendations. Positive controls were used in each staining run ("sausage block" with tonsil, appendix, pancreas, and liver). First, a standard streptavidin-biotin-peroxidase technique was used, and later the DAKO EnVision Flex+ System. Diaminobenzidine was used as the chromogen and haematoxylin as the counterstain.

PROLIFERATION INDEX EVALUATION

The immunostained sections were scanned using a 40X objective with an eye grid for the areas with the highest density of labelled tumour cells (hot spots). At least 1000 tumour cells, or alternatively three high power fields (HPF) were

examined. Only immunoreactive tumour cell nuclei were counted. Areas with necrosis and/or vascular endothelium were excluded. The Ki-67/MIB-1 PI was defined as the percentage of immunoreactive tumour cell nuclei among the total number of cells.

PAPER II AND III

All tumour samples were fixed in buffered formalin. Paraffin sections (3-µm-thick) were cut, mounted on Superfrost Plus glass and dried at 60°C for 60 minutes.

Immunohistochemical staining for IDH1 R132H was done on BenchMark Ultra fully automated tissue-staining system (Ventana Medical Systems, Inc., Tucson, AZ) using validated protocols. The staining procedure included pre-treatment with Cell Conditioner 1 (Ventana Medical Systems). The sections were incubated with mouse monoclonal IDH1-R132H antibody (clone H09, Dianova, 1:100) or ATRX antibody (polyclonal, Sigma Aldrich, 1:500), followed by incubation with OptiView HQ Universal Linker and OptiViewHRP Multimer antibody reagent (both Ventana Medical Systems). For signal amplification OptiView Amplification kit (Ventana Medical Systems) was used. Antigen detection was performed using OptiView DAB (Ventana Medical Systems). Tissues were counterstained with haematoxylin.

Two pathologists scored ATRX and IDH1 R132H independently. For IDH1 R132H only moderate and strong cytoplasmic staining was considered positive (18). A known IDH1 R132H positive anaplastic oligodedroglioma was used as positive control. For ATRX only nuclear staining was considered for evaluation, and cases with more than 10% positive tumour cells were considered positive (116). Endothelial cells and neurons served as internal positive control. Heterogeneous immunoreaction was observed in some cases, especially around crush artefacts in small biopsies, but only areas with highest staining were considered.

### 3.7.2 Fluorescent in-situ hybridization

Fluorescent in-situ hybridization (FISH) is a technique for visualizing copy number alterations or structural gene rearrangements in formalin-fixed, paraffin-embedded tissues (117). In short, denaturation of DNA strands and "renaturation" or hybridization with labelled DNA probes allows for visualization of chromosomal integrity while observing the morphology of the tissue. In neuropathology, FISH is used for assessment of chromosomal integrity in gliomas to look for the genetic hallmark of oligodendrogliomas; 1p19q co-deletion. This co-deletion is the result of an unbalanced whole-arm translocation between chromosomes 1 and 19, resulting in the loss of one derivative chromosome [der(1;19)(p10;q10)], with retention of the reciprocal der(1;19)(q10;p10) chromosome.

In paper II and III we used a commercially available Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (Abbott) and Histology FISH Accessory Kit (Dako). The signal ratio was assessed individually for chromosomes 1 and 19. Target signals (red) and control signals (green) were counted in at least 100 adjacent, non-overlapping nuclei. The ratio between red and green signals was calculated, and a ratio of <0.85 was needed to conclude with deletion (118). The counting was done independently by a pathologist and a researcher experienced in the evaluation of FISH.

### 3.7.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method were several copies of a specific DNA segment is created and then exponentially amplified. Using both primers which are short single strand DNA fragments (oligonucleotides) that are complementary to the target DNA region and a DNA polymerase, the reaction goes through a series of heating and cooling known as thermal cycling. The two strands of the DNA double helix are separated and the primers (forward or rear primer) then bind to the complementary sequences of DNA. These DNA strands then become templates for DNA polymerase that extends in the 5' to 3' direction

on each strand. As PCR progresses, original template strands plus all newly generated strands are used as a template for replication and the original DNA template is exponentially amplified.

In paper II and III DNA was isolated using Qiagen QIAamp DNA FFPE Tissue kit. A fragment of 129bp corresponding to IDH1 ex4 where IDH1 mutation hotspot R132 is located was PCR amplified using the primer pair: 5'-CGGTCTTCAGAGAAGCCATT-3'and 5'- GCAAAATCACATTATTGCCAAC-3'. A fragment of 236 bp corresponding to IDH2 ex6 where the IDH2 hotspot mutation sites R140 and R172 are located was PCR amplified using the following primer: 5´- GCTGCAGTGGGACCACTATT-3´ and 5´-GTGCCCAGGTCAGTGGAT-3´. A universal primer pair: 5´-CACGACGTTGTAAAACGAC-3´ and 5´-CAGGAAACAGCTATGACC- 3´was attached to the IDH1 and IDH2 primers, respectively. The PCR reaction mix consisted of 1XGeneAmp PCR Gold buffer (Applied Biosystems), MgCl2 (1.5mM), dNTP (0.4mM), 0.6uM of each primer, 1.25U AmpliTaq Gold DNA polymerase, in a total volume of 25ul. The amount of 30ng genomic DNA was applied for each PCR reaction with following program: Denaturation at 950C for 10 min followed by 40 cycles of incubation at 950C for 30 seconds, 560C for 30seconds, 720C for 30 seconds and final extension at 700C for 10 min. The PCR product was purified with illustra ExoStar kit.

### 3.7.4 Sanger sequencing

Sanger sequencing is a method of reading single bases in a DNA or RNA molecule. The process involves copying single-stranded DNA with chemically altered bases called di-deoxynucleotide triphosphates. These altered bases terminate the chain selectively at A, C, G, or T, and are fluorescently labelled creating multiple fragments separated by one base. By using capillary electrophoresis, the order of the fragments and subsequent base order may be established.

In paper II and III the sequencing reaction was performed using the universal primers and Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit. The sequencing products were analysed on Applied Biosystems 3130 genetic analyser.

### 3.7.5 Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a technique by which up to 45 different sequences can be targeted in a single, semiquantitative PCR-based experiment (119). For paper II SALSA MLPA probe mix P088-C1 oligodendroglioma 1p-19q (MRC-Holland) was used in one of the cohorts. This probe mix contains multiple probes for the 1p and 19q chromosomal arms and mutation specific probes for four of the most common IDH mutations. All samples were run in duplicates.

## 3.8 ALGORITHM FOR MOLECULAR SUBTYPING

Paper II was planned before the release of the revised WHO classification of 2016, and an algorithm for molecular subtyping had to be developed. Below is the algorithm used at the two University Hospitals in paper II and III. Subgroup 1 corresponds to oligodendroglioma, IDH-mutated,1p19q co-deleted. Subgroup 2 corresponds to astrocytoma, IDH-mutated. Subgroup 3 corresponds to astrocytoma IDH wild type.

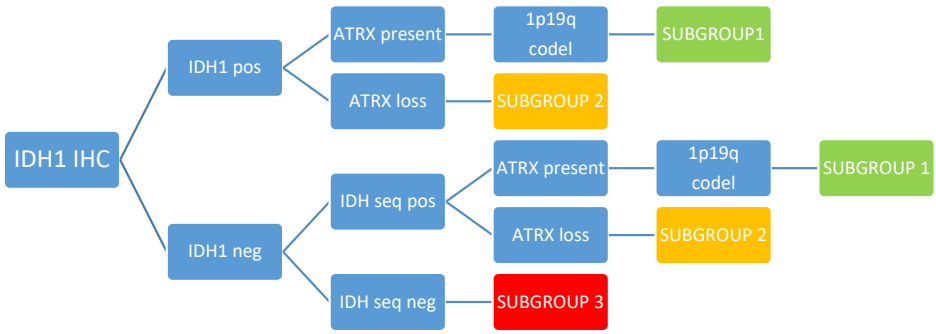


*Figure 2: Algorithm for molecular subtyping used at St. Olavs hospital, Trondheim University Hospital in paper II and III.*

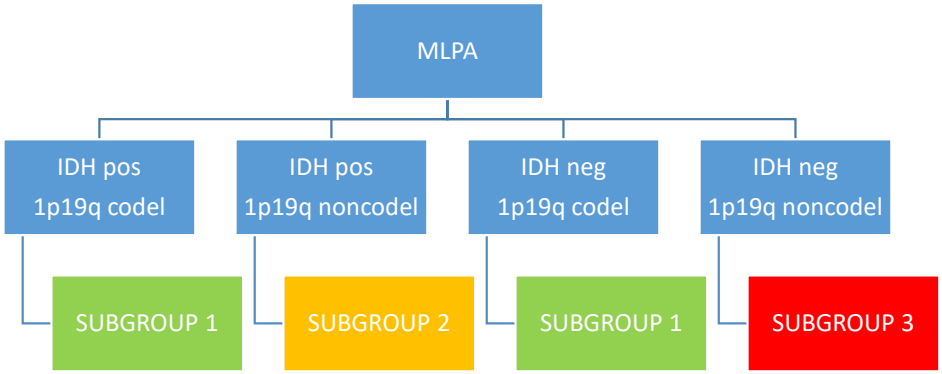


*Figure 3: Algorithm for molecular subtyping used at University Hospital of North-Norway in paper II and III*

## 3.9 STATISTICAL ANALYSIS

All statistical analyses were performed using IBM SPSS Statistics (IBM Corp Armonk, NY). Statistical significance was set to $p< 0.05$.

In paper I the Mann-Whitney U test was applied to estimate differences in the PIs between groups of tumours.

In paper II GraphPad Prism version 6 was used in addition to SPSS. Fisher's exact test was used for comparing results from 2× 2 tables. For other categorical data, the $\chi 2$ test was used. For continuous data, comparison of groups was carried out with independent samples t-test. Overall survival is presented as Kaplan–Meier plots and the log-rank test was used for between groups comparison. Cox multivariable survival analysis was carried out to adjust for important prognostic factors, including molecular markers.

In paper III Q-Q plots were used to assess normal distribution in continuous data. The $\chi 2$ test was used for comparison analysis of categorical variables. Nonparametric Mann-Whitney U and Kruskal-Wallis test were used for comparison of continuous variables.

# 4 SUMMARY

**Paper I Ki-67/MIB-1 immunostaining in a cohort of human gliomas**

Histopathological malignancy grading of human gliomas is limited by subjective interpretation of the morphological criteria. Assessment of mitotic activity is a cornerstone of grading these tumours, but mitotic figures can be hard to identify in haematoxylin-eosin stained sections. The aim of this study was to analyse and evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas. The study showed that Ki-67/MIB-1 PIs correlated well with histological malignancy grade in all glioma subtypes, but a considerable overlap of PIs was observed between the malignancy groups. Consequently, Ki-67/MIB-1 immunostaining alone is not sufficient to adequately determine the malignancy grade and future work is needed.

**Paper II Surgical resection versus watchful waiting in low-grade gliomas**

The effect of up-front surgery on diffuse low-grade gliomas has been controversial and the impact of molecular biology on the effect of surgery is unknown. The long-term results of upfront surgical resection compared with watchful waiting in two molecularly defined cohorts was investigated. The population-based parallel cohorts were followed from two Norwegian university hospitals with different surgical treatment strategies and defined geographical catchment regions. In region A watchful waiting was favoured while early resection was favoured in region B. The inclusion criteria were histopathological diagnosis of supratentorial low-grade gliomas from 1998 through 2009 in patients 18 years or older. Follow-up ended 1 January 2016. Making regional comparisons, the primary end-point was overall survival. A total of 153 patients (66 from region A, 87 from region B) were included. Early resection was carried out in 19 (29%) patients in region A compared with 75 (86%) patients in region

B. Overall survival was 5.8 years (95% CI 4.5-7.2) in region A compared with 14.4 years (95% CI 10.4-18.5) in region B ($P < 0.01$). Molecular markers were determined for all patients and classified according two three risk groups; i: oligodendroglioma, 1p19q co-deleted, ii: astrocytoma, IDH mutated and iii: astrocytoma, IDH wild type. The three groups displayed different long-term survival, with poorest survival in astrocytoma, IDH wild type group. The effect of surgical strategy remained after adjustment for these molecular markers ($P = 0.001$).

**Paper III Is the anatomical distribution of low-grade gliomas linked to regions of gliogenesis?**

According to the stem cell theory, two neurogenic niches in the adult human brain may harbour cells that initiate the formation of gliomas: The larger subventricular zone (SVZ) and the subgranular zone (SGZ) in the hippocampus. We wanted to explore whether defining molecular markers in diffuse low-grade gliomas are related to distance to the neurogenic niches. Patients treated at two Norwegian university hospitals with population-based referral were included. Eligible patients had histopathological verified supratentorial low-grade glioma. IDH mutational status and 1p19q co-deletion status was retrospectively assessed. 159 patients were included, and semi-automatic tumour segmentation was done from pre-treatment T2-weighted or FLAIR images. 3D maps showing the anatomical distribution of the tumours were then created for each of the three molecular groups; i: oligodendroglioma IDH mutated/1p19q co-deleted, ii: astrocytoma IDH mutated and iii: astrocytoma IDH wild type. The study showed that low-grade gliomas are more often found closer to the SVZ than the SGZ, but IDH wild type tumours are more often found near SGZ. Our study suggests that the origin of IDH wild type and IDH mutated low-grade gliomas may be different.

# 5 DISCUSSION

## 5.1 DISCUSSION OF MAIN FINDINGS

### 5.1.1 MIB-1 and WHO as prognostic markers

The WHO grade is based on how abnormal cells and tissue look in the microscope, and these changes are used to predict response to therapy and outcome (120). Patients with WHO grade II tumours (low-grade glioma) may typically survive for >5 years and patients WHO grade III tumours survive for 2-3 years (12). The designation into a grade II or III tumour often determines the choice of whether to give adjuvant therapy in the form of radiation and/or chemotherapy.

The distinction between a grade II and a grade III tumour is based on morphological features such as cellularity, cellular/nuclear atypia, degree of mitotic activity, architectural patterns and vascular properties. Distinguishing between a grade II and II tumour can be difficult, and the histopathologic criteria are subjective and a source of inter-observer variability (121, 122). The MIB-1 proliferating index is therefore an additional tool to more objectively determine the ‘increased mitotic activity’ requirement for grade III tumours. In paper I we found a positive correlation between WHO grade and MIB-1 proliferative index, but with considerable overlap across grades. No clear cut-off values for MIB-1 have been developed for the distinction between grade II and grade III tumours (49) and there have also been conflicting results regarding MIB-1 as a prognostic marker (47, 123, 124).

Even though the use of MIB-1 has been advocated as a means of overcoming inter-observer variability in histopathological diagnosis, there is also inter-observer variability in the reporting of proliferating indices and interlaboratory differences in both tissue processing and evaluation methodology (125). Recent attempts with digital image analysis have also yielded conflicting results, with some reporting good correlation (126), while others report substantial inter-observer variability between pathologists and for pathologists versus digital

analysis (127). However, the studies are not completely comparable as different modes of reporting variability was used (intraclass correlation coefficient and kappa-statistics, respectively).

### 5.1.2 MIB-1 and WHO after the WHO 2016 Classification

Paper I was conducted before the implementation of IDH1/2 analysis in our department and FISH analysis for 1p19q co-deletion was not routinely done on all cases. The IDH mutation is now seen in most low-grade gliomas (except IDH wild type astrocytomas) and 1p19q co-deletion is the hallmark of oligodendrogliomas (12). Before the implementation of the WHO 2016 classification the astrocytomas grade II and III could harbour tumours where 20% were without an IDH mutation (13) and 40% of the diffuse and anaplastic oligodendroglial tumours (both oligodendrogliomas and oligoastrocytomas) were lacking 1p19q co-deletions (128). We now know that a substantial subset of IDH wild type tumours may display the same characteristics in clinical course as glioblastomas WHO grade IV (129). Supratentorial IDH wild type gliomas of grade II and III should therefore be assessed for EGFR amplification, combined loss of chromosome10/ gain of chromosome 7 or TERT promoter mutations (130). These molecular features point to a more aggressive clinical behaviour and should be designated as “diffuse astrocytic glioma, IDH wild type, with molecular features of glioblastoma, WHO grade IV” even though the histopathological appearance may suggest a low-grade glioma WHO grade II. In the absence of these markers, MYB/MYBL alterations suggest a more indolent clinical course (130, 131).

Publications conducted with the incorporation of molecular markers such as IDH mutation and 1p19q co-deletion now suggest that mitotic activity and proliferation indices are probably not as relevant for outcome among IDH-mutant gliomas (132) but more important in IDH wild type gliomas (133). A recent French multicentre study have suggested a small niche for the utility of MIB-1 PI in the rare anaplastic oligodendrogliomas where an index above 15%

was associated with poor survival (126). However, the study did not report on CDKN2A status. Homozygous deletion of CDKN2A causes uncontrolled cell proliferation and has been shown to be correlated with both mitotic index and MIB-1 proliferation index (134). CDKN2A is another example of genetic alterations that outweighs the prognostic value of the WHO grade. This deletion has such a strong negative impact on survival that a new diagnostic entity of oligodendroglioma WHO grade IV has been carefully suggested (134). New copy-number alteration-based molecular subtypes, which are independent of WHO grading, as well as predictive of clinical outcome have been suggested as an alternative to WHO grade in IDH mutant gliomas (135, 136). The introduction of an adjusted WHO-grade based on prognosis after treatment has previously been deemed too confusing (120) but it is anticipated that the future WHO classification will incorporate a molecular grade.

### 5.1.3 Surgical resection

Maximal safe resection is now considered the best option for most diffuse gliomas (85). The dominating study method in neurosurgery has historically been retrospective cohorts were surgeons have been evaluating their own work. Randomized controlled trials in neurosurgery in general have obvious challenges with patient inclusion, surgical selection bias, appropriate control group, defining clinically relevant outcomes and perceived lack of equipoise (137). Randomized trials in low grade glioma surgery has even been deemed unlikely because of the rarity of the disease, presumed long survival with need for long follow-ups and local differences in treatment.

Technical innovations in neurosurgery include neuronavigation, intraoperative imaging and neurophysiological monitoring, which has facilitated an aggressive surgical approach in many centres. This is also true at our institution, where the development of 3D ultrasound based neuronavigation (138) led to improved patient survival (139). Partly because of this, two different neurosurgical

treatment strategies existed in two neighbouring health regions in Norway, and this forms the background for paper II.

Paper II was a follow-up paper of the landmark paper comparing surgical strategies of diffuse glioma in these two regions with opposite surgical management traditions (2); one hospital (A) preferred a wait and scan strategy after biopsy whereas another hospital (B) favoured early resections. In paper II we reported molecular markers and long-term survival data. We showed that median overall survival was 5.8 years in hospital A as opposed to 14.4 years in hospital B. Adjustment for molecular markers did not alter results and the findings strongly advocates early resections in low-grade gliomas.

### 5.1.4 Surgery as a prognostic factor

In paper II some patients underwent resection in the region with a wait and scan strategy, and some had biopsy only in the region advocating early resection. The observed survival benefit of early surgical resection is therefore maybe a conservative measure. We were not able to provide data on extents of resection (EOR) in this study due to the lack of postoperative MRI and lack of digitalization of images before 2005–2006 in one of the regions. Extent of resection is considered an important prognostic factor (140) in gliomas. Smith et al. found greater overall survival (OS) and progression free-survival (PFS) with >90% resection and that residual tumour volume negatively impacts OS (141). Wijnenga et al. also found greater overall survival in molecularly defined low grade gliomas and showed that even small remnants, especially in IDH mutated tumours, had a negative impact on survival (93), a finding also supported by Dedev et al. (142). Preventing new permanent neurological deficits needs to be balanced against extents of resection because diffuse gliomas are diffusely infiltrating tumours by definition and cannot be cured by surgery. Bø et al. showed in a resection probability map that the probability for resection is lowest in eloquent regions, such as primary motor areas, language areas, basal

ganglia and brain stem (88). This is not surprising as preserving neurological functions in these areas are important for quality of life. Surgery is often a balancing act between extent of resection and risk of postoperative deficits (143). To complicate matters, many brain functions are not assessable during surgery, even in awake surgery settings. Some postoperative deficits are transient and may recover with time and/or rehabilitation. Although early resection has been deemed favourable in terms of quality of life determined by questionnaires (144), the impact of deficits on quality of life is also complex (143). Because of this, it is difficult to estimate the risk of permanent loss of quality of life and functions following brain tumour surgery. If functions are preserved at the cost of lower extents of resection, the preservation of functions may be temporary if later progression leads to loss of functions.

Paper II showed that an overall strategy with early surgical resection resulted in survival benefit, even after adjustment for molecular markers. However, we still do not know if early surgery is more effective in all molecular subgroups. To date, no studies exist that have long enough follow-up, volumetric measurements on extent of resection, incorporated 2016 classification and have a large enough study population to include subgroup analysis.

### 5.1.5 Molecular markers

As the cohorts in paper II were originally diagnosed using the WHO 2007 Classification a molecular update was needed, but the WHO Classification of 2016 was, at the time, not yet published. We therefore developed a molecular algorithm as outlined in section 3.8. of this thesis. In paper II the cohort showed differences in overall survival among the different molecular groups. In oligodendrogliomas, 1p19q co-deleted, median survival was not reached. In astrocytomas, IDH mutated, median survival in the region advocating biopsy was 5.6 years compared with 10.2 year in the region advocating early resection. In astrocytomas, IDH wild type, median survival in the region advocating biopsy was 1.4 year compared with 5.3 year in the region advocating early resection.

IDH MUTATION

The distribution of IDH1 and IDH2 mutations found in paper II are in line with previous findings (13). Interestingly, we found two novel IDH2 mutations not previously described in low grade gliomas, G145R and P167L.

*Table 6: IDH mutations in paper II. The two novel IDH2 mutations found are highlighted.*

| IDH mutation | Number of cases | Region A (*n*=64) | Region B (*n*=81) |
|---|---|---|---|
| IDH1 R132H | 98 | 47 | 51 |
| IDH1 R132C | 2 | 1 | 1 |
| IDH1 R132G | 1 | 0 | 1 |
| **IDH2 G145R** | **1** | **0** | **1** |
| IDH2 R172M | 1 | 0 | 1 |
| **IDH2 P167L** | **1** | **0** | **1** |
| IDH wild type | 41 | 16 | 25 |

The IDH2 G145R mutation has been reported in early gastric cancer (145) and its relevance in gliomas is unknown. G145 is located close to the assumed nucleotide binding site (http://www.uniprot.org/uniprot/P48735) and to the active-site arginine residues of IDH2; R172 and R140. The mutation we found involves a substitution from glycine to arginine. Arginine is both a larger and positively charged amino acid and is therefore likely to perturb the catalytic activity of the enzyme.

The IDH2 P167L mutation has previously been described in a glioblastoma (146). The P167 mutation it is located close to the binding site of the allosteric inhibitor (http://www.rcsb.org/pdb/explore/images.do?structureId=4JA8). The allosteric inhibitor is itself located at the dimerization interface. A mutation affecting this region is likely to affect the catalytic activity of the enzyme, following the same mechanism of an allosteric inhibitor. The mutation involves

the substitution of proline, an amino acid with a known structural role, to leucine. In theory, this could affect both the structure and the flexibility of this region, leading to a change in the catalytic activity of the enzyme, but it is unclear how this would affect the enzyme activity.

Both the position and the nature of the amino acid substitutions are strong indicators of a role on the activity of the IDH2 enzyme. Of note, the patients with these mutations reached median survival and analysing these patients as either IDH mutated or as IDH wild type did not alter the results. One could argue that they probably do not have a role as they are not directly affecting the nucleotide binding site. Since the publishing of paper II, no new studies have been found to confirm the presence of these mutations in other glioma cohorts, but IDH2 mutations are inherently rare. Further research in the form of in silico modelling, in vitro experiments and animal models is needed to confirm the true role of these substitutions in the IDH2 enzyme.

### ATRX

For paper II and III ATRX immunohistochemistry was used as a surrogate marker for 1p19q co-deletion. This may have led to a slight underestimation of oligodendrogliomas. The interpretation of ATRX immunohistochemistry proved difficult in some cases as crush artefacts and a few completely negative samples were observed. This has also been reported among other institutions (147). The reason for this is unknown, and one could speculate on both preanalytical and analytical causes (see section 5.2 below) as this was not observed in all slides from the same staining batch. Repeat immunohistochemistry resolved the problem and this was not observed with IDH1 R132H immunohistochemistry.

1p19q CO-DELETION

Two different methods were used for detection of 1p19q co-deletion in paper II and paper III as previously discussed. MLPA is able to detect whole arm chromosomal losses. A limitation with FISH is that it only detects small areas on the chromosome arms and does not distinguishes between partial and whole arm loss. In addition, polysomy (relative deletion) could have been reported. A study by Chen et al. confirmed that polysomy detected by FISH is associated with adverse outcome and short survival in tumours with 1p/19q co-deletion, but has no prognostic value in tumours with 1p/19q is retained (148). There are however subtleties in this dichotomous classification as a universal definition of chromosome deletion is lacking (117, 149). Also, false-negative samples may be an issue for samples with truncation artefacts, low burden of tumour cells in a particular sample and extensive autofluorescence. The presence of polysomy and/or imbalanced aneuploidy may complicate interpretation of signals, but a way to overcome this is using a cut-off probe ratio.

### 5.1.6 Tumour heterogeneity

Historically, histology has been considered the ‘gold standard’ in diagnosing gliomas. However, there are major concerns regarding interobserver variation of the histological diagnosis of glioma, with reports of disagreement in about 40% of cases, which was considered serious in 9% (122). Although the introduction of molecular markers may reduce the problem, tissue diagnosis depends on biopsies which are subject to sampling bias. A diffuse and infiltrative growth pattern is characteristic for gliomas of grade II-IV, and there are spatial differences in cellular phenotype and malignancy grade (150). For high-grade gliomas, even cells from the same tumour may harbour different mutations or exhibit distinct phenotypic or epigenetic states, a presumed cause of treatment failure and disease recurrence (151).

### 5.1.7 Tumour location as a prognostic marker

In paper III we found that gliomas with different IDH mutation status have different distribution patterns within the brain. The location of a glioma does matter. As an example, frontal tumours are associated with a better prognosis. This is probably due to several factors: frontal tumours have higher resectability compared to other locations (152), extent of resection is associated with outcome (140) and frontal tumours tend to be oligodendroglial tumours, which have an intrinsically better prognosis (153). The anatomic location of low-grade gliomas may therefore provide a crude indication of molecular status and hence prognosis.

The pattern of distribution of gliomas are almost invariably reported as lobar (frontal, parietal, temporal, occipital) in previous studies. This way of anatomical location has nothing to do with embryological development of the brain itself or functional classification of the brain. Another way of reporting is relationship to presumed eloquent regions of the brain. These are regions with a role in basic neurologic function such as sensimotor regions, language area, basal ganglia or large white matter tracts (154), and neuroimaging/ neuromapping techniques are often used to identify these regions prior or during surgery. But not all brain functions are accessible to pre- or intraoperative testing, and what is functional brain and what is non-functional? Not all brain functions completely understood.

In paper III we wanted to address location in a different way. Few studies have looked at the global distribution pattern (not restricted to cerebral lobes) based on molecularly defined gliomas. A study by Wang et al. used voxel-based lesion-symptom mapping analysis and found that IDH1 R132-mutated low-grade gliomas are located preferentially along the rostral extension of the lateral ventricles (77). This was subsequently confirmed by Tejada Neyra et al. (155) using the same method of voxel-based lesion-symptom mapping analysis. Tejada Neyra et al. found that IDH-mutated glioblastomas had the same preferential location, an interesting finding which could suggest the same origin for these tumours. A recent study by Wijnenga et al. used an estimated 3D reconstruction of gliomas to generate probability maps of tumour location for

different molecular subtypes of low-grade gliomas (156). Wijnenga et al. found IDH wild type astrocytomas more frequently in the basal ganglia. These three above-mentioned studies used different ways of molecular characterization; DNA pyrosequencing, methylation-based array and next generation sequencing analysis respectively. These methods are not completely comparable and NGS is considered superior to both pyrosequencing and methylation-based arrays (157). Although these studies do not prove causality, one may speculate if region specific growth of certain subtypes provide hints of origin? The neurogenic niches (SVZ and SGZ) have been suggested at as possible origin of gliomas (59). In paper III the IDH mutation status was correlated to distance to neurogenic niches in the brain, and IDH wild type tumours were more often found near SGZ. The SVZ is a large structure and the SGZ is comparatively small. We did not analyse different regions of the subventricular zone, but considering there are ultrastructural differences (158) there may also be functional differences. One might argue that with such a large structure it would be close to any tumour and opposite, that a large tumour might show proximity to any region. However, the IDH wild type gliomas in our cohort were smaller than IDH mutated gliomas, and tumour volume does not explain the shorter distance from the SGZ to IDH wild type lesions. The frontal predilection of IDH mutated tumours may in part explain our findings as the distance to the SGZ is intrinsically longer for this group. Another possible explanation for proximity to the neurogenic niches, as discussed in the introduction, is that these niches support stem cell maintenance and differentiation and that tumours simply appropriate this niche by taking advantage of an existing system that promotes proliferation and migration (67). Proximity does not prove point of origin. Also, it is important to realise that the neural stem cell is not a single cell, but probably various cell types with different transcriptional and epigenetic profiles, and how this translates to a glioma remains a major area of investigation (159, 160).

### 5.1.8 Prognostic or not

The papers in this thesis explored selected tumour-related prognostic factors in diffuse low-grade gliomas, but to what extent all factors are truly independent prognostic factors is not known. There is likely interactions and associations between factors. As previously discussed, tumour size, location, functional level, and surgical results are often linked. Tumour location is also associated with molecular markers as seen in paper III. It may be difficult to distinguish prognostic factors from predictive factors, and some are considered both, such as ATRX and 1p19q co-deletion. Are some prognostic factors modifiable or driven by treatment selection? The prognostic value of age may for example be coloured by ageism or nihilism in treatment of elderly patients as well as different treatment regimens across age groups. Hegi et al. found that age is no longer considered a prognostic factor in glioblastoma when adjusted for MGMT-status (43). Although observational studies in cohorts where treatment is homogenous are ideal to assess the prognostic value of single factors, cohorts may be influenced by both case selection and treatment given.

### 5.1.9 High risk and low risk

Treatment of newly diagnosed low-grade glioma (LGG) is a controversial area in neuro-oncology. With molecular markers predicting longer survival in some subgroups, there are concerns regarding morbidity of treatment resulting in a lack of consensus regarding the timing and extent of surgery, timing of radiotherapy, and role of chemotherapy. As discussed in the introduction, the clinical concept of ‘high risk’ low-grade glioma (age >40 or incomplete resection) are patients who require postoperative management without delay. With the arrival of a molecular classification, the clinical concept of ‘high risk’ is changing as seen with the entity ‘diffuse astrocytoma, IDH wild type, with molecular features of glioblastoma WHO grade IV’. Also, IDH mutation status is also a more accurate prognostic marker than the Pignatti score (161).

## 5.2 VALIDITY OF MOLECULAR SUBTYPING AND MARKER ASSESSMENT

### 5.2.1 Preanalytical factors

FFPE archival tissue was used for all papers in this thesis. This has been a method of choice for decades due to its ability to maintain morphology, but this method negatively impacts the quality of DNA, RNA and proteins (162). Factors such as tissue collection, processing, storage, and preparation may also differ as the tissue has been collected over several years (163). For paper II all tissue sections were cut at the same time and stored in the freezer to minimize antigenicity loss. FISH-sections were stored in the fridge while awaiting analysis. For PCR analysis, any contamination of DNA might have affected the result.

In all the studies in this thesis the inclusion criteria was based on histopathology. This alone may introduce bias as some tumours may not have been operated at all, thus not receiving a histopathological diagnosis. To minimize classification bias, a blinded review was carried out in paper II where discordant diagnoses were settled in a consensus meeting. Paper I and the additional patients in paper III did not have a blinded review, but two pathologists reviewed the slides independently. In case of discrepant results cases were discussed and consensus was reached. As previously discussed, the histopathological diagnosis of low-grade glioma is associated with considerable interobserver variability (122). The diagnostic accuracy of biopsy versus resection is also possible source of bias (164).

### 5.2.2 Analytical factors

Analytical factors such as reagent choice, incubation time and conditions, washing steps and choice of antigen retrieval are all factors that may affect immunohistochemical markers. Many of these variations may be bypassed using an automated platform and validation protocols (162), both which was used in all studies in this thesis. In paper I two different antibodies were used

which might have influenced the result. To reduce random error, the average PI of 3 HPF were used in paper I. Also, in paper I and III not all sections were stained at the same time. The primers used for PCR can anneal non-specifically to similar, but not identical sequences to target DNA. In addition, incorrect nucleotides can be incorporated into the PCR sequence, to counteract this a part of the samples were run in duplicates in paper II. A weakness in paper II is also that molecular data was not assessed centrally or using the same methodology. For all laboratory analysis used in this thesis internal validation protocols were carried out with the use of standardized lab-specific procedures by experienced laboratory staff.

### 5.2.3 Reporting molecular markers

With the increasing incorporation of molecular markers in routine practice there is still a lacking international consensus on both method and cut-off values being used, as previously discussed. Although the WHO classification suggests methods for determining 1p19q and IDH mutation, there is no mandate for the use of a particular method. There are also no recommendations for determining i.e. MGMT methylation, which often guides therapy in the elderly. In a recent international survey of 146 neuropathologist in 24 countries the most commonly used methods of determining MGMT status was methylation specific PCR (37%) and pyrosequencing (34%) (165). The survey revealed both differences in laboratory method and more importantly differences in clinically relevant cut-off levels. Although a recent study by Hegi et al. suggest a lower safety margin for determining MGMT with the use of methylation specific PCR (166), there is still a need for international standards in reporting molecular markers.

# 6 FUTURE PERSPECTIVES

Gliomas are considered a systemic disease of the brain, and knowledge of both origin, prognostic and predictive factors are of essence in understanding and treatment of this this disease.

Molecular genetics have revolutionized the diagnosis of gliomas and uncovered many prognostic markers. However, the process of diagnosing gliomas has also become more complex. Capper et al. have suggested a DNA methylation-based classification of *all* central nervous system tumours, not just gliomas, to improve diagnostic precision (167). With this method Capper et al. showed a change in diagnosis in up to 12%. However, a substantial number of tumours fall into the "diffuse leptomeningeal glioneuronal tumour" category. Patients with tumours that do not fit a category is still a patient in need of optimal treatment.

As the next WHO classification is might be based solely on molecular classification, this transition needs to be translated into routine clinical practice at both acceptable costs and feasibility. Several different technologies, many of whom are discussed in this thesis, are currently needed to assess genomic and epigenomic alterations making diagnosing laborious. A recent survey by showed differences in access to biomarkers and molecular techniques across geographic regions and between high-income and low-income countries (168). To solve this Euskirchen et al. demonstrated affordable, same-day detection of structural variants, point mutations, and methylation profiling relevant for the diagnosis of central nervous system tumours using nanopore sequencing (169). Another competing technique called single-molecule real-time sequencing offers many of the same advantages (170).

New image data analysis techniques, such as texture data analyses, may provide information that could affect the surgical decision (171, 172). However, good enough non-invasive prediction of molecular status has yet not been demonstrated. The new simultaneous multi-parametric quantification of radiological images, termed radiomics, is thought to enable image-based clustering of neoplasms and might become one of the modalities used in the

future (173). There are also attempts at digital imaging analysis aimed at helping with quantitative assessment of gliomas (174). More recently, a study suggested omitting the neuropathologist entirely, with a combination of stimulated Raman histology, a label-free optical imaging method and deep convolutional neural networks that aims to predict diagnosis intraoperatively in just a few minutes (175).

Although non-randomized retrospective cohort studies can detect associations with outcome at best, and can by definition never demonstrate effect or impact, paper II is probably the closest one will ever get to a randomized trial comparing surgery to watchful waiting in low grade gliomas. Research on central nervous system tumours in general, and gliomas in particular, is a challenge as there are more than 100 subtypes, making the incidence of different subtypes low. Consequently, the number of randomized controlled trials pertaining to primary tumours of low WHO grade is much lower than that of high-grade gliomas and metastatic tumours, and of this reason randomized controlled trials on low-grade gliomas may simply not be feasible (176).

Most paper in glioma research including the papers in this thesis, have reductionistic viewpoint, that is a simplified model with focus on specific aspects of tumour biology. Pathologists tend to look at molecular markers in relation to survival, radiologists do the same with an imaging features and survival. Studies from oncologists tend to lack neurosurgical parameters, and neurosurgeons are often omitting detailed parameters related to radiotherapy or chemotherapy. Separately viewing these parameters from one's own area of expertise does not make them independent. Many of the parameters are linked: IDH mutation status and age, extent of resection and radiation volume, symptoms and location, location and IDH mutation status. In addition to publication bias, which is a concern in the medical literature in general, academic viewpoint may contribute to systematic bias as demonstrated by Hirshman et al. Here, meta-analysis of the high-grade glioma literature showed that radiation oncologists often revealed no reduction in the hazard of death after gross total resection,

while neurosurgeons often reported that gross total resection was associated with a significant reduction in the hazard of death (177).

There is a need for more complex models in glioma research where both clinical, quantitative radiological, oncological, genetic, molecular and histopathological markers are seen in context, and prospective registry-based observational studies might be the solution.

# 7 CONCLUSIONS

This thesis explored prognostic markers in patients with gliomas, with emphasis on proliferation, molecular markers and tumour location which are known tumour-related prognostic factors. The following conclusions can be drawn:

- Proliferative index as measured by MIB1 is significantly associated with WHO grade but considerable overlap exists between grades. Proliferative index is an ancillary tool, but not sufficient to adequately determine WHO grade in glioma subtypes.

- Molecular risk groups according to WHO classification of 2016 have different survival rates.

-The effect of a surgical strategy was not changed by molecular subtyping in a population-based cohort.

- Two potential new IDH-mutations, previously not described in low-grade gliomas were discovered, but future studies are needed to confirm these findings.

- Gliomas with different IDH mutation status have different distribution patterns within the brain.

- Gliomas with IDH mutation may have different anatomic origin than gliomas with IDH wild type gliomas.

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## PUBLICATIONS

# PAPER I

**Ki-67/MIB-1 immunostaining in a cohort of human gliomas.**

Skjulsvik, A. J., Mørk, J. N., Torp, M. O., & Torp, S. H. (2014). International journal of clinical and experimental pathology, 7(12), 8905–8910.

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*Original Article*

# Ki-67/MIB-1 immunostaining in a cohort of human gliomas

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**Abstract:** Histopathological malignancy grading of human gliomas is limited by subjective interpretation of the morphological criteria. Assessment of mitotic activity is a cornerstone of grading these tumours, but mitotic figures can be hard to identify in haematoxylin-eosin stained sections. Thus, determining proliferative activity by means of Ki-67/MIB-1 immunostaining has become a useful supplement. However, this method has drawbacks, so continuous testing and evaluation are required for optimization and standardization. The aim of this study was to analyse and evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas. We found that Ki-67/MIB-1 PIs correlated well with histological malignancy grade in all glioma subtypes, but a considerable overlap of PIs was observed between the malignancy groups. Consequently, Ki-67/MIB-1 immunostaining alone is not sufficient to adequately determine the malignancy grade. Therefore, future work is necessary to clarify the role of this immunostaining in the histopathological diagnosis of human gliomas.

**Keywords:** Astrocytoma, brain tumour, diagnosis, glioblastoma, immunohistochemistry, proliferation

## Introduction

Histopathological classification and malignancy grading of human gliomas are based on criteria issued by the World Health Organization (WHO) [1]. However, these criteria are encumbered with subjective interpretations, giving rise to inter- and intra-observer variability [2, 3]. Because proliferation is a basic process in gliomagenesis, mitotic counting constitutes a cornerstone in the grading of these tumors. Since identification and counting of mitotic figures in haematoxylin-eosin stained sections can be difficult, glioma grading is imprecise and may unfavorably impact prognosis, treatment, and follow-up.

Immunohistochemical determination of proliferative activity is a useful supplement for establishing the histopathological diagnosis of glioma. Ki-67/MIB-1 immunostaining is most commonly used and has been shown to correlate positively with tumor grade and prognosis [4-6]. Despite its widespread use, the procedure still has many uncertain and limiting factors, including problematic overlap of indices between different glioma grades and inherent problems in the immunohistochemical analysis [5-9]. Thus, publishing data on Ki-67/MIB-1 immunostaining in human gliomas is still worthwhile in order to optimize this method, with the superior goal of achieving a standardized procedure. The aim of this study was to evaluate the Ki-67/MIB-1 proliferative indices (PIs) in a series of gliomas and critically evaluate the findings and procedure.

## Materials and methods

### *Patients*

This study includes a series of gliomas in adults (over 16 years of age) who underwent operations at St. Olavs University Hospital in Trondheim, Norway, during the time period 1998-2013. Both the histopathological diagnosis (according to the WHO classification system) and determination of the Ki-67/MIB-1 PI were

**Table 1.** Ki-67/MIB-1 proliferative indices for glioma subtypes

| Diagnosis (abbreviations) | WHO grade | n | Median age, years (range) | Median Ki-67/ MIB-1 PI[a] (range) | Statistical analysis[b] |
|---|---|---|---|---|---|
| Pilocytic astrocytoma (PILOCYT) | I | 12 | 38 (17-65) | 1.9 (0.7-8.1) | vs. diffuse astrocytoma: $P = 0.004$ |
| Diffuse astrocytoma (A) | II | 57 | 44 (18-78) | 5.2 (0.5-16.6) | vs. oligodendroglioma: $P = 0.218$<br>vs. oligoastrocytoma: $P = 0.287$<br>vs. anaplastic astrocytoma and glioblastoma: $P < 0.001$ |
| Oligo-astrocytoma (OA) | II | 13 | 42 (26-73) | 4.0 (1.0-13.3) | vs. anaplastic oligoastrocytoma: $P = 0.006$ |
| Oligodendroglioma (O) | II | 27 | 44 (21-72) | 4.4 (1.0-22.0) | vs. oligoastrocytoma: $P = 0.798$<br>vs. anaplastic oligodendroglioma: $P < 0.001$ |
| Anaplastic astrocytoma (AA) | III | 28 | 52 (19-82) | 13.1 (2.1-39.1) | vs. anaplastic oligoastrocytoma: $P = 0.643$<br>vs. anaplastic oligodendroglioma: $P = 0.122$<br>vs. glioblastoma: $P = 0.002$ |
| Anaplastic oligoastrocytoma (AOA) | III | 7 | 53 (33-71) | 12.7 (6.3-39.0) | vs. anaplastic oligodendroglioma: $P = 0.006$ |
| Anaplastic oligodendroglioma (AO) | III | 12 | 49 (31-78) | 21.7 (2.4-41.2) | |
| Glioblastoma (GBM) | IV | 89 | 65 (30-89) | 19.4 (2.2-80.0) | vs. anaplastic oligodendroglioma: $P = 0.867$<br>vs. anaplastic oligoastrocytoma: $P = 0.035$ |
| Sub-ependymoma (SUBEP) | I | 9 | 38 (22-62) | 1.0 (0.1-8,5) | vs. ependymoma: $P = 0.126$ |
| Ependymoma (EP) | II | 13 | 55 (27-74) | 2.0 (0.6-25.3) | |
| Anaplastic ependymoma | III | 0 | | | |

[a]PI = proliferation index; [b]Significance was determined using the Mann-Whitney U test.

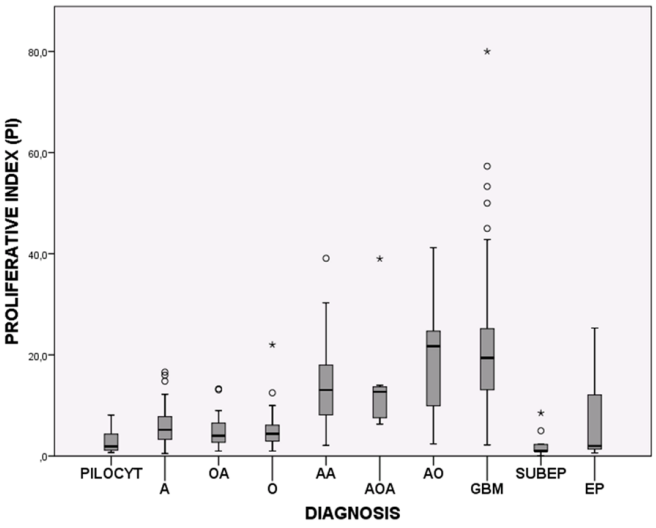


**Figure 1.** Box plots showing the distribution of Ki-67/MIB-1 PIs among the glioma subtypes. PILOCYT: Pilocytic astrocytoma, A: Diffuse astrocytoma, OA: Oligoastrocytoma, O: Oligodendroglioma, AA: Anaplastic astrocytoma, AOA: Anaplastic oligoastrocytoma, AO: Anaplastic oligodendroglioma, GBM: Glioblastoma, SUBEP: Subependymoma and EP: Ependymoma.

performed in collaboration by AJS and SHT. All patients were found by searching the electronic patient data files of the pathology department. Patients were included at primary diagnosis and all cases were diagnosed based solely on WHO classification system, and in addition the Ki-67/MIB-1 PIs were continuously registered in a spreadsheet. Diagnosis were made independent of Ki-67/MIB-1 PIs, but in cases were the PI was unusually high, a comment was made in the diagnosis. However, this did not change the WHO grade.

*Immunohistochemistry*

All tumor samples were fixed in buffered formalin, usually for not more than 24 hours, and then embedded in paraffin. Paraffin sections (3-µm-thick) were cut and mounted on Superfrost glass slides, deparaffinized, and dehydrated. Different antigen retrieval methods were used during the study period, including pressure cooking, microwave oven, and water bath. The Ki-67/MIB-1 antibody was supplied by Immunotech (Hamburg, Germany) and by DAKO (Glostrup, Denmark). The working dilution was 1:100 or 1:600 depending on the detection system used. The sections were incubated for 40 min at room temperature.

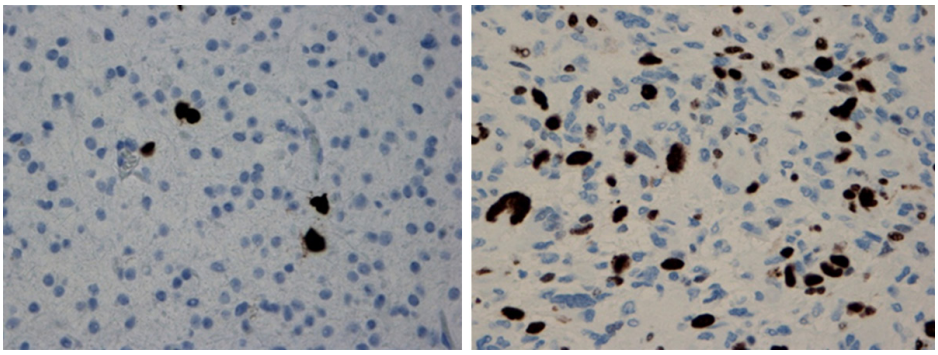

**Figure 2**. Ki-67/MIB-1 immunostaining showing low PI (~4%) in a grade II astrocytoma (left) and high PI (~30%) in a glioblastoma (right). Magnification ×400.

Automatized immunohistostainers and detection systems were purveyed by DAKO (TechMate 500, Autostainer Plus, Autostainer Link 48). The staining procedures were performed according to the manufacturer's recommendations. Positive controls were used in each staining run ("sausage block" with tonsil, appendix, pancreas, and liver). First, a standard streptavidin-biotin-peroxidase technique was used, and later the DAKO EnVision Flex+ System. Diaminobenzidine was used as the chromogene and haematoxylin as the counterstain.

*Proliferation index evaluation*

The immunostained sections were scanned using a 40× objective with an eye grid for the areas with the highest density of labeled tumor cells (hot spots). At least 1000 tumor cells, or alternatively three high power fields (HPF) were examined. Only immunoreactive tumor cell nuclei were counted. Necrotic areas and vascular endothelium were excluded. The Ki-67/MIB-1 PI was defined as the percentage of immunoreactive tumor cell nuclei among the total number of cells.

*Statistical analyses*

Statistical analyses were performed using IBM SPSS Statistics version 21. The Mann-Whitney U test was applied to estimate differences in the PIs between groups of tumors. $P < 0.05$ was considered significant.

## Results

A total of 267 glioma subtypes were examined: 186 astrocytomas, 39 oligodendrogliomas, 20 mixed gliomas, and 22 ependymal tumors. **Table 1** shows all data and statistical correlations for the tumor subtypes. The Ki-67/MIB-1 PIs are graphically illustrated in **Figure 1**.

In general, the quality of the Ki-67/MIB-1 immunostaining was good (**Figure 2**). Some variation in staining intensity was observed, however, only distinctly labeled tumor cell nuclei were counted. Normal brain tissue did not show any immunoreactivity. Various distribution patterns were observed for the labeled tumor cells, both homogenous dispersion throughout the tumor tissue and hot spots, with the latter being more frequent in high grade tumors.

The Ki-67/MIB-1 PI correlated significantly with tumor grade for each glioma type. However, considerable overlap was observed between the malignancy groups (**Figure 2**). No significant difference was found between glioma types of the same tumor grade. Anaplastic oligodendrogliomas and anaplastic oligoastrocytomas had indices comparable to glioblastomas.

## Discussion

In our material we found that the Ki-67/MIB-1 PIs correlated significantly with increasing tumor grade in all types of gliomas but an overlap occurred between the malignancy groups.

The positive correlations between Ki-67/MIB-1 PI and tumor grade in our series of gliomas are in agreement with the literature [10-14]. We found that indices were comparable between gliomas of similar malignancy grade, and indices for high-grade gliomas (grade III/IV) were

significantly higher than in low-grade (grade I/II) tumors. Thus, Ki-67/MIB-1 is useful for differentiating between high and low-grade gliomas, but differentiating between grade I and grade II or grade III and grade IV is more problematic due to the overlap of values between the different tumor grades. This overlap is a main limitation of this immunostaining. For this reason, Ki-67/MIB-1 should not be used alone as a marker of tumor grade but in conjunction with histological features [15, 16].

Histological grading and estimation of Ki-67/MIB-1 PI are subjected to heterogeneity-induced sampling errors, limiting their diagnostic accuracy, especially in small specimens such as stereotactical biopsies [17]. Tumor histology can appear discordant with the observed Ki-67/MIB-1 PI. In cases with histologically anaplastic glioma tissue in which mitotic figures can be difficult to find, a high index may support the high grade diagnosis. On the other hand, a low index in a cellular lesion may indicate a reactive condition (e.g., gliosis, microglial response) rather than a neoplasm [5, 16]. If the index is elevated for a glioma with an otherwise benign histology, a more aggressive tumor may be indicated. Such a setting should not lead to a change in tumor grade but a remark in the biopsy report saying "with elevated Ki-67/MIB-1 PI, see comment" [5, 16]. In these cases one should consider step sections as well as to correlate to radiological images and clinical history [5, 16].

Ki-67/MIB-1 immunostaining to distinguish gliosis and low-grade gliomas should be interpreted with caution [5]. Normally, reactive astrocytes do not exhibit proliferative activity, but in some non-neoplastic conditions reactive astrocytes may have a proliferation rate of 1-5% [18]. In such cases, immunohistochemical analyses for mutated p53 and isocitrate dehydrogenase (IDH) proteins can be useful, though p53 immunoreactivity may occur in both settings, and there are gliomas without IDH mutation [19-21].

The procedure for Ki-67/MIB-1 immunostaining is not standardized and has various analytical and clinical elements of uncertainty [7]. Nevertheless, the method is regarded as being robust [8, 9], which is also in accordance with our experience during several years with both clinical and experimental use [14, 22, 23]. The recommended fixative is buffered formalin, and storage time, delay in fixation and fixation time does not seem to substantially affect the staining results [8, 24, 25]. Loss of immunoreactivity has been described if cut sections are exposed to room air for some months [8]. A prerequisite for satisfactory immunostaining is adequate antigen retrieval [24-26]. Various antibodies against the Ki-67 antigen are commercially available, but MIB-1 is the predominant antibody [22, 27]. Counting procedures vary across studies. Usually counting is performed in areas with the highest immunoreactivity ("hot spots"), and approximately 1000 cells are counted using the 40× objective. The PI is calculated as the percentage of labeled tumor cell nuclei to the total number of tumor cells [5, 9]. As the expression of the Ki-67 antigen changes during the cell cycle [28], the intensity of nuclear staining will vary; principally, all types of staining should be regarded as positive [8, 9]. Counting can be done manually or by digitalized image analysis systems, but manual counting has turned out to be applicable for most diagnostic purposes [5]. Defining a cut-off value is also a topic of interest due to its impact on the determination of patients classified as "high Ki-67", which is indicative of a poorer outcome. Generally, these patients will receive more aggressive treatment. However, the definition of threshold value is not straightforward mostly due to inter-/intra-observer variability and counting procedures. Accordingly, extrapolating values from other laboratories can be deceptive; thus, Ki-67/MIB-1 immunostaining should be interpreted in the context of one's own practice [5]. Each pathology department should regularly adjust its Ki-67/MIB-1 PIs by tumor grade and survival and develop its own in-house policy. Such a work-up will constitute an important part of a department's quality assurance and accreditation programs [29]. For astrocytomas, a cut-off of approximately 10% has appeared clinically feasible [6, 16]. However, the predictive value of Ki-67/MIB-1 is ambiguous [7, 30].

This study also has limitations inherent to the Ki-67/MIB-1 immunohistochemistry, including definition of immunoreactive tumor cell nuclei, sampling error and counting procedures. In addition, no statistical analysis of intra- or inter-observer variability was done. The statistics may also be influenced by the fact that not all glioma cases during the study period were immunostained.

Overall, Ki-67/MIB-1 immunostaining is a useful supplement to the histopathological diagnosis of human gliomas. However, the procedure cannot be used alone, but should be used in combination with established histopathological features of malignancy. The analytical and clinical performance of Ki-67/MIB-1 immunostaining in glioma diagnosis is not sufficiently determined. This limits its clinical utility and underlines the need for further research and standardization of procedures between laboratories [7]. To improve the diagnostics for human gliomas, a battery of proliferation markers might be considered [23]. Progress has been made in the recent years towards introducing molecular genetics in glioma diagnosis [31]. This has the potential to move us towards a more personalized medicine in the care of glioma patients.

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## Disclosure of conflict of interest

None.

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# PAPER II

**Surgical resection versus watchful waiting in low-grade gliomas.**

Jakola, A. S., Skjulsvik, A. J., Myrmel, K. S., Sjåvik, K., Unsgård, G., Torp, S. H., Aaberg, K., Berg, T., Dai, H. Y., Johnsen, K., Kloster, R., & Solheim, O. (2017). Annals of oncology: official journal of the European Society for Medical Oncology, 28(8), 1942–1948.
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ORIGINAL ARTICLE

# Surgical resection versus watchful waiting in low-grade gliomas

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**Background:** Infiltrating low-grade gliomas (LGG; WHO grade 2) typically present with seizures in young adults. LGGs grow continuously and usually transform to higher grade of malignancy, eventually causing progressive disability and premature death. The effect of up-front surgery has been controversial and the impact of molecular biology on the effect of surgery is unknown. We now present long-term results of upfront surgical resection compared with watchful waiting in light of recently established molecular markers.

**Materials and methods:** Population-based parallel cohorts were followed from two Norwegian university hospitals with different surgical treatment strategies and defined geographical catchment regions. In *region A* watchful waiting was favored while early resection was favored in *region B*. Thus, the treatment strategy in individual patients depended on their residential address. The inclusion criteria were histopathological diagnosis of supratentorial LGG from 1998 through 2009 in patients 18 years or older. Follow-up ended 1 January 2016. Making regional comparisons, the primary end-point was overall survival.

**Results:** A total of 153 patients (66 from *region A*, 87 from *region B*) were included. Early resection was carried out in 19 (29%) patients in *region A* compared with 75 (86%) patients in *region B*. Overall survival was 5.8 years (95% CI 4.5–7.2) in *region A* compared with 14.4 years (95% CI 10.4–18.5) in *region B* ($P < 0.01$). The effect of surgical strategy remained after adjustment for molecular markers ($P = 0.001$).

**Conclusion:** In parallel population-based cohorts of LGGs, early surgical resection resulted in a clinical relevant survival benefit. The effect on survival persisted after adjustment for molecular markers.

**Key words:** astrocytoma, brain neoplasm, low-grade glioma, population based, survival, treatment outcome

## Introduction

Infiltrating low-grade gliomas (LGG) are slow growing brain tumors typically presenting with seizures in young or middle-aged adults. LGGs grow continuously and usually transform to higher grades of malignancy, eventually causing progressive disability and premature death [1].

The management of LGGs has long been controversial, both with respect to surgical and oncological management and timing of treatment [1, 2]. Although case-series have reported associations between extent of surgical resection and survival, a causal relationship is impossible to establish from such uncontrolled studies [3, 4]. Due to concerns if clinical equipoise exists [1, 4–8], and need for long follow-ups [9], a randomized controlled trial comparing surgery to no surgery is not to be expected. Also, patients hesitate to enroll in randomized controlled trials with radically different options involving brain tumor surgery [10]. Our population-based parallel cohort study comparing outcomes in two Norwegian regions with

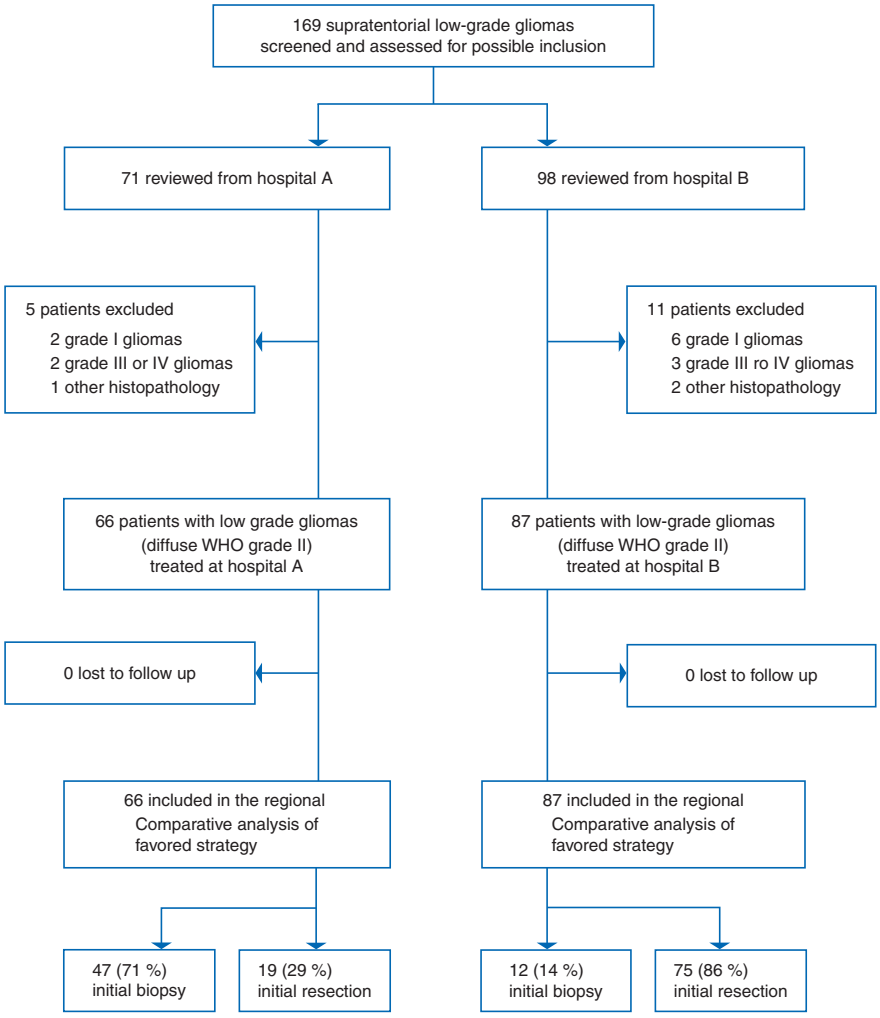


**Figure 1.** Flow chart of patient inclusion.

opposite surgical management traditions was a landmark paper in surgical management of LGG [5]. The study demonstrated a marked survival advantage in favor of early surgical resection compared with watchful waiting. Although practice changing (including in the region that used to favor watchful waiting), criticism included risk of histopathological sampling bias when comparing stereotactic biopsies to tissue samples from resection [11]. Even though the population-based setting presumably would ensure well-balanced groups from the two regions, more tumors from the region advocating early and extensive surgery had a favorable histopathological subtype (i.e. containing oligo-component) [5, 8, 9, 12]. Also, since median survival was not reached in one of the cohorts, it still remains unknown how much surgery improves survival in the longer term [8].

The recently updated WHO classification system now incorporates molecular markers in LGG classification [13]. With molecular characterization the possibility of diagnostic sampling errors is much reduced, as these are early, common events with homogenous distribution within the tumor [14]. 1p19q codeletion in combination with *IDH* mutation now define oligodendroglioma, a diagnosis that used to be associated with considerable uncertainty based on morphological classification alone [15]. Also, although *IDH* wild-type LGGs are still classified as LGGs, they frequently present a much more malignant phenotype [16, 17]. However, the impact of surgery in the recently defined molecular subgroups is still unknown [13, 16, 17].

In the present study, we now provide long-term survival data and assess molecular markers in our population-based parallel cohorts of LGG.

**Table 1. Comparisons of baseline factors and molecular markers between cohorts**

| | Region A (*n*=66) | Region B (*n*=87) | *P*-value |
|---|---|---|---|
| Age, mean (SD) | 45 (15) | 44 (16) | 0.67 |
| Gender, *n* (%) | | | 0.33 |
| Female | 25 (38) | 40 (46) | |
| Male | 41 (62) | 47 (54) | |
| KPS ≥80, *n* (%) | 51 (77) | 71 (82) | 0.55 |
| Contrast enhancement, *n* (%) | 13 (20) | 15 (17) | 0.83 |
| Histopathology, *n* (%) | | | 0.19 |
| Astrocytoma | 55 (83) | 62 (71) | |
| Oligodendroglioma | 6 (9) | 16 (19) | |
| Oligoastrocytoma | 5 (8) | 9 (10) | |
| Tumor >6 cm in diameter, *n* (%) | 19 (29) | 24 (28) | 1.00 |
| Tumor crossing midline, *n* (%) | 10 (15) | 11 (13) | 0.81 |
| Neurological deficit, *n* (%) | 17 (26) | 25 (29) | 0.72 |
| *IDH* status, *n* (%) | | | 0.46 |
| Mutated | 48/64 (75) | 56/81 (69) | |
| Wild-type | 16/64 (25) | 25/81 (31) | |
| Undetermined/missing | 2 | 6 | |
| 1p19q codeletion, *n* (%) | 23/64 (36) | 20/81 (25) | 0.14 |
| Molecular-risk group, *n* (%) | | | 0.33 |
| Low | 23 (36) | 20 (25) | |
| Intermediate | 25 (39) | 36 (44) | |
| High | 16 (25) | 25 (31) | |

Contrast enhancement indicates all types, including subtle patchy or diffuse contrast enhancement and should not be confused with only significant nodular or ring-like contrast enhancement. The molecular risk-groups are as follows: (i) low risk infers *IDH* mutated and 1p19q codeleted; (ii) intermediate risk infers *IDH* mutated and 1p19q non-codeleted; and (iii) high-risk infers *IDH* wild-type.
KPS, Karnofsky performance status; IDH, isocitrate dehydrogenase.

**Table 2. Treatment related factors in the parallel cohorts**

| | Region A (*n*=66) | Region B (*n*=87) | *P*-value |
|---|---|---|---|
| Early resection, *n* (%) | 19 (29) | 75 (86) | <0.001 |
| Number of new/repeated resections, *n* (%) | | | 0.11 |
| 0 | 42 (63) | 49 (56) | |
| 1 | 18 (27) | 24 (28) | |
| 2 | 1 (2) | 8 (9) | |
| 3 or more | 5 (8) | 6 (7) | |
| Ever resection, *n* (%) | 36 (55) | 77 (89) | <0.001 |
| Early chemotherapy, *n* (%) | 14 (21) | 18 (21) | 1.00 |
| Ever chemotherapy, *n* (%) | 44 (67) | 42 (48) | 0.32 |
| Early radiotherapy, *n* (%) | 20 (30) | 37 (43) | 0.13 |
| Ever radiotherapy, *n* (%) | 50 (76) | 57 (66) | 0.21 |
| Early radio- and chemotherapy, *n* (%) | 11 (17) | 13 (15) | 0.82 |
| Early radiotherapy and PCV, *n* (%) | 2 (3) | 8 (9) | 0.19 |

Early chemotherapy indicates treatment within 6 months following histopathological diagnosis. PCV denotes procarbazine, CCNU (lomustine) and Vincristine. Combined radio- and chemotherapy means concomitant or succeeding treatment upfront.

## Methods

### Study design and patients

In a retrospective population-based parallel cohort study, we assessed survival in patients with LGGs treated at two Norwegian university hospitals with completely different surgical treatment strategies, as described earlier [5]. The two hospitals served exclusively in defined geographical catchment regions. The hospital in *region A* favored biopsy and watchful waiting while early 3D ultrasound guided resection in general anesthesia was the preferred strategy in *region B*. Thus, the treatment strategy in individual patients highly depended on their residential address.

The inclusion criteria were histopathological verification of supratentorial LGG in adult patients (18 years or older) in the period from 1998 through 2009 using the WHO 2007 classification [18]. To minimize classification bias a blinded review was carried out where a neuropathologist from *region A* reviewed all LGGs diagnosed at *region B* and vice versa. Discordant diagnoses were settled in a consensus meeting [5, 6]. Our final sample included 153 consecutive patients (66 from *region A* and 87 from *region B*) with LGGs as seen from flow chart in Figure 1.

### Assessment of molecular markers

The assessment of molecular markers aimed at assigning patients to one of three molecular groups: (i) the low-risk group being *IDH* mutated, 1p19q codeleted, (ii) the intermediate-risk group being *IDH* mutated and 1p19q non-codeleted, and (iii) the high-risk group being *IDH* wild-type [16]. At the hospital in *region A* the 1p19q codeletion and *IDH* status were determined using multiplex ligation-dependent probe amplification (MLPA) directly since the limited amount of tissue available did not allow for step-wise integrated approach [19]. Samples classified as *IDH* wild-type after MLPA assessment were subject to PCR and DNA sequencing for *IDH1* and *IDH2* mutations. At hospital in *region B* an integrated approach was used [19], with immunohistochemistry for *IDH1* R132H and alpha thalassemia/mental retardation syndrome X-linked (ATRX) protein expression. In this initial step, if simultaneous *IDH* mutation and ATRX loss were observed no further analyses were carried out; and patients were classed as IDH mutated, 1p19q non-codeleted. Samples classified as *IDH* wild-type after immunohistochemistry were subject to PCR and DNA sequencing for *IDH1* and *IDH2* mutations. In samples with *IDH* mutation and ATRX presence, we carried out fluorescence *in situ* hybridization (FISH) to confirm the 1p19q codeletion. However, in three cases we assumed 1p19q codeletion based on IDH and ATRX presence, but without FISH confirmation since no additional tissue was available. Further details on the assessment of molecular markers are available in supplementary material, available at *Annals of Oncology* online.

### Follow-up and outcomes

All Norwegian citizens have a unique identification number making them traceable in the Norwegian population registry. With the use of this registry the patients' status (dead/alive) and date of death was verified in all patients. Follow-up ended 1 January 2016. No patients were lost to follow up with respect to the primary end-point. The primary end-point was overall survival making direct regional comparison between cohorts (i.e. analyzing strategy, not introducing selection bias).

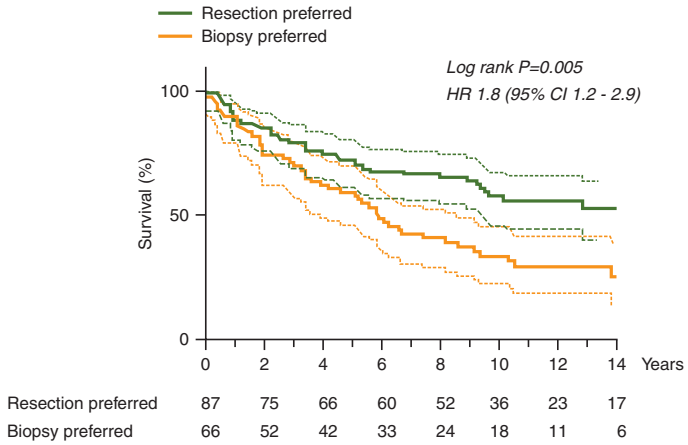


**Figure 2.** Survival analysis comparing cohorts, where *region A* preferred biopsy while *region B* preferred early resection. In *region A* the median survival was 5.8 years (95% CI 4.5–7.2) compared with 14.4 years (95% CI 10.4–18.5) in *region B*.

### Statistical analyses

For analyses, we used GraphPad Prism version 6 and SPSS version 21.0. We used Fisher's exact test for comparing results from 2× 2 tables. For other categorical data, we used the $\chi^2$ test. For continuous data, comparison of groups was carried out with independent samples *t*-test. Overall survival is presented as Kaplan–Meier plots and the log-rank test was used for between groups comparison. Cox multivariable survival analysis was carried out to adjust for important prognostic factors, including molecular markers. All tests are two-sided and statistical significance was set to $P < 0.05$.

### Statements

The Regional Committee for Medical Research in Central Norway Ethical approved the study (reference: 2014/1674). The committee waived the need for informed consent. The study is reported based on criteria from the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement [20].

## Results

### Characteristics of patients and tumors at baseline

The age adjusted incidence rate was 1.2 per 100 000 in both regions. Baseline characteristics were presented in the initial study [5], and the most important ones are presented again along with *IDH* mutation status and 1p19q codeletion status in Table 1. More detailed analyses of molecular profile in relation to clinical factors are available in supplementary Tables S1–S3 and Figures S1 and S2, available at *Annals of Oncology* online. There were neither significant differences in known prognostic factors such as age, tumor size, contrast enhancement, functional level, nor histopathological subtypes. In 64 out of 66 patients (97%) from *region A* and in 81 out of 87 patients (93%) from *region B* we had available tissue for molecular analyses. The molecular markers were not significantly different between cohorts. Interestingly, the trend toward overrepresentation of oligodendroglial tumors in *region B* as defined from the 2007 WHO classification system changed when analyzed according to 1p19q codeletion status.

### Treatment-related factors

As seen from Figure 1 and Table 2, early surgical resection was carried out in 19 patients (29%) in *region A* compared with 75 patients (86%) in *region B* ($P < 0.001$). As seen in Table 2, there were no regional differences in administration of either early or late radio- or chemotherapy, including early radiotherapy and PCV. Furthermore, the fraction of patients undergoing later surgical resections was similar between cohorts.

### Survival

Overall survival was significantly worse in *region A* advocating watchful waiting (Figure 1, $P = 0.005$) with a median survival of 5.8 years (95% CI 4.5–7.2) compared with 14.4 years (95% CI 10.4–18.5) in *region B* advocating early resections. As seen in Figure 2, adjustment for molecular factors did not alter the results. In supplementary Tables S4, available at *Annals of Oncology* online, there is an overview of 2, 5, 7 and 10 years actual and estimated survival rates in both cohorts.

As seen in Figure 3, the survival benefit of the active surgical strategy remained after adjusting for molecular-risk group ($P = 0.001$). In sensitivity analyses of survival presented in supplementary Figures S3 and S4, available at *Annals of Oncology* online we analyzed younger patients with seizures only and we adjusted for molecular-risk group in addition to a widely used clinical LGG risk-score (i.e. the Pignatti score) [12]. In sum, these additional analyses did not alter results. Adding year of treatment to the models also did not alter results (data not shown).

## Discussion

In this updated analysis of our unique population-based parallel cohorts we demonstrate that early resection is associated with a

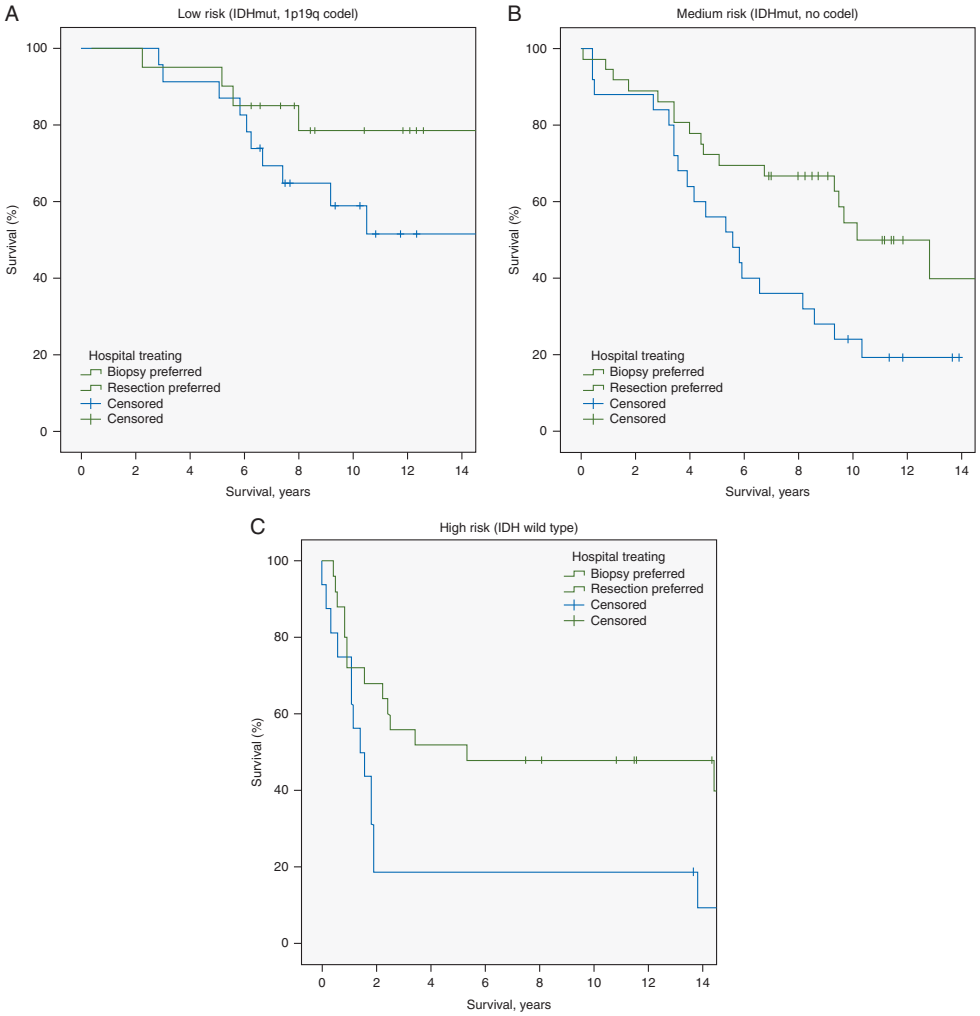


**Figure 3.** Survival in cohorts (A–C) with adjustment for molecular risk-group (log-rank test, $P = 0.001$). Results are presented stratified according to risk groups (A) low-risk (B) medium-risk and (C) high-risk group. (A) *IDH mutated,* 1p19 codeleted LGGs ($n$ =43). Median survival was not reached. (B) *IDH mutated,* non-codeleted LGGs ($n$ =61). Median survival in *region A* was 5.6 years (95% CI 3.5–7.6) compared with 10.2 year (95% CI 6.9–13.4) in *region B*. (C) *IDH wild-type* LGGs ($n$=41). Median survival in *region A* was 1.4 year (95% CI 0.6–2.2) compared with 5.3 year (95% CI 0.0–20.0) in *region B*.

clinically relevant survival benefit when compared with watchful waiting in LGGs. Cohorts were balanced at baseline and adjustment for molecular markers did not alter results. Thus, our findings are in line and strengthen the results from our previous publication [5].

A more definitive effect size assessment can better guide decision-making for physicians and patients that need to estimate the risks and benefits of surgery in a given case. However, since some patients underwent resection in *region A* and some had biopsy only in *region B*, the observed survival benefit of early surgical resection is presumably a conservative measure. The debate on the extent of surgical resection needed in order to result in a clinically relevant survival benefit is not settled. Retrospective uncontrolled studies report a clear advantage with radiological complete resection, although often not achievable [3, 4, 21]. Others have emphasized that a residual tumor volume <15 ml must be achieved to have a beneficial survival effect [22]. However, extent of resection is not random and selection bias may clearly be an issue in such studies. Unfortunately, we are not able to provide data on extents of resection in this study due to the lack of postoperative MR and lack of digitalization of images before 2005–2006 in *region B*. However, based on the observed large effect on a

population level it seems safe to conclude that aiming at early and extensive surgical resections should be considered in the vast majority of patients with suspected LGGs. Post hoc analyses also demonstrated a benefit of early surgical resection on young patients with seizures only, a patient group where some still advocate watchful waiting.

It should be acknowledged that some LGGs are not eligible for a meaningful extent of resection with an acceptable risk. However, an overall treatment strategy in favor of watchful waiting cannot be recommended in patients eligible for resection and should only be done with informed consent, and preferably within clinical trials. Also, the logic behind postponing treatment until growth is detected can be questioned since LGGs always grow, although some grow so slowly that growth is not detected with crude measures and relatively short follow-up times, while others transform without causing additional symptoms [23–25]. Finally, malignant transformation usually occurs with time but extensive surgical resection may delay this process [5].

With modern surgical tools and techniques, including intraoperative MRI [21], 3D-ultrasound guided resection (as used in *region B* in the present study) [5], and mapping techniques [1], morbidity and surgical extent of resection is perhaps more predictable than earlier. Also there was no difference in health-related quality of life in patients still alive from the two cohorts, as reported previously [7].

The fraction of patients harboring LGGs with *IDH* mutations is comparable or slightly lower than recently published clinical studies, including a large Chinese population-based study [9, 26, 27]. Thus, our population-based cohorts seems representative of the LGG reported in other clinical settings as well. In many of our *IDH* wild-type cases no further tissue was available, and consequently we did not perform additional analyses on *TERT* mutation to further differentiate between the *IDH* wild-type tumors between regions [17]. Although one should be careful of reading too much into small subgroup analyses, the exploratory subgroup analyses indicate that surgical resection is effective in all molecular subgroups.

## Limitations

The methodological concept behind our study is outlined in detail in our previous publication [5]. The main limitation of our study is the lack of randomization, but as emphasized earlier a randomized study is highly unlikely to ever be carried out. With this study there is even less clinical equipoise to this topic. The retrospective assessment is also a limitation with respect to baseline variables and nonstandardized documentation, but the primary end-point (i.e. overall survival) is robust regardless of this. As described earlier, disease-specific death was not assessed and with a long follow-up some patients may die of unrelated causes [5]. However, in Norway the difference between overall and disease-specific survival for adults with primary brain tumors does not exceed 2% during the first 15 years of observation [28]. Another criticism we faced was that the cohort from *region A* did so poorly that they could not be representative for a LGG cohort, but this speculation is refuted after molecular classification. In fact, survival in *region A* compares well to historical Surveillance, Epidemiology, and End Results (SEER) data from the years before extensive surgery was as often attempted or achieved and from other studies studying biopsies as a surgical policy [22, 29, 30].

Our population-based data from two geographical regions served by two different neurosurgical departments with highly different treatment traditions ensured comparable and balanced groups. Also, we have analyzed results conservatively with only regional comparisons to avoid introducing selection bias to our study. Diagnostic sampling bias may be an issue when comparing biopsy to surgical resection [11], but this would be unavoidable in any study comparing biopsy with resection in a histopathological defined group. After assessment of the molecular markers that are early, common events we are now reassured that tumors were comparable from a biological point of view, as expected in a population-based study [14]. Thus, the potential risk of histopathological sampling bias that may result when comparing stereotactic biopsies to tissue samples from resection is much reduced by analyzing molecular markers. However, the molecular markers were assessed differently between regions and due to the approach in region B there could be a slight underestimation of 1p19q codeleted tumors.

In conclusion, there is a considerable and sustained survival advantage associated with early resection compared with a strategy of watchful waiting in unselected patients with LGGs. The survival benefit remained after adjustment for molecular markers.

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## Disclosure

All authors have completed the *ICMJE* uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare that no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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# SUPPLEMENTARY MATERIAL PAPER II

**Surgical resection versus watchful waiting in low-grade gliomas.**

Jakola, A. S., Skjulsvik, A. J., Myrmel, K. S., Sjåvik, K., Unsgård, G., Torp, S. H., Aaberg, K., Berg, T., Dai, H. Y., Johnsen, K., Kloster, R., & Solheim, O. (2017). Annals of oncology: official journal of the European Society for Medical Oncology, 28(8), 1942–1948.
https://doi.org/10.1093/annonc/mdx230

**Surgical resection versus watchful waiting in low-grade gliomas**

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**Supplementary material**

**Molecular markers**

Assessment of molecular markers in Region A

*DNA isolation*

DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue sections using the DNA-arrow kit according to the supplier's protocol (DiaSorin). For some samples, selected tumor areas were macro dissected using a scalpel in order to increase the fraction to neoplastic cell to >50 %. We used Nanodrop to determine the DNA concentration.

*Analysis of 1p19q codeletion and IDH1/IDH2 mutation status*

The copy number status of 1p and 19q was determined using the Multiplex ligation-dependent probe amplification (MLPA) with SALSA MLPA probemix P088-C1 oligodendroglioma 1p-19q (MRC-Holland). This probe mix contains multiple probes for the 1p and 19q chromosomal arms, as well as 14 reference probes that are used for calculation of relative peak high ratios. The probe mix also contains mutation specific probes for the following 4 *IDH* mutations; *IDH1* c.395G>A p.(Arg132His), *IDH1* c.394C>T p.(Arg132Cys), *IDH2* c.515G>A p.(Arg172Lys) and *IDH2* c.515G>T p.(Arg172Met). DNA from three normal samples was included in all MLPA set-ups in order to allow for relative quantification of peak

high ratios of 1p and 19q probes. A total of 50 ng was used for each sample and all patient samples were run in duplicate.

MLPA products were separated by capillary electrophoresis on a ABI3130 genetic analyzer (Life Technology, Applied Biosystems, Foster City, CA) and analyzed using GeneMapper v. 5.0 software. The peak heights of the 1p and 19q probes were normalized to the heights of the control probes in excel, and the peak high ratios were calculated and compared to the mean peak height ratio of the 3 normal samples. Only samples showing consistent peak height ratio of <0.75 for the 1p and 19q probes were scored as codeleted. For several samples MLPA indicated partial loss of 1p or 19q, or even chromosomal gains. In line with studies assessing copy number status using assay methods, these were not scored as codeleted.[1]

The presence of *IDH1* and *IDH2* mutations was determined from the electropherograms as described in the MLPA protocol. All samples that were negative on the MLPA *IDH* probes were subjected to PCR and DNA sequencing of the relevant codons of *IDH1* and *IDH2*. Primer sequences for PCR were *IDH1* F1 (5'-CGGTCTTCAGAGAAGCCATT-3')/IDH1 R1 (5'-CACATACAAGTTGGAAATTTCTGG-3') and *IDH2* F1 (5'-TTCTGGTTGAAAGATGGCG-3') /*IDH2* R1 (5'-CAGGTCAGTGGATCCCCTC-3'). [PCR conditions: 95 °C 07:00, followed by 35 cycles of 95 °C/00:45, 60 °C/00:45, 72 °C/01:30]

Internal validation prior to study:

In lab at *Region A* the performance of the MLPA probemix P088-C1 oligodendroglioma 1p-19q (MRC-Holland) has been internally validated on a panel of 23 gliomas that also had been tested by FISH (Vysis 1p36/1q25 and 19q13/19p13 FISH probe kit) as well as by sequencing of *IDH1* and *IDH2*.

Assessment of molecular markers in Region B

Immunohistochemistry

All tumor samples were fixed in buffered formalin. Paraffin sections (3-µm-thick) were cut, mounted on Superfrost Plus glass and dried at 60°C for 60 minutes.

Immunohistochemical staining for *IDH1* R132H was done on BenchMark Ultra fully automated tissue-staining system (Ventana Medical Systems, Inc., Tucson, AZ) using validated protocols. The staining procedure included pretreatment with Cell Conditioner 1 (Ventana Medical Systems). The sections were incubated with mouse monoclonal IDH1-R132H antibody (clone H09, Dianova, 1:100) or ATRX antibody (polyclonal, Sigma Aldrich, 1:500) followed by incubation with OptiView HQ Universal Linker and OptiViewHRP Multimer antibody reagent (both Ventana Medical Systems). For signal amplification OptiView Amplification kit (Ventana Medical Systems) was used. Antigen detection was performed using OptiView DAB (Ventana Medical Systems). Tissues were counterstained with hematoxylin.

Two investigators (AJS, SHT) scored ATRX and *IDH1* independently. For *IDH1* only moderate and strong cytoplasmic staining was considered positive. A known *IDH1* R132H positive anaplastic oligodedroglioma was used as positive control. For ATRX only nuclear staining was considered for evaluation, and cases with more than 10% positive tumor cells were considered positive. Endothelial cells and neurons served as internal positive control. Heterogeneous immunoreaction was observed in some cases, but only areas with highest staining were considered.

1p19q codeletion assessed using FISH

For all cases tumor areas were pre-selected under the light microscope. FISH analysis of 1p/19q status was performed using the Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (Abbott Molecular Inc., Abbott Park, Illinois, USA) and Histology FISH Accessory Kit (Dako, Glostrup, Denmark) using a validated protocol. Briefly, 4-μm-thick formalin-fixed, paraffin-embedded sections were mounted on Superfrost Plus glasses. They were then incubated at 37°C for 60 minutes. The slides were then deparaffinized, treated with saline sodium citrate and digested in pepsin solution according to manufacturer's instructions. The probe mix (10 μl) was added to each slide. Target DNA and probes were codenatured at 73°C for 5 minutes and incubated at 37°C overnight in a humidified hybridization chamber (Dako Hybridizer or Abbott Molecular ThermoBrite™). Post-hybridization washes were performed according to manufacturer's instructions. Finally, the slides were air dried and counterstained with Fluorescence Mounting Medium with DAPI (4′,6-diamidine-2-phenylindole). Sections were viewed using a Nikon Eclipse 90i with CytoVision software version 3.7 (Applied Imaging International Ltd, Newcastle-upon-Tyne, UK). Two investigators (AJS and HYD) independently scored cases. The signal ratio was assessed individually for chromosomes 1 and 19. Target signals (red) and control signals (green) were counted in at least 100 adjacent, non-overlapping nuclei. The ratio between red and green signals was calculated, and our lab-specific cut-off is a ratio of <0.85 to conclude with deletion. Consensus was reached in all cases that had sufficient material for evaluation.

PCR and Sequencing of *IDH1* and *IDH2*

DNA was isolated using Qiagen QIAamp DNA FFPE Tissue kit. A fragment of 129bp corresponding to *IDH1* ex4 where IDH1 mutation hotspot R132 is located was PCR amplified using the primer pair: 5'-CGGTCTTCAGAGAAGCCATT-3'and 5'-GCAAAATCACATTATTGCCAAC-3'. A fragment of 236 bp corresponding to *IDH2* ex6 where the *IDH2* hotspot mutation sites R140 and R172 are located was PCR amplified using the following primer: 5´- GCTGCAGTGGGACCACTATT-3´ and 5´-GTGCCCAGGTCAGTGGAT-3´. A universal primer pair: 5´-CACGACGTTGTAAAACGAC-3´ and 5´-CAGGAAACAGCTATGACC- 3´was attached to the *IDH1* and *IDH2* primers, respectively. The PCR reaction mix consisted of 1XGeneAmp PCR Gold buffer (Applied Biosystems), MgCl2 (1.5mM), dNTP (0.4mM), 0.6uM of each primer, 1.25U AmpliTaq Gold DNA polymerase, in a total volume of 25ul. The amount of 30ng genomic DNA was applied for each PCR reaction with following program: Denaturation at 950C for 10 min followed by 40 cycles of incubation at 950C for 30 seconds, 560C for 30seconds, 720C for 30 seconds and final extension at 700C for 10 min. The PCR product was purified with illustra ExoStar kit. The sequencing reaction was performed using the universal primers and Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit. The sequencing products were analyzed on Applied Biosystems 3130 genetic analyzer.

**IDH mutated gliomas**

Since IDH wild-type low-grade gliomas may be heterogeneous tumors because of the high inter-rater problem with morphological classification this subgroup may theoretically consist of both high-grade gliomas and indolent lesions. We lacked tissue for exploring regional differences in *TERT* mutation that could further separate *IDH* wild-type patients with a fairly good prognosis from those with a dismal, glioblastoma-like prognosis.[2] However, we further explored the *IDH* mutant subgroup with respect to baseline variables and treatment as seen in table S1. Further, table S2 summarize the *IDH* mutations observed in our cohorts and table S3 explored patterns of surgical treatment in the molecular subtypes..

The IDH2 P167L mutation has previously been described in a glioblastoma,[3] while the IDH2 G145R mutation has not been described in gliomas, although it has previously been reported in early gastric cancer.[4] G145 is located close to the assumed nucleotide binding site (http://www.uniprot.org/uniprot/P48735) and to the active-site arginine residues of IDH2; R172 and R140. The mutation involves a substitution from glycine to arginine, the latter being a larger and positively charged amino acid and this is therefore likely to perturb the catalytic activity of the enzyme. P167 is located close to the binding site of the allosteric inhibitor (http://www.rcsb.org/pdb/explore/images.do?structureId=4JA8).[5] The allosteric inhibitor is itself located at the dimerization interface. A mutation affecting this region is likely to affect the catalytic activity of the enzyme, following the same mechanism of an allosteric inhibitor. In this case the mutation involves the substitution of proline, an amino acid with a known structural role, to leucine. In theory, this could affect both the structure and the flexibility of this region, leading to a change in the catalytic activity of the enzyme. In summary, both the position and the nature of the amino acids mutated are strong indicators of a role on the activity of the IDH2 enzyme, but further studies are needed which

is beyond the scope of this article and their relevance in gliomas remains unknown. Survival in the *IDH* mutated cohorts are shown in figure S1. Also, if adjusting this homogenous group of IDH mutated gliomas for the only known oncological therapy to significantly prolong overall survival, namely early combined radiotherapy and chemotherapy, the association between early resection and survival remained unaltered (data not shown, $P$=0.05).

The two novel IDH mutations were observed in LGG morphologically classified as WHO grade II astrocytomas, and 1p19q was not detected in either. Since their role in gliomas are uncertain, we consequently performed the adjusted survival analyses with coding these as both IDH mutations (as presented) and as wild-type (sensitivity analysis) and this did not influence the results (data not shown). Also, since these two patients were classified as IDH mutated, non-codeleted we here additionally present the survival analysis excluding these two patients from this specific subgroup (similar to figure 1B, but now N=59). As seen in supplemental figure 2, how we classify these two patients does not influence the clinical results in our study.

**Sensitivity analyses: Survival**

In addition to the molecular subgroups, we explore clinical relevant phenotypes in figure S3 since molecular markers are unavailable at time of surgical decision-making. In addition, in figure S4 we performed a Cox multivariable analysis adjusting for both molecular markers and a validated clinical prognostic score. Table S4 provides data on actual and estimated survival rates at 2, 5, 7 and 10 years after procedure in the cohorts.

## References


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**Supplemental figure legends**

**Supplement figure 1:** Overall survival in IDH mutated gliomas (*n*=104) according to treatment policy (i.e. regional comparison). Median survival was 7.4 years (95 % CI 4.5 to 10.3) at hospital favoring biopsies compared to 14.6 years (95 % CI 10.4 to 18.8), *P*=0.05.

**Supplementary figure 2**: Survival in IDH mutated, non-codeleted patients excluding the two patients with novel, IDH mutations with uncertain clinical relevance. Median survival in region A preferring biopsy was 5.6 years (95 % CI 3.5 to 7.6) compared to 10.2 years (95 % CI 6.7 to 13.6) in region B, *P*=0.03.

**Supplement figure 3 (A-B):** Survival in patients younger than 40 years (A) and younger than 40 years and presenting with seizure only (B). These analyses were performed to meet the difficult clinical situation deciding for surgery or not when facing a young individual that is neurological intact.

A: Median survival in cohort where biopsy was preferred was 7.4 y (95 % CI 2.8 to 12.1) compared to not reached in the cohort preferring resection (*p*=0.003, *n*=68).

B: Median survival in cohort where biopsy was preferred was 8.2 y (95 % CI 4.7 to 11.7) compared to not reached in the cohort preferring resection (*p*=0.06, *n*=42).

**Supplement figure 4:** Cox multivariable analysis adjusting for molecular status and LGG prognostic score (i.e. the Pignatti score).[6] Biopsy only as preferred initial strategy was associated with a HR for death of 2.0 (95 % 1.3 to 3.2, *P*=0.002).

**Supplement table 1:** IDH mutated gliomas in respective regions; baseline factors and treatment provided

| | *Region A* (*N*=48) | *Region B* (*N* =56) | *P*-value |
|---|---|---|---|
| Age, mean (SD) | 44 (13) | 44 (14) | 0.78 |
| Gender, *N* (%) | | | 0.10 |
| Female | 15 (31) | 27 (48) | |
| Male | 33 (69) | 29 (52) | |
| Contrast enhancement, *N* (%) | 8 (17) | 11 (20) | 0.80 |
| Histopathology*, *N* (%) | | | 0.09 |
| Astrocytoma | 38 (79) | 35 (63) | |
| Oligodendroglioma & mixed | 10 (21) | 21 (38) | |
| 1p19q codeleted, *N* (%) | 23 (48) | 20 (36) | 0.24 |
| Tumor > 6 cm in diameter, *N* (%) | 13 (27) | 17 (30) | 0.83 |
| Tumor crossing midline, *N* (%) | 6 (13) | 6 (11) | 1.00 |
| Early resection, *N* (%) | 17 (35) | 53 (95) | <0.001 |
| Ever resection, *N* (%) | 31 (65) | 54 (96) | <0.001 |
| Early radiotherapy, *N* (%) | 13 (27) | 25 (45) | 0.07 |
| Ever radiotherapy, *N* (%) | 37 (77) | 40 (71) | 0.65 |
| Early chemotherapy, *N* (%) | 9 (19) | 13 (23) | 0.64 |
| Ever chemotherapy, *N* (%) | 34 (71) | 31 (55) | 0.16 |
| Early radio- and chemotherapy, *N* (%) | 6 (13) | 11 (20) | 0.43 |

*according to WHO 2007 classification

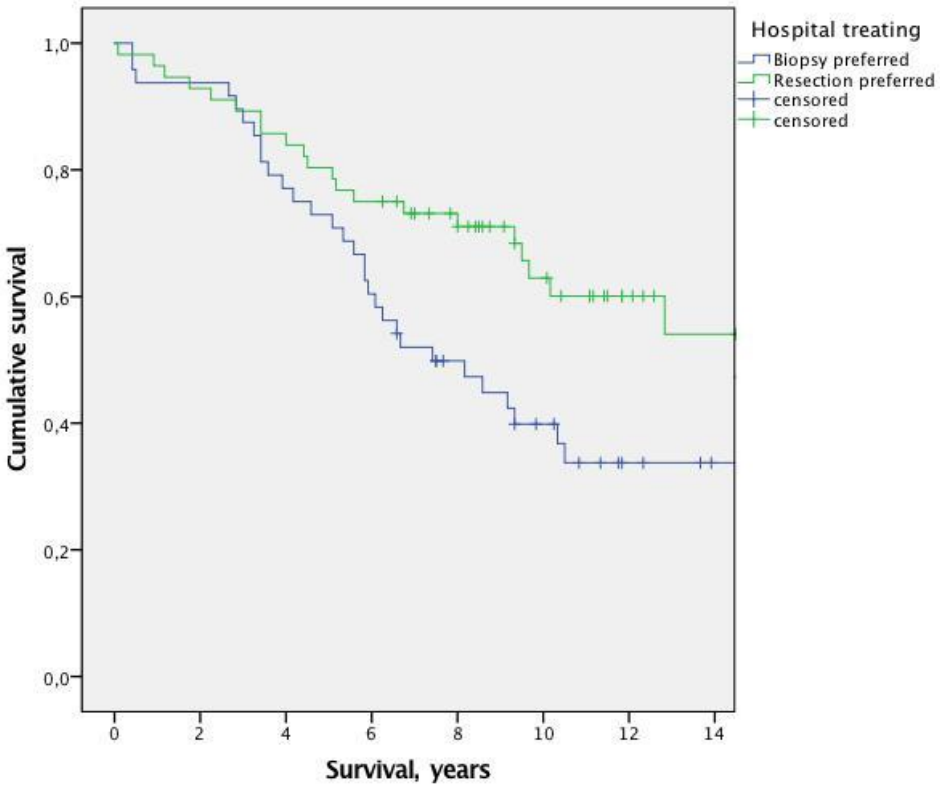
**Supplementary table 2:** IDH mutations in *region A* and *region B*.

| | Region A (*n*=64) | Region B (*n*=81) |
|---|---|---|
| IDH1 R132H | 47 | 51 |
| IDH1 R132C | 1 | 1 |
| IDH1 R132G | 0 | 1 |
| IDH2 G145R* | 0 | 1 |
| IDH2 R172M | 0 | 1 |
| IDH2 P167L* | 0 | 1 |
| IDH wild-type | 16 | 25 |

**Supplementary table 3:** Initial surgical strategy in relation to molecular risk-group

| | *Region A* (*n*=64) | | *Region B* (*n*=81) | |
|---|---|---|---|---|
| | Biopsy | Resection | Biopsy | Resection |
| IDH mut, 1p19q codel | 15 | 8 | 2 | 15 |
| IDH mut, 1p19q non-codel | 16 | 9 | 1 | 38 |
| IDH wild-type | 14 | 2 | 7 | 18 |

**Supplementary figure 1:**



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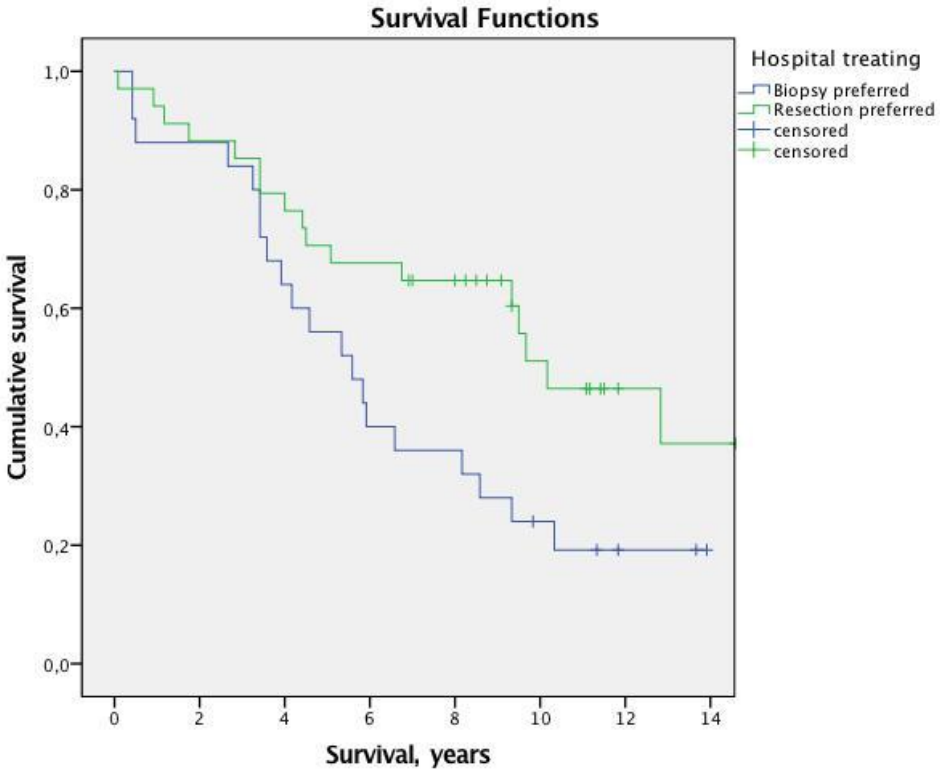
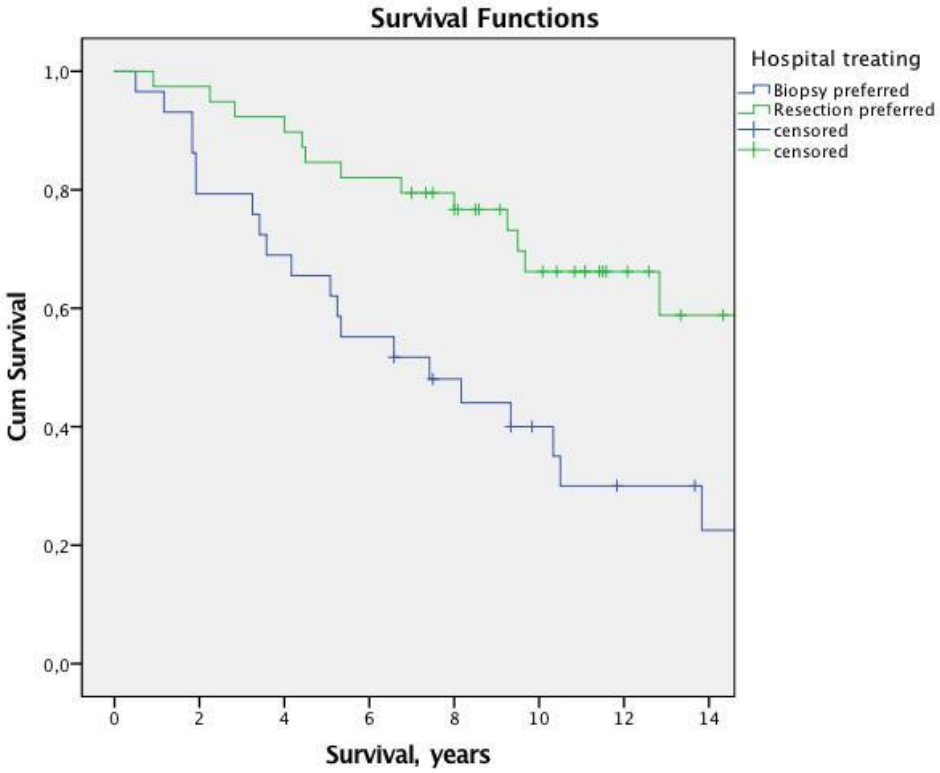
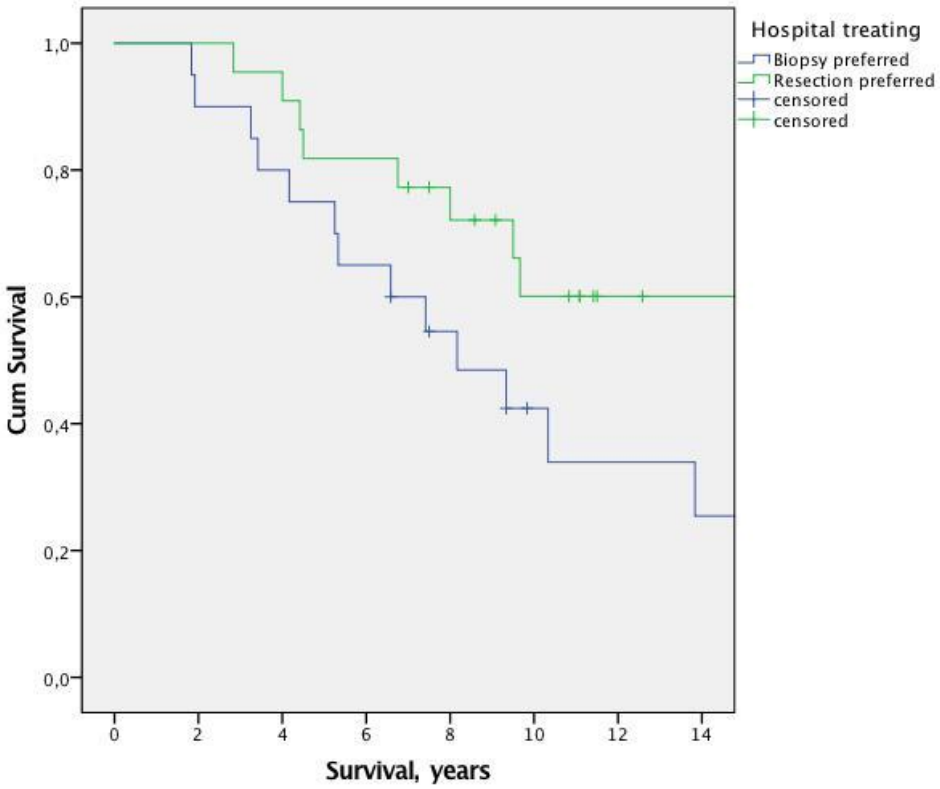


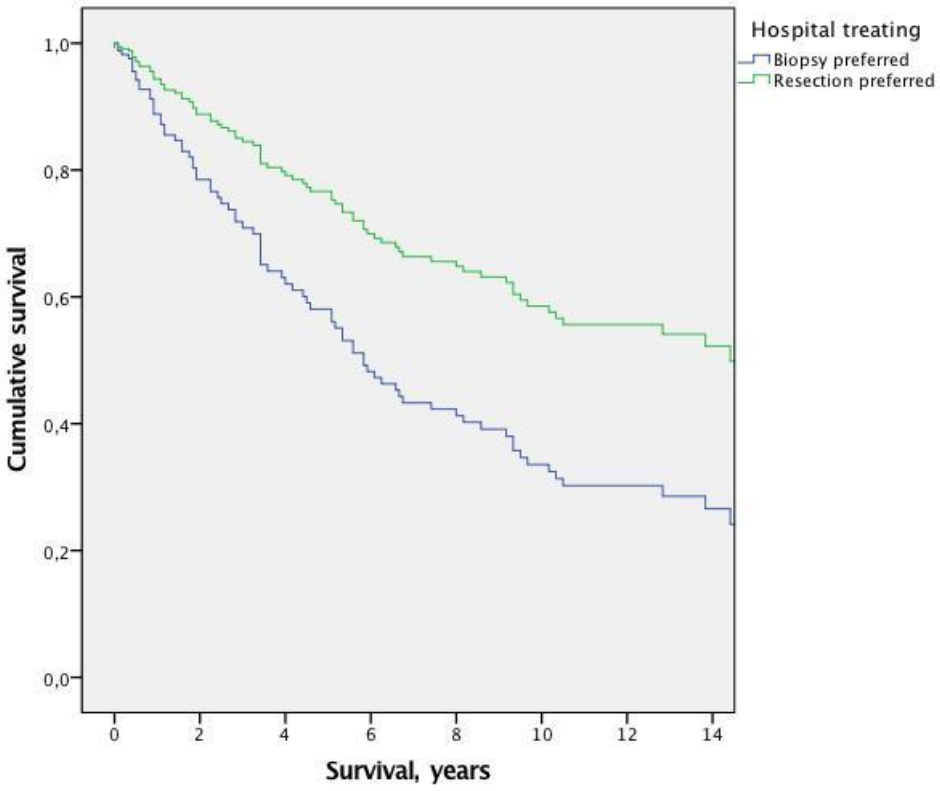
**Supplementary figure 3A:**



**Supplementary figure 3B:**



**Supplementary figure 4:**



# PAPER III

**Is the anatomical distribution of low-grade gliomas linked to regions of gliogenesis?**

Skjulsvik, A. J., Bø, H. K., Jakola, A. S., Berntsen, E. M., Bø, L. E., Reinertsen, I., Myrmel, K. S., Sjåvik, K., Åberg, K., Berg, T., Dai, H. Y., Kloster, R., Torp, S. H., & Solheim, O. (2020). Journal of neuro-oncology, 147(1), 147–157. https://doi.org/10.1007/s11060-020-03409-8