Chapter 6
Discussion; answers to questions
Four questions underlying our work were introduced in paragraph 1.3. Subsequently, answers to these questions were provided by the studies described in chapters 2 to 5. In this discussion we provide a synopsis of the answers to the four questions as well as future perspectives.

1. ARE THERE COPY NUMBER ABERRATIONS (CNAS), OTHER THAN 1P/19Q CO-DELETION, WITH PROGNOSTIC VALUE FOR PATIENTS WITH LGG? (CHAPTERS 2 AND 3)

A wide variation in survival of patients with LGG requires markers with classifying properties to tailor timing and nature of postoperative treatments. Co-deletion of 1p and 19q in LGG can select patients with a more favorable prognosis, but is insufficient to adequately tailor treatment for all patients. There is a need for additional markers that provide better distinction between the good, the bad and the ugly.

Two studies in this thesis (chapters 2 and 3) focused on the prognostic value of CNAs. Both assessed the genome in similar detail, albeit with different techniques, and two cohorts were included in both studies (with a third, confirmatory cohort in chapter 3). Yet, the results of downstream analysis of the data do not completely overlap. In the first study (chapter 2), losses at 11p and 19q are put forward to have an independent, unfavorable association with prognosis, while the second study (chapter 3) resulted in the identification of distal loss at 10q to have an unfavorable association (175;184). These apparently differential results may be partly explained by dissimilarities in the analytical approach to the data. In chapter 2 the cohort was classified into five CNA-subgroups in a supervised manner and these were associated to clinical features such as age and survival. In chapter 3 unsupervised analysis was performed; all CNAs were tested for their prognostic value. The benefit of unsupervised analysis as opposed to supervised classification of major subgroups is the opportunity to find novel markers. Survival analysis of high volume data requires statistical correction for multiple testing, however, and therefore only markers with very strong prognostic value can be identified with this approach. The use of archival material in chapter 3 enabled retrospective collection of patients with long clinical follow up and due to this wide distribution in survival distal loss of 10q could be identified with unsupervised analysis. The clinical relevance of the 1p/19q co-deletion was confirmed independent of the differential preselection and statistical approach used in the two studies. The discrepancy between the consistently identified 1p/19q co-deletion and the variable outcome for losses of 11p, isolated 19q and distal 10q may be explained by the relatively low detection rate of prognostic unfavorable CNAs. Molecular alterations associated with tumor growth are likely to reside only in more aggressive subclones of a tumor and may have been missed in the single sample analysis which was applied in these studies. 1p/19q co-deletion, however, is an early event, homogeneously present throughout all tumor cells.
The current WHO classification based on histopathology (155) provides some guidance to the neuro-oncologist towards timing of treatment as oligodendroglial components are associated with a more favorable survival compared to LGG with primarily astrocytic features (305). However, inter-observer variability of histopathological diagnosis is a complicating factor, also in distinguishing oligodendrocytic from astrocytic features (306). Molecular markers such as CNAs could be the answer to this problem, although their implementation depends on well-validated, and preferably uniform technical and statistical analysis (159;307). The upcoming revised WHO classification of diffuse glioma will include molecular markers and the value of discriminating WHO grade II from grade III glioma is reconsidered (308). Histological classification is based on the area with the highest malignancy grade, but therefore highly dependent on the quantity of provided tissue for the pathologist. While in the past anaplastic (WHO grade III) diffuse gliomas were, together with glioblastomas (WHO grade IV) considered as high-grade gliomas, more recently WHO grade II and III gliomas have been grouped as ‘lower-grade glioma’ for research purposes (309). There are several shared molecular features with comparable clinical relevance in lower grade gliomas, such as the similar association with survival of the IDH1 mutation, independent of WHO grade (310;311). Validation of the CNAs presented in this thesis in cohorts in which the traditional malignancy grades II and III are combined, could result in improved classification. In a preliminary setting, we performed survival analysis of distal loss of 10q in WHO grade III glioma in the TCGA cohort and the prognostic unfavorable value observed in grade II (chapter 3) could be confirmed (Cordes and Ylstra et al. personal communications). Detailed elucidation of tumor biology is not always required for application of a marker in daily clinical practice (exemplified by the unknown oncogenic mechanism associated with co-deletion of 1p and 19q). Still, identification of a causative gene in the distal 10q region could be of interest to design an agent specifically targeting the alteration in order to halt tumor progression. The smallest region of overlap of loss (10q25.2-10qter) covers 148 genes. PTEN (10q23.3) is a gene frequently impaired by copy number loss of 10q in higher grade glioma and has been introduced as a potential marker which stratifies the subgroups defined by 1p/19q co-deletion and mutation of IDH1 and/or PTEN (304;312;313). Combined loss of 10q and gain of chromosome 7 are frequently detected in higher-grade gliomas and has been speculated to activate mutations in major oncogenes. In the LGG cohort of 98 patients, five samples with loss of 10q including the PTEN region showed concurrent gain of whole chromosome 7. Survival in four out of five patients exceeded the median survival time of patients with LGG. Also, PTEN is located more proximal to the centromere and is not covered by the smallest region of overlap of distal 10q, as delineated in chapter 3 using strict statistical conditions. The prognostic significance of distal 10q loss was decreased
if the region was expanded since this would require exclusion of those patients with an LGG with loss of a smaller region from the survival analysis. Our data suggest that another gene located within the smaller region is involved. *MGMT* (10q26.3) is covered by the smallest region of interest of distal 10q loss and has been studied extensively in glioma-research, it nowadays considered a favorable marker and deserves attention in this context. Promoter methylation of *MGMT* results in decreased expression (314), and activity of the gene can be further compromised by heterozygous loss (202). *MGMT* promoter methylation is predictive for treatment with the alkylating agent temozolomide (TMZ) in high-grade glioma (226) and has been associated with longer survival in elderly patients with anaplastic astrocytoma, independent of treatment (315). These predictive and prognostic qualities of *MGMT* promoter methylation have not been confirmed in LGG (316;317). It seems unlikely that compromised activity of *MGMT* results in a paradox of positive predictive and negative prognostic features, and other mechanisms behind the prognostic value of distal 10q loss have to be considered in future research.

It is important to emphasize that CNAs do not provide sufficient prognostic features for all patients with LGG. In both of the presented studies the survival of a substantial number of patients could not be predicted on the basis of clinically relevant CNAs, i.e. loss of 11p, 19q or 1p/19q co-deletion (chapter 2, 35% of cases) or 10q and/or 1p/19q co-deletion (chapter 3, 42% of cases). Integration of other types of molecular alterations, such as gene mutations, translocations and epigenetic changes may help to stratify this subgroup within the wide spectrum of survival times of patients with LGG. In the time span 2013-2015, several other molecular markers have been introduced or further validated. As a result, two recent landmark papers consolidated the clinical value of *IDH* mutation, 1p/19q co-deletion and *TERT* mutation in the molecular classification of glioma, including the superiority of these markers to histological diagnosis (188;318).

One very important – and rather spectacular- conclusion of these studies is that lack of *IDH* mutation in a glioma histologically classified as an LGG should prompt the neuro-oncologist to consider treating this patient as having a glioblastoma. It is mandatory, however, to perform additional molecular tests on BRAF and mitotic index in these patients, since a small subgroup of patients with *IDH* wild type lower grade astrocytoma have a more favorable outcome and could consequently be subjected too soon to postoperative treatment with serious long-term side-effects. In contrast, H3F3A K27M mutated (apparently) lower grade glioma have a worse prognosis compared to *IDH* wild type variants without this mutation (311;318).
2. WHAT CAN WE LEARN FROM SPATIAL AND TEMPORAL ANALYSIS OF CNAS IN LGG AND COULD THESE ANALYSES PROVIDE INSIGHT INTO EVOLUTION TO A HIGHER MALIGNANCY GRADE? (CHAPTER 3)

Patients with LGG may live for extended periods of time, up to 20 years, in relatively good health until tumor progression occurs. At that time, an MRI scan shows new tumor mass or enhancement of a previously non-enhancing tumor area. These changes can be considered to be preceded by tumor progression on a cellular level. Preferably, the neuro-oncologist will anticipate this development and act as soon as this early progression at a cellular level starts. Such a strategy would require identification of markers associated with tumor progression in the surgical tissue of the initial tumor. Several studies revealed that tumor progression involves the development of subclones with distinct molecular features (149;151), referred to in this thesis as spatial and temporal heterogeneity. A subset of molecular markers is detected in all tumor cells and is therefore regarded as an early, and often clonal event, for example 1p/19q co-deletion and mutation of \textit{IDH}. They are considered the first steps in oncogenesis of glioma, and \textit{IDH} mutations only rarely disappear in a subset of the tumor cells (320).

We observed extensive spatial, and temporal heterogeneity with regard to CNAs in the study described in chapter 3. Distal loss of 10q is a putative marker for tumor progression because of the association with shorter survival combined with subclonal localization and a higher frequency in recurrent tumors. Subclones with distal loss of 10q seem to have a competitive advantage. Still, the sample size in this study was too small to provide us with solid arguments. If independent studies would support this finding, laboratory studies should analyze if the copy number loss has a direct effect on malignant progression or, in fact, only functions as a passenger alteration. A classical approach using \textit{in vitro} analysis may not be applicable in LGG. The slow-growing characteristics of this tumor type complicate culturing of the cells.

In addition to the study on LGG evolution in chapter 4, recent published investigations on the evolution of primary glioblastoma address molecular events involved in glioma recurrence (321). It is inapt to extrapolate the results directly since the molecular signatures of LGG and primary glioblastoma differ substantially, reflected by the very low incidence of the clonal \textit{IDH} mutation and 1p/19q co-deletion in the latter group. Still, it does build knowledge on glioma evolution in general. The authors primarily focused on gene mutations, but they do address amplification of \textit{MDM2} which was associated to an increased fraction of subclonal mutations in the primary tumors. These findings could not be reproduced in the cohort of 98 patients in chapter 3 since no \textit{MDM2} amplifications were observed at all.
Spatial heterogeneity implies that the presence of clinically relevant CNAs, such as distal loss of 10q, can be underestimated with current diagnostics of diffuse glioma which is based on one or a few random samples. Missing a marker could result in under- or overtreatment of the patient. Alternatively, the proportion of tumor cells with a specific marker could be related to prognosis, i.e. patients suffering from LGG with a low fraction of cells with distal loss of 10q (subclonal) might have a better prognosis than patients suffering from LGG with clonally present distal loss of 10q. The abovementioned glioblastoma-evolution study supports this hypothesis indirectly; a higher fraction of subclonal gene mutations was associated with longer event-free survival in patients aged <55 years. Not detecting an unfavorable marker due to single sample analysis may therefore be less catastrophic than intuitive reasoning implies. Obviously, it is mandatory to study an association between the fraction of subclonal molecular alterations (gene mutations as well as CNAs) and prognosis in larger cohorts. The cohort of chapter 3 was too small to assess the prognostic value of the fraction of subclonal CNAs. Suggestions on how to study clonal evolution in LGG as well as non-invasive approaches are provided in the future perspective below.

3. ARE DNA REPAIR DEFICITS INVOLVED IN TMZ-ASSOCIATED HYPERMUTATION OF LGG? (CHAPTER 4)

Postoperative treatment with the alkylating agent TMZ is beneficial for survival in glioblastoma (322). In a subset of patients treated with TMZ, hypermutation of tumor DNA is observed. The term ‘TMZ-associated hypermutation’ is applied due to the predominance of C>T/G>A mutations which can be attributed to TMZ, and the fact that this exorbitant high number of mutations is not detected in glioma not treated with TMZ (150;151;321). In glioblastoma, deficient mismatch repair has been reported to be involved in this potential resistance mechanism (323). Chapter 4 focused on LGG with astrocytic features and their recurrences (324). In this study, whole exome analysis revealed loss of heterozygosity and/or mutations of genes involved in DNA repair in five out of six recurrences with the TMZ-associated hypermutation after TMZ treatment, concomitant with an increase in the methylation level of the \textit{MGMT} promoter in all six recurrences. In a subset of the initial tumors of these recurrences DNA repair was compromised through loss of heterozygosity prior to TMZ treatment. Also, a trend was observed towards a slightly higher level of \textit{MGMT} methylation in these initial tumors compared to initial tumors without hypermutation at time of recurrence.

The results demonstrate that sequential acquisition of DNA repair deficits occurs. The exact order of events remains elusive, since it is unclear if alterations occur before or
after TMZ administration between surgeries of the initial and recurrent tumor. The findings in chapter 4 incite the following hypothesis: first, DNA repair is compromised in initial tumors by heterozygous deletion of *MGMT* and/or an MMR gene and a high level of *MGMT* methylation. After surgery of the initial tumor and prior to TMZ treatment, continued positive selection of *MGMT* hypermethylated cells and a corresponding decrease in *MGMT* expression further predispose a tumor cell to persistent O6-methylguanine lesions and acquisition of MMR gene mutation(s). These alterations enable hypermutation in the tumor caused by subsequent rounds of TMZ treatment. A large cohort of initial and recurrent tumors is required to confirm this theory and possibly identify markers to select LGG with a propensity to hypermutation (see future perspective).

The previously mentioned glioblastoma-evolution study also provides food for thought on this topic, since the authors’ report findings contradictory to our results in chapter 4 (321). Spatial analysis of a single recurrent tumor of a primary glioblastoma with a TMZ-associated hypermutation profile revealed that mutations in MMR genes were present in a subclonal fashion, and the authors suggest that not DNA repair, but mutations in receptor tyrosine kinases are key elements in hypermutation. However, *MGMT* methylation was assessed using a binary outcome, as opposed to quantitative, more differentiated outcome we used in chapter 4, and three out of five recurrent tumors ‘lost’ their *MGMT* methylation status.

Chapter 4 also encourages evaluation of the clinical relevance of TMZ-associated hypermutation. In the present study, recurrences with a TMZ-associated hypermutation phenotype were all histologically classified as secondary glioblastoma. Combined with their highly disrupted genomic code, an unfavorable outcome for the patient is not unthinkable. Survival and the number of administrated TMZ cycles were variable in these six patients and can therefore not be compared to patients without the TMZ-associated hypermutator phenotype. Hypothetically, TMZ-associated hypermutation may actually even prove to be beneficial instead of disadvantageous for patients with glioma in terms of subsequent treatment. Metastatic melanoma and non-small cell lung carcinoma are characterized by a high mutation level, associated with environmental factors (325). In diffuse glioma, TMZ could be considered such an ‘external factor’. Mutation rate and immunotherapy efficiency seem to be correlated. This favorable correlation may be explained by concurrent, somatic mutations in DNA repair and increased proliferation resulting in a higher production of so called neoantigens in tumor cells. The immune system would be naïve and therefore activated to such neoantigens, encoded by mutant DNA, and hence display a higher anti-tumor activity.
4. **CAN SHALLOW WHOLE GENOME SEQUENCING FOR CNAS IN VARIOUS GLIONEURONAL TUMORS (GNT) HELP TO ESTABLISH A LESS OBSERVER-DEPENDENT CLASSIFICATION? (CHAPTER 5)**

In chapter 5, 114 GNT were analyzed using shallow whole genome sequencing. The aim of this study was to contribute to a new classification which reduces the inter-observer variability related to histopathological diagnosis. GNT includes several subgroups with a wide range of morphologic characteristics (279). Only the two most frequent histological subgroups of GNT were selected; ganglioglioma and dysembryoplastic neuroepithelial tumors. Samples were revised according to WHO 2007 classification. As expected, there was still substantial variability in morphology and immunoreactivity scores within these two subgroups. The presented copy number landscape of ganglioglioma and dysembryoplastic neuroepithelial tumors did not result in a classification superior to histopathological analysis, but did shed further light on molecular characteristics of these tumors. Recurrent gain of chromosome 5 and/or 7 suggests that there is a shared genomic origin for ganglioglioma and dysembryoplastic neuroepithelial tumors, supported by the discriminative value of gain of whole chromosome 5 present in diffuse dysembryoplastic tumors and absence of this marker in a cohort of WHO grade II diffuse astrocytoma.

Chapter 5 provides directions for future research in this subtype of glioneuronal neoplasms. First of all, most GNT are indolent tumors consisting of a mixture of neoplastic and inflammatory cells. A low proportion of neoplastic cells was observed in GNT samples complicating detection of somatic alterations, as shallow genome-wide sequencing requires a tumor cell percentage (as estimated by the pathologist) of at least 60%. Indeed, a substantial number of samples in our study did not show any CNAs, also confirmed by validation using array comparative genomic hybridization and FISH. Shallow whole genome sequencing is not the optimal technique to study CNAs in GNT. To pursue a study focused on CNAs, a solution would be deep sequencing (326). This technique enables detection of molecular markers present in a smaller subset of the cells, an approach that becomes increasingly feasible now that the costs of such analyses are going down.

Shallow whole genome sequencing, however, may be beneficial to identify spatial heterogeneity in GNT. Similar to the LGG cohort of chapter 3, variability was observed in copy number levels within individual chromosomal copy number profiles. This suggestion of molecular heterogeneity in spatially distinct regions may add another layer to the complex architecture of GNT of neoplastic cells intermingled with non-neoplastic components. Clonal and subclonal markers could be indicative of initiating
events and consequent evolution of GNT. To identify the few low-grade GNT which do recur after initial surgery, however, a cohort of initial and recurrent tumors should be collected for comprehensive genomic analysis. This future study could be troubled by the very low incidence of these tumors, a problem which can be solved by an effort to organize a large, global consortium. Alternatively, markers present in malignant GNT can be tested in low-grade GNT. A small cohort of initial malignant GNT and their matched recurrent tumors is currently under investigation in our laboratory.

CNAs are obviously not the only molecular alterations in GNT. In addition to the frequent BRAFV600e mutation (327), other studies have revealed that alterations of genes involved in epigenetic regulation are particularly prevalent in pediatric brain tumors compared to adult gliomas. The number of low-grade GNT included in these studies, however, was marginal (328;329), while the study in chapter 5 was mainly focused on the question whether information on copy number landscape of GNTs might aid in reducing inter-observer variability in classification.

New questions and future perspective

The studies presented in chapters 2 to 5 contribute to the unraveling of the complex molecular landscape of low-grade glial neoplasms. Despite the consistent and strong prognostic impact of the 1p/19q co-deletion, more specific stratification is warranted for patients with LGG. Such new markers will eventually contribute to a decision tree for clinical decision making in a multidisciplinary counsel. In this decision tree histopathological and molecular features shall be combined with clinical information (205), and preferably also applies when parameters of an opposite association concur in the same patient. Examples of such simultaneously occurring parameters are diagnosis of a low-grade oligodendroglial tumor at an older age, or co-occurrence of distal loss of 10q and 1p/19q co-deletion.

When constructing such a decision tree, the main task will be to find a balance between comprehensiveness and practicality. As a first step, regression analysis might help in selecting the most relevant parameters. In the next paragraph, a conceptual study design is presented which aims to further refine the current state-of-the-art stratification by 1p/19q co-deletion, and mutations of IDH, ATRX and TERT (313;330). This can be enforced through the implementation of additional molecular markers such as distal 10q loss, knowledge on spatial and temporal evolution, and eventually information on predisposition to TMZ-associated hypermutation.

The clinical value of promising molecular markers can be tested in archival material of existing cohorts under the condition that the applied techniques have been validated for formalin-fixed paraffin-embedded (FFPE) material (331). For example, the prognostic
significance of distal loss of 10q could be tested in the cohort of the EORTC study 22033-26033 (332). This trial compared postoperative irradiation to TMZ treatment in patients with LGG at risk for tumor progression.

Meanwhile, prospective collection of data and specimens provides an opportunity to systematically study clonal evolution and apply techniques that are less suitable for FFPE materials. Such a prospective study will meet several challenges. The first challenge will be to reach adequate patient numbers and events. Prospective trials in patients with LGG are by nature long lasting due to the relatively long median survival. The minimal duration of follow up described in the study of chapter 3 was set at 6 years, the estimated median survival of patients with LGG. A balance will have to be found between practical consequences and long follow-up to optimize clinical value. The low incidence and the lack of standardized postoperative treatment are additional complicating factors. International collaboration of expert neuro-oncology centers is pivotal to reach adequate patient numbers, and comparison of treatment strategies.

Recently, a European network of expertise centers for the diagnosis and treatment of LGG, including the VUmc Brain Tumor Center Amsterdam, has been established. It may be expected that this network will facilitate future studies.

The second challenge will be to achieve representative molecular information on the tumor. In order to evaluate subclonal evolution, ideally multiple samples should be obtained during each surgery, i.e. at initial diagnosis and of recurrent tumors. The minimal number of samples required to model a tumor’s architecture will vary, but four to six, equally distanced samples per surgery is common in tumor evolution studies presented in literature to ensure a fairly representative overview of molecular variability (figure 1) (333). MRI guidance would enable documentation of anatomical location as well as follow up of tumor growth in a specific direction, e.g. highly malignant subclones have a tendency to migrate to the contralateral side of the brain. Many cancer studies use fresh frozen tumor samples as well as a matched blood sample to enable identification of germline alterations. The technique applied in chapter 2 illustrates that somatic copy number aberrations can be identified using tumor samples only (154). Once a substantial cohort has been built and followed, (epi)genomic data can be generated and analyzed using state of the art techniques. In a situation where whole genome or -exome sequencing would be too costly, a panel of markers can be tested in these samples (334). An alternative approach to assess subclonal features of tumors is deep sequencing of ‘liquid biopsies’, i.e. circulating free tumor DNA derived from platelets and exosomes (335).

Superiority with regard to prognostic significance between candidate markers can be evaluated and will finally result in a diagnostically applicable set which, together with clinical and radiological features, can then be implemented in the LGG-decision tree.
Several hypotheses inspired by this thesis can be tested using such a valuable dataset, suggestions are provided in box 1.

**BOX 1: Questions for future research**

**Tumor evolution:**
- Does the degree of genomic divergence between spatially distinct samples correlate to prognosis?
- Does the degree of genomic divergence between spatially distinct samples correlate to their physical distance?
- Is the proportion of tumor cells affected by a prognostic marker associated with prognosis?

**TMZ-associated hypermutation phenotype:**
- What is the incidence of TMZ-associated hypermutation in TMZ treated recurrent tumors of LGG?
- Does compromised DNA repair prior to TMZ administration predispose LGG to TMZ-associated hypermutation?
- Are all recurrent tumors with the TMZ-associated hypermutation phenotype histologically diagnosed as secondary glioblastoma.
- Is there a significant difference in (progression free and overall) survival in patients with and without the TMZ-associated hypermutation phenotype.

**Decision tree**
- Is the prognosis of a patient with an LGG that shows concurrent distal loss of 10q and 1p/19q co-deletion worse compared to 1p/19q codeletion without distal loss of 10q?
- Does loss of 11p trump astrocytic histological features as a marker for an unfavorable prognosis?

**Fig. 2** Schematic representation of sample acquisition from tumor to study clonal evolution
To conclude this thesis: four years of research on low-grade glial neoplasms have 1. resulted in putative prognostic markers for patients with LGG, 2. provided insight into LGG evolution, and 3. revealed part of the mechanism behind TMZ associated hypermutation in LGG. Furthermore, this research has 4. contributed to insight into the molecular landscape of GNT. Combined with study results of other research groups, and the establishment of accessible databases such as The Cancer Genome Atlas (TCGA), the behavior of diffuse glioma is getting more predictable. At the time of LGG diagnosis, 1p/19q co-deletion and \textit{IDH1} mutation already provide prognostic information. The addition of new molecular markers will refine classification. However, an excess of markers with comparable clinical relevance could hamper application in daily practice. This may be prevented by continuous evaluation, underlining the importance of keeping genomic data that are generated accessible for the research community through public databases, as we have done with the datasets produced in this thesis (336). Low incidence of LGG and GNT, and the wide variation in survival of patients with LGG remain obstacles for the design and execution of studies aimed at improving the management of patients suffering from these neoplasms. Endurance as well as creativity are required from clinicians, molecular biologists and bio-informaticians to make further progress in this field. The work presented here was only possible because of close interaction between the disciplines just mentioned. Only if we continue to ask questions we can adjust timing and type of therapies and shed light into the life of patients with LGG.