# Prognostic significance of genome-wide DNA methylation profiles within the randomised, phase 3, EORTC CATNON trial on non-1p/19q deleted anaplastic glioma

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Abstract

Background.

Survival in patients with *IDH1/2* mutant (*mt*) anaplastic astrocytomas is highly variable. We have used the prospective phase 3 CATNON trial to identify molecular factors related to outcome in *IDH1/2mt* anaplastic astrocytoma patients.

Methods.

The CATNON trial randomized 751 adult patients with newly diagnosed 1p/19q non-codeleted anaplastic glioma to 59.4 Gy radiotherapy +/- concurrent and/or adjuvant temozolomide. The presence of necrosis and/or microvascular proliferation was scored at central pathology review. Infinium MethylationEPIC BeadChip arrays were used for genome-wide DNA methylation analysis and the determination of copy number variations (CNV). Two DNA methylation-based tumour classifiers were used for risk stratification. Next-generation sequencing (NGS) was performed using one of two glioma-tailored NGS panels. The primary endpoint was overall survival measured from date of randomization.

Results.

Full analysis (genome-wide DNA methylation and NGS) was successfully performed on 654 tumours. Of these, 432 tumours were *IDH1/2mt* anaplastic astrocytomas. Both epigenetic classifiers identified poor prognosis patients that partially overlapped. A predictive prognostic Cox proportional hazards model identified that independent prognostic factors for *IDH1/2mt* anaplastic astrocytoma patients included; age, mini-mental state examination score, treatment with concurrent and/or adjuvant temozolomide, the epigenetic classifiers, *PDGFRA* amplification, *CDKN2A/B* homozygous deletion, *PI3K* mutations and total CNV load. Independent recursive partitioning analysis highlights the importance of these factors for patient prognostication.

Conclusion.

Both clinical and molecular factors identify *IDH1/2mt* anaplastic astrocytoma patients with worse outcome. These results will further refine the current WHO criteria for glioma classification

Keywords

IDH mutant; anaplastic glioma; 1p/19q non-codeleted; DNA methylation profiling; patient prognostication.

Key Points

1. DNA methylation profiles have prognostic relevance for *IDH1/2mt* anaplastic astrocytoma patients.
2. Integration of clinical data, DNA methylation profiles, and genetic data is needed for accurate prognostication of *IDH1/2mt* anaplastic astrocytoma patients.

Importance of the Study

Grading of diffuse *IDH1/2mt* glioma is still done by classical morphology, apart from homozygous deletion of *CDKN2A/B* that is correlated with a poor outcome and which is proposed by the cIMPACT-NOW committee as a criterion for *IDH1/2mt* astrocytoma, grade 4. Identifying other molecular factors associated with outcome within tumour entities potentially allows for more precise prognostication of histologically similar tumours. We used the randomized CATNON trial to test the prognostic significance of several alterations within a homogeneously treated group of patients diagnosed with *IDH1/2mt* anaplastic astrocytoma. Tumour related factors associated with an inferior outcome were identified by two different DNA methylation classifiers, as well as homozygous deletion of *CDKN2A/B*, *PDGFRA* amplification, *PI3K* mutations and total CNV load. In contrast to histological indicators of high-grade, these molecular markers were significant in multivariable analysis and therefore can be used to identify patients with poor prognosis.

Introduction

The diagnosis of gliomas has shifted from histology alone towards the incorporation of molecular data in the revised World Health Organization (WHO) 2016 classification of brain tumours, initially with isocitrate dehydrogenase 1 and 2 (*IDH1/2*) mutational status and the presence or absence of combined loss of the 1p and 19q chromosomal arms (1p/19q codeletion). This classification was found to be more objective, reproducible, and correlated better with outcome compared to classical histology.[1-3] Still, even within the well-defined molecular subsets of glioma, significant and clinically relevant differences in outcome exist. Homozygous deletion of cyclin-dependent kinase inhibitor 2 A and B (*CDKN2A/B*) has recently been identified in *IDH1/2* mutant (*mt*) astrocytoma as a first molecular marker of grade 4 clinical behaviour.[4-6] Numerous other genetic markers have been proposed to further prognosticate *IDH1/2mt* astrocytoma patients, in particular retinoblastoma 1 (*RB1*) pathway alterations, tyrosine-protein kinase Met (*MET*) amplification (*amp*), N-myc proto-oncogene (*MYCN*) amplification, platelet derived growth factor receptor alpha (*PDGFRA*) amplification, phospoinositide-3-kinase (*PI3K*) mutations and total copy number variation (CNV) load.[4-10] At the epigenetic level, tumour specific genome-wide cytosine-phosphate-guanine (CpG) methylation patterns have been identified in diffuse glioma. Two classification systems have been reported that make use of the epigenetic profile to diagnose brain tumour subtypes: 1. a DNA methylation-based classification of all central nervous system (CNS) tumours by Capper et al. (named here: 'the CNS tumour classifier'), and 2. a DNA methylation-based classification of gliomas by Ceccarelli et al. which can be supplemented with a risk to progression stratification from glioma CpG island methylator phenotype (G-CIMP)-high to G-CIMP-low as reported by de Souza et al. (combined, named here: 'the glioma classifier').[11-14] The accuracy of these molecular characteristics in relation to outcome has thus far not been clinically validated in homogeneously treated cohorts of patients. We therefore aimed to test and evaluate the importance of reported prognostic genetic and epigenetic molecular markers within the CATNON trial, the largest prospective study conducted on anaplastic astrocytoma patients.[15] These validated subtypes can be used for prognostication and results can be considered for the grading of tumours of the CNS.

## Methods

### Study design and participants

The CATNON trial is a 2x2 factorial design, non-blinded, multicentre (n=137), randomised clinical trial in adult patients (n=751) with primary 1p/19q non-codeleted anaplastic gliomas conducted by the European Organisation for Research and Treatment of Cancer (EORTC).[15] After local diagnosis of an anaplastic glioma, tumour samples were submitted prior to randomization for central pathology review of the tumour grade, and for central testing of $O^6$-methylguanine DNA methyltransferase (*MGMT*) promoter methylation status. Absence of combined 1p/19q loss was either determined by local testing or at central review. Patients were computer-generated randomised (1:1:1:1) to radiotherapy alone (59.4 Gy in 33 fractions of 1.8 Gy), radiotherapy with concurrent temozolomide (75 mg/m$^2$ daily, max 7 weeks), radiotherapy with adjuvant temozolomide (12 4-week cycles: 150-200 mg/m$^2$ on day 1-5), or radiotherapy with both concurrent and adjuvant temozolomide.

### Compliance with ethical standards

All institutions obtained ethics approval from their institutional review boards or ethics review committees before enrolment started. All patients gave written informed consent according to local, national, and international guidelines.

### Procedures

DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumour samples as described previously.[16] *IDH1/2*, B-Raf proto-oncogene serine/threonine-protein kinase (*BRAF*), H3.3 histone A (*H3F3A*), *CDKN2A/B*, *RB1*, *PI3K* subunit alpha (*PIK3CA*), and *PI3K* regulatory subunit 1 (*PIK3R1*) mutation status were determined from either one of two glioma-tailored NGS panels as described previously.[17,18] DNA methylation profiling was performed with the Infinium MethylationEPIC BeadChip according to the manufacturer's instructions after using the Infinium FFPE DNA Restoration Kit. CNV data (*CDKN2A/B* homozygous deletion (HD), *RB1* HD, cyclin-dependent kinase 4 (*CDK4*) *amp*, cyclin-dependent kinase 6 (*CDK6*) *amp*, *METamp*, *MYCNamp*, *PDGFRAamp*) were derived from the DNA methylation data. *MGMT* promoter status was assessed with the MGMT-STP27 algorithm.[19] Samples that did not pass quality control, i.e. the signal detection p value was < 0.01 in more than 5% of the probes were excluded from further analysis. Homozygous deletions and amplifications were defined as ±0.60 log2 intensity differences. CNV differences between ±0.60 and ±0.30 log2 intensity were visually inspected for CNV calling, blinded to all other molecular or clinical data. DNA

methylation-based epigenetic subtyping was established using random forest modelling using a classifier described by Capper et al., and one classifier described by Ceccarelli et al. [11,12]. The latter classifier was supplemented with the risk of progression from G-CIMP-high to G-CIMP-low based on beta-values of seven specific CpGs as described previously (named here: 'risk tumours' and 'no-risk tumours').[13] Total CNV load was calculated as described previously with a threshold for loss and gain of ±0.10 log2 intensity on the CNV plot and a cut-off of 350 Mb.[20] Two dedicated neuropathologists scored the presence or absence of necrosis and microvascular proliferation at the central pathology review (JMK: European and Australian samples, KA: North-American samples). Clinical data such as survival, sex, age at enrolment, use of corticosteroids at enrolment (no use vs stable/decreasing dose), type of surgery (biopsy vs resection), mini-mental state examination score (MMSE) at enrolment (<27 vs 27-30) and treatment regimen were collected from the study entry forms.

Statistical analysis

The primary endpoint for all correlations of the epigenetic subtypes, DNA mutations and CNV was overall survival (OS), measured from the date of randomisation until the date of death or censored at the date of last follow-up. Survival curves were created using the Kaplan-Meier technique and compared with the log-rank test. The Cox regression model was used for univariable and multivariable analysis to determine hazard ratios (HR) with 95% confidence intervals (CI), and significance was assessed with the likelihood ratio test. Factors with likelihood ratio test p values ≤ 0.10 and at least two subgroups with n > five were taken into consideration for multivariable analysis. Principal component analysis (PCA) was performed on the 20,000 most variable probes between samples. The t-distributed stochastic neighbour embedding (t-SNE) plot was created with all significant probes at detection p value < 0.01 except those with lower overall reliability as previously described by Zhou et al. [21]. The prognostic prediction models were made using stepwise backward elimination. Schoenfeld's test was utilized to assess proportional hazard (PH) assumptions of Cox PH models. Harrell's concordance index (C-index) was calculated and bootstrapped by 1,000 iterations for the final models. Recursive partitioning analysis was performed on the significant factors from univariable analysis with 10 cross-validations in which every leaf had n > 20 patients. For all non-specified analyses, p values below 0.05 were considered significant. Statistical analysis was performed using R version 3.6.3 and packages minfi, stats, rms, survival, Rtsne, and rpart. This trial is registered with ClinicalTrials.gov, number NCT00626990. Upon completion of all ongoing analyses, all data will be made available upon request at EORTC headquarters.

Results

Cohort description

Comparison of the patient cohorts with and without molecular data identified that those able to be analysed were younger (median age: 41 years vs 47 years, p=0.006) and more likely to have had debulking surgery (82.6% vs 58.8%, p<0.0001), but all other factors were similar (*Sup. Table 1*). Genome-wide DNA methylation analysis was successfully performed on 654 samples of the 751 patients (19 samples were of low quality, and for 78 patients there was insufficient tumour tissue available, *Sup. Figure 1* and *Sup. Figure 2*). Of these, 440 (67.3%) were *IDH1/2mt*, 204 (31.2%) were *IDH1/2* wildtype (*wt*) and for ten the *IDH1/2* status could not be determined (*Figure 1A*, *Figure 1B*, and *Sup. Figure 1*). Copy number assessment identified eight (1.2%) *IDH1/2mt* anaplastic oligodendroglioma leaving 432 *IDH1/2mt* anaplastic astrocytomas. Ten *IDH1/2wt* tumours had an *H3F3A K27M* mutation and four had an *H3F3A G34R* mutation. Three *IDH1/2wt* tumours had a *BRAF* mutation (two *V600E* and one *R509Q*) and one *IDH1/2mt* tumour had a *BRAF* mutation (*K601E*). Central histology review identified necrosis and/or microvascular proliferation, consistent with grade 4 tumours, in 95 (14.5%) samples (38 *IDH1/2wt*, 57 *IDH1/2mt*; in 40 necrosis, and in 79 microvascular proliferation; *Figure 2F* and *Sup. Figure 3A*). PCA of the DNA methylation data primarily showed division between *IDH1/2wt* and *IDH1/2mt* gliomas in the first primary component accounting for 51% of variance in all samples (*Figure 1B*). Clinical characteristics of the cohort are listed in *Table 1*.

At the time of database lock (May 7, 2019), 31 *IDH1/2wt* anaplastic astrocytoma patients (15.2%), 288 *IDH1/2mt* anaplastic astrocytoma patients (66.7%), and eight *IDH1/2mt* anaplastic oligodendroglioma patients (100%) were still alive. Median OS was 1.7 years (95% CI 1.4-1.9) for *IDH1/2wt* anaplastic astrocytoma patients and 8.2 years (95% CI 7.1-not reached) for *IDH1/2mt* anaplastic astrocytoma patients (HR 6.95, 95% CI 5.51-8.78; p<0.0001).

DNA methylation analysis of all samples

The majority of samples were assigned by 'the CNS tumour classifier' to lower-grade (WHO 2 or 3) *IDH1/2mt* astrocytomas (A_IDH, n=317) or high-grade (WHO 3 or 4) *IDH1/2mt* astrocytomas (A_IDH_HG, n=113). Other diagnoses according to 'the CNS tumour classifier' were methylation class family *IDH1/2wt* glioblastomas (MTCF_GBM, n=161), *H3F3Amt G34R* gliomas (GBM_G34, n=5), *H3F3Amt K27M* gliomas (DMG_K27, n=11), 1p/19q codeleted gliomas (O_IDH, n=12), and 35 tumours with other, more rare diagnoses.

Of note, not all classifications were considered equally reliable: in 74 samples the main class calibrated score was below 0.836, which has been described as the lower bound for an optimal sensitivity and specificity.[12] When only samples meeting this calibration score were taken into account, the classifications correlated even better with molecular features. For example, all four DMG_K27 tumours with a reliability score below 0.836 did not show an *H3F3A K27M* mutation in the NGS analysis, whereas the mutation was confirmed in all seven tumours with a calibrated score ≥ 0.836 (*Figure 1D*).

The different epigenetic subtypes are highly correlated with patient survival: median OS in patients with A_IDH not reached (95% CI 8.0-not reached), with A_IDH_HG 5.6 years (95% CI 4.0-not reached), with DMG_K27 3.3 years (95% CI 2.3-not reached), with GBM_G34 1.4 years (95% CI 1.0-not reached), with MTCF_GBM 1.4 years (95% CI 1.3-1.8), with O_IDH 9.1 years (too few events to estimate the CI), and with the combination of other diagnoses 2.4 years (95% CI 1.8-5.5; *Figure 2A and 2B*). The difference in OS between patients with the molecularly highly similar tumours A_IDH and A_IDH_HG tumours was highly significant (A_IDH_HG vs A_IDH: HR 2.43, 95% CI 1.73-3.40; p<0.0001).

We next assigned the 654 tumours into subgroups based on 'the glioma classifier', to 409 G-CIMP-high tumours, 19 G-CIMP-low tumours, 18 codel tumours, 107 mesenchymal-like tumours, 48 classic-like tumours, and 53 pilocytic astrocytoma (PA)-like/glioblastoma-like glioma methylation group 6 ('LGm6-GBM') tumours. The latter sub-diagnosis is combined as it requires additional histology review to determine presence or absence of necrosis and/or microvascular proliferation for further separation. After this central histology review, the subgroup is further separated in 41 PA-like tumours, and 12 LGm6-GBM tumours. The PA-like tumours were not a single nosological entity; in this set we identified nine tumours with *H3F3A* mutations (seven *K27M*, two *G34R*) and one *BRAF* mutated tumour (*R509Q*). The LGm6-GBM tumours also contained two tumours with *H3F3A* mutations (*K27M*) and one tumour with a *BRAF* mutation (*V600E*).

'The glioma classifier' tumour subtypes were also strongly associated with patient survival: OS in patients with G-CIMP-high 9.5 years (95% CI 7.5-not reached), with G-CIMP-low 2.8 years (95% CI 2.0-not reached), with codel 9.1 years (too few events to estimate the CI), with mesenchymal-like 1.3 years (95% CI 1.2-1.7), with classic-like 1.6 years (95% CI 1.4-1.8), and with PA-like/LGm6-GBM 2.8 years (95% CI 2.1-3.7; *Figure 2C and 2D*). Particularly notable was the difference in OS between G-CIMP status subgroup patients (G-CIMP-low vs G-CIMP-high: HR 4.12, 95% CI 2.37-7.18; p<0.0001). PA-like/LGm6-GBM tumour patients had better outcome than mesenchymal-like and classic-like tumour patients (HR 0.42, 95% CI 0.28-0.62; p<0.0001, and

HR 0.38, 95% CI 0.24-0.59; p<0.0001, respectively), suggesting a positive prognostic epigenetic subgroup within *IDH1/2wt* astrocytomas. This survival benefit remained for both the PA-like and the LGm6-GBM tumours after separation by central histology review of this group (*Sup. Figure 3B*). Using the G-CIMP-low risk to progression profile, we classified 116 of the 409 G-CIMP-high tumours (28.4%) as 'risk tumours' (*Sup. Figure 4*). Patients harbouring 'risk tumours' had a worse outcome compared to 'no-risk tumour' patients: median OS of 6.9 years (95% CI 5.7-not reached) in risk tumour patients vs not reached (95% CI 8.0-not reached) in no-risk tumour patients (risk vs no-risk: HR 1.64, 95% CI 1.13-2.37; p=0.01; *Figure 2E*).

Although both classifiers were generated independently, there is a large overlap between the epigenetic subtypes of 'the CNS tumour classifier' and 'the glioma classifier' (*Figure 1A, 1C* and *1D*). For example, every G-CIMP-low tumour was classified as an A_IDH_HG tumour, and 11/12 (91.7%) O_IDH tumours were classified as codel tumours. All classic-like tumours and 100/107 (93.5%) mesenchymal-like tumours were MTCF_GBM tumours, whereas 404/409 (98.8%) G-CIMP-high tumours were A_IDH or A_IDH_HG tumours. 'Risk tumours' (n=61) were mainly A_IDH_HG tumours and most 'no-risk tumours' (n=258) were A_IDH tumours (odds ratio 9.47, 95% CI 5.46-16.73; p<0.0001). The major difference between the two classifiers are those tumours that 'the CNS tumour classifier' does not identify as MTCF_GBM, A_IDH, A_IDH_HG or O_IDH (n=51), or tumours with a main diagnosis reliability score < 0.836 (n=74): most of these tumours (n=41 and n=31 respectively) were labelled PA-like/LGm6-GBM tumours.

To explore potential prognostic differences between 'the CNS tumour classifier' and 'the glioma classifier', we stratified 'the CNS tumour classifier' classes by 'the glioma classifier' subtypes and vice versa (*Sup. Figure 5*). Within the A_IDH_HG tumours, those assigned to G-CIMP-low had a significantly worse OS compared to those assigned to G-CIMP-high regardless of progression risk. There were, however, no differences in OS when either A_IDH or A_IDH_HG tumours were stratified by risk to G-CIMP-low progression (log-rank test between G-CIMP-high: No-risk and G-CIMP-high: Risk of *Sup. Figure 5A* and *5B*: p=0.33 and p=0.42, respectively). Stratifying 'risk tumours' and 'no-risk tumours' into 'the CNS tumour classifier' subgroups showed a significant OS difference in both A_IDH and A_IDH_HG tumours (HR 1.80 95% CI 1.01-3.21, p=0.06; and HR 2.00 95% CI 1.06-3.77, p=0.03, respectively).

Analysis of the *IDH1/2mt* anaplastic astrocytoma cohort

We set out to build a prognostic model specifically for *IDH1/2mt* anaplastic astrocytoma patients. Although a t-SNE plot of only the 432 *IDH1/2mt* anaplastic astrocytomas does not separate these epigenetic subtypes, they do have a distinct spatial distribution within this plot (*Figure 3A* and *Sup. Figure 6*). We further performed NGS and CNV analysis and identified 43 *IDH1/2mt* anaplastic astrocytomas with *CDKN2A/B* HD, 6 with *RB1* HD, 25 with *CDK4amp*, 22 with *PDGFRAamp*, 7 with *METamp*, 7 with *MYCNamp*, 4 with *CDK6amp*, and 20 with *PI3K* mutations (14 *PIK3R1*, 6 *PIK3CA*). The total CNV load was classified as high in 185 (42.8%) *IDH1/2mt* anaplastic astrocytomas based on an algorithm by Shirahata et al. [20].

Univariable analysis of the *IDH1/2mt* anaplastic astrocytomas showed that most of the clinical, molecular and histological factors were associated with OS i.e. age groups, WHO performance score, MMSE, type of surgery, use of corticosteroids, treatment with adjuvant temozolomide, treatment with concurrent temozolomide, presence of necrosis and/or microvascular proliferation, 'the CNS tumour classifier', 'the glioma classifier', *CDK4amp*, *METamp*, *PDGFRAamp*, *CDKN2A/B* HD, *PI3K* mutations, and total CNV load (*Sup. Table* 2, *Figure 2F*, *Sup. Figure 7*, and *Sup. Figure 8*). Only patient sex, *MGMT* promoter methylation, *MYCNamp*, and *RB1* HD were not associated with OS at univariable analysis (with the latter two factors present in only a few cases). The presence of the prognostic molecular and histological markers correlated with DNA methylation classes A_IDH_HG tumours, G-CIMP-low tumours, and G-CIMP-low progression risk (*Figure 3B*). Moreover, several of the prognostic molecular and histological markers showed significant co-occurrence.

Cox PH models made by stepwise backward elimination of significant factors from univariable analysis resulted in three multivariable models with independent significant factors: 1. the clinical/histological model (using the clinical and histological factors), 2. the tissue-based model (using the molecular and histological factors), and 3. the combined model (using clinical, molecular and histological factors). The final clinical/histological model included age groups, WHO performance score, treatment with adjuvant temozolomide, presence of necrosis and/or microvascular proliferation, and type of primary surgery and had a bootstrap corrected C-index of 0.626, while the final tissue-based model included 'the CNS tumour classifier', 'the glioma classifier', *PDGFRAamp*, *CDKN2A/B* HD, *PI3Kmt*, and total CNV load with a bootstrap corrected C-index of 0.665. However, the predictive final combined model performed markedly better than both the clinical/histological and the tissue-based model with a bootstrapped C-index of 0.709, and included age group, MMSE, treatment with concurrent temozolomide, treatment with adjuvant temozolomide, 'the CNS tumour classifier', 'the glioma classifier',

*PDGFRAamp*, *CDKN2A/B* HD, *PI3Kmt*, and total CNV load. The final clinical/histological and combined models are illustrated as Forest plots in *Figure 4*. Schoenfeld's tests for individual variables and the total model of the combined model were non-significant (*Sup. Figure 9*). The final tissue-based model is illustrated in *Sup. Figure 10*.

Finally, we performed recursive partitioning analysis on the significant factors from univariable analysis to create a prognostic decision tree for *IDH1/2mt* anaplastic astrocytoma patients. The first split of the tree is made on presence/absence of *CDKN2A/B* HD highlighting the importance of this molecular marker for prognostication. Additional branches are made by treatment with adjuvant temozolomide, WHO performance score, and 'the CNS tumour classifier', resulting in three clinically relevant subgroups with either a fair outcome, intermediate outcome or poor outcome (*Figure 5*). Recursive partitioning analysis of solely the *IDH1/2mt* anaplastic astrocytoma cohort that was treated with adjuvant temozolomide did not result in a stable model and is therefore not shown here.

## Discussion

Our study used tissue samples from the prospective randomised CATNON trial to validate the prognostic relevance of DNA methylation-based epigenetic subtyping by both 'the CNS tumour classifier' and 'the glioma classifier' in a cohort of homogenously treated anaplastic glioma patients. We demonstrate that, in *IDH1/2mt* anaplastic astrocytoma patients, both DNA methylation classifiers, alone or in combination with clinical data as well as other molecular markers (*PI3K* mutation status, and CNV analysis of *PDGFRA*, *CDKN2A/B*, and CNV load) exhibit prognostic significance. Our study validates the OS difference of A_IDH and A_IDH_HG tumour patients, the negative prognostic impact of the G-CIMP-low methylation profile, and the negative prognostic impact of the risk to G-CIMP-low progression score.

This validation is important, as both 'the CNS tumour classifier' and 'the glioma classifier' are available and can be easily used to diagnose and prognosticate new tumour samples with locally assessed DNA methylation data [12,22]. The clear prognostic importance of the DNA methylation-based subgroups in *IDH1/2mt* anaplastic astrocytoma suggests consideration for implementation in standard glioma diagnostics. Importantly, the added predictive effect of *PDGFRAamp*, *CDKN2A/B* HD, total CNV load, and *PI3K* mutation status can be considered as an integral part of prognosticating ('grading') *IDH1/2mt* anaplastic astrocytomas. For example, both the G-CIMP-low methylation profile and HD of *CDKN2A/B* were shown to be negative prognostic markers, yet only

in patients harbouring tumours with the combination of these markers does the OS resemble the outcome in grade 4 *IDH1/2wt* glioma patients as described in previous research.[23]

Within *IDH1/2wt* anaplastic astrocytoma, the prognostic PA-like/LGm6-GBM subgroup of 'the glioma classifier' was assessed as well. Both *BRAF* mutations and *H3F3A* mutations were identified in these tumours showing heterogeneity in this subgroup, since these mutations are indicative of both better and worse outcome patients.[3,24,25] This heterogeneity is further emphasized by the overlap with sporadic diagnoses from 'the CNS tumour classifier' with low reliability scores. These findings show that some tumours of this group cannot be classified by epigenetics and they require classification based on NGS and/or CNV data. Further analysis of *IDH1/2wt* anaplastic gliomas of the CATNON trial and outcome to treatment will be reported separately elsewhere.

The limitations of this study are predominantly the limited number of survival events within the *IDH1/2mt* anaplastic astrocytoma patients, a lack of longitudinal tissue analysis, absence of external validation of our predictive prognostic model, and the limited number of several investigated molecular markers. Although the OS of the epigenetic subtypes in the present dataset so far confirms previous observations, a longer-term follow-up of this study will aim to validate our findings.[11,13] Longitudinal analysis is required to determine how many tumours at risk to progression to G-CIMP-low indeed have progressed to this subtype. Though there is no external validation of our combined predictive model, the model has been generated in the largest cohort of *IDH1/2mt* anaplastic astrocytoma patients to date and all evaluated prognostic markers were previously identified in *IDH1/2mt* astrocytomas. Another limitation is the focus of the CATNON trial on grade 3 tumours, as one might argue that conclusions of this study cannot be extrapolated to grade 2 and 4 *IDH1/2mt* astrocytomas. However, the histological criteria used for grading are prone to interobserver and intraobserver variability, and most samples were reviewed by two central pathologists who at times disagreed about the grade. Additionally, some centres were allowed to enter patients on a local diagnosis from which the central reviewers deviated. As a consequence, the study enrolled cases that either bordered on grade 2 or on grade 4 tumours, and even cases with some necrosis and/or microvascular proliferation were enrolled. Although necrosis and microvascular proliferation are criteria for grade 4 tumours, the dedicated central neuropathologists diagnosed some tumours as grade 3 in the presence of these histological abnormalities. This apparent discrepancy reflects the difficulty in tumour grading and therefore this dataset represents a broader range of tumours than strict grade 3.

In short, DNA methylation-based epigenetic subtyping by 'the glioma classifier' and 'the CNS tumour classifier' in combination with clinical and genetic data has added value in prognosticating and diagnosing *IDH1/2mt* anaplastic astrocytomas. These epigenetic subtypes and molecular markers should therefore be considered for incorporation within the next WHO classification of CNS tumours.

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Conflict of interest

MJvdB: grants Dutch Cancer Foundation, Brain Tumor Charity, Strijd van Salland, MSD; personal fees Carthera, Nerviano, Bayer, Celgene, Agios, Abbvie, Karyopharm, Boston Pharmaceuticals, Genenta. PMC: personal fees Bristol-Meyers Squibb, Abbvie, Merck Serono, MSD, Vifor Pharma, Daiichi Sankyo, LEO Pharma, AstraZeneca, Takeda. MAV: royalty rights and indirect equity Infuseon Therapeutics, personal fees Tocagen, Celgene. AKN: grants AstraZeneca, Douglas Pharmaceuticals, travel funding AstraZeneca, Boehringer Ingelheim, consulting fees Douglas Pharmaceuticals, Bayer Pharmaceuticals, Pharmabcine, Atara Biotherapeutics, Trizell Ltd., payment to institution Douglas Pharmaceuticals, Atara Biotherapeutics, steering committee fees Roche Pharmaceuticals, Boehringer Ingelheim. OLC: personal fees Abbvie, non-financial support Abbvie, Immatics, BMS. RR: advisory board fees UCB, Novocure. MW: grants Abbvie, Adastra, Dracen, MSD, Merck, Novocure, personal fees Abbvie, MSD, Merck, Basilea, Bristol-Meyers Squibb, Celgene, Medac, Nerviano Medical Sciences, Orbus, Philogen, Roche, Tocagen. AvD: antibody patent $IDH1^{R132H}$, $BRAF^{V600E}$. HJD: personal fees Abbvie. BGB: personal fees Roche, non-financial support Roche. Other no competing interests.

Authorship

Study design: CMST, MJvdB, AvD, BGB, TG, HN, PJF. Data collection: CMST, MJvdB, MS, WW, AAB, PMC, SCE, MAV, AKN, JFB, WPM, HW, OLC, SG, MG, LR, WT, RR, MW, CM, MEvL, IdH, WFJvIJ, RWWB, KA, RBJ, HJD, JMK, PW, KJC, VG, BGB, HN, PJF. Data analysis: CMST, MJvdB, IdH, TSS, YH, HN, PJF. Data interpretation: CMST, MJvdB, TSS, TG, PJF. Writing/approving final manuscript: all authors.

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Figure legends

**Figure 1: Epigenetic subtyping of all CATNON samples that had molecular data available and passed quality control. A: DNA methylation heatmap of discriminative probes for 'glioma classifier' subtypes shows clear overlap between the different classifiers, and a strong correlation with *IDH1/2* status, *MGMT* promoter status, and patient age. B: Principal component analysis of the 20,000 most variable probes illustrates separation of the epigenetic data on *IDH1/2* status. C: Circle plot shows a high degree of correlation between 'glioma classifier' and 'CNS tumour classifier' diagnoses. D: Venn diagrams showing the correlation between 'the glioma classifier', 'the CNS tumour classifier' and genetic changes.** CNS = central nervous system. *IDH1/2* = isocitrate dehydrogenase 1 and 2. *MGMT* = methylguanine methyltransferase. G-CIMP = Glioma-CpG Island Methylator Phenotype. No-risk = no-risk of progression to G-CIMP-low. Risk = risk of progression to G-CIMP-low. Codel = 1p/19q codeleted. PA-like/LGm6-GBM = pilocytic astrocytoma-like/glioblastoma-like glioma methylation group 6. A_IDH = *IDH1/2mt*-like astrocytomas lower-grade. A_IDH_HG = *IDH1/2mt*-like astrocytomas high-grade. O_IDH = *IDH1/2mt*-like oligodendrogliomas. *IDH1/2mt* = *IDH1/2* mutant. GBM_G34 = *H3F3Amt G34R*-like tumour. MTCF_GBM = methylation class family *IDH1/2wt* glioblastomas. DMG_K27 = *H3F3Amt K27M*-like tumour. *H3F3Amt* = H3.3 histone A mutant. RT = radiotherapy. RT->TMZ = radiotherapy with adjuvant temozolomide. RT/TMZ = radiotherapy with concurrent temozolomide. TMZ/RT->TMZ = radiotherapy with concurrent and adjuvant temozolomide. NGS = next generation sequencing. GBM_RTK_1 = glioblastoma, receptor tyrosine kinase 1 alterations. GBM_RTK_2 = glioblastoma, receptor tyrosine kinase 2 alterations. GBM_MES = glioblastoma, mesenchymal. CONTR_HEMI = control tissue, hemispheric cortex. PLEX_PED_B = plexus tumour, paediatric subtype B. SUBEPN_SPINE = subependymoma, spine. PXA = pleomorphic xanthoastrocytoma. LGG_GG = low grade glioma, ganglioglioma. HGNET_BCOR = high grade neuroepithelial tumour with *BCOR* alteration. *BCOR* = *BCL6* corepressor. CNS_NB_FOXR2 = CNS neuroblastoma with *FOXR2* activation. *FOXR2* = forkhead box R2. ANA_PA = anaplastic pilocytic astrocytoma. CONTR_WM = control tissue, white matter. CONTR_INFLAM = control tissue, inflammatory tumour microenvironment. GBM_MYCN = glioblastoma, *MYCN* alterations. *MYCN* = N-myc proto-oncogene. GBM_MID = glioblastoma, midline. *BRAF* = B-Raf proto-oncogene serine/threonine-protein kinase. CNV = copy number variation.

**Figure 2: Kaplan-Meier curves of CATNON stratified by DNA methylation class demonstrates prognostic relevance of epigenetic profiling. A: Overall survival of A_IDH, A_IDH_HG, and O_IDH tumour patients. B: Overall survival of DMG_K27, GBM_G34, MTCF_GBM and other diagnosis tumour patients. C: Overall survival of codel, G-CIMP-high, and G-CIMP-low tumour patients. D: Overall survival of classic-like, mesenchymal-like, and PA-like/LGm6-GBM tumour patients. E: Overall survival of G-CIMP-high: No-risk, G-CIMP-high: Risk, and G-CIMP-low tumour patients. F: Overall survival of *IDH1/2mt* anaplastic astrocytoma patients: necrosis and/or microvascular proliferation vs no necrosis or microvascular proliferation.** CNS = central nervous system. A_IDH = *IDH1/2mt*-like astrocytomas lower-grade. A_IDH_HG = *IDH1/2mt*-like astrocytomas high-grade. O_IDH = *IDH1/2mt*-like oligodendrogliomas. *IDH1/2mt* = isocitrate dehydrogenase 1 and 2 mutant. DMG_K27 = *H3F3Amt K27M*-like tumour. GBM_G34 = *H3F3Amt G34R*-like tumour. *H3F3Amt* = H3.3 histone A mutant. MTCF_GBM = glioblastoma. Codel = 1p/19q

codeleted. G-CIMP = Glioma-CpG Island Methylator Phenotype. PA-like/LGm6-GBM = pilocytic astrocytoma-like/glioblastoma-like glioma methylation group 6. No-risk = no-risk of progression to G-CIMP-low. Risk = risk of progression to G-CIMP-low.

**Figure 3: Detailed molecular analysis of *IDH1/2mt* anaplastic astrocytomas of CATNON. A: t-distributed stochastic neighbour embedding plot of all *IDH1/2mt* anaplastic astrocytomas. Samples are coloured by 'CNS tumour classifier' subgroup with shapes representing 'glioma classifier' subgroups. Although no subgroups are clearly distinguishable in this plot, specific classifier subgroups show spatial segregation. B: Correlation plot of DNA methylation data, genetic data, and histology. A_IDH_HG and G-CIMP-low tumours correlate with both molecular and histological markers of malignancy. Non-significant correlations (i.e. Fisher's exact test ≥ 0.10) were removed from this plot.** CNS = central nervous system. A_IDH = *IDH1/2mt*-like astrocytomas lower-grade. A_IDH_HG = *IDH1/2mt*-like astrocytomas high-grade. *IDH1/2mt* = isocitrate dehydrogenase 1 and 2 mutant. CONTR_HEMI = control tissue, hemispheric cortex. CONTR_INFLAM = control tissue, inflammatory tumour microenvironment. GBM_RTK_1 = glioblastoma, receptor tyrosine kinase 1 alterations. O_IDH = *IDH1/2mt*-like oligodendrogliomas. PLEX_PED_B = plexus tumour, paediatric subtype B. Codel = 1p/19q codeleted. G-CIMP = Glioma-CpG Island Methylator Phenotype. No-risk = no-risk of progression to G-CIMP-low. Risk = risk of progression to G-CIMP-low. PA-like = pilocytic astrocytoma-like. *amp* = amplification. *CDK4* = cyclin dependent kinase 4. *CDK6* = cyclin dependent kinase 6. *MET* = tyrosine-protein kinase Met. *MYCN* = N-myc proto-oncogene. *PDGFRA* = platelet derived growth factor receptor alpha. HD = homozygous deletion. *CDKN2A/B* = cyclin dependent kinase inhibitor 2 A and B. *RB1* = retinoblastoma 1. *mt* = mutant. *PIK3CA* = phospoinositide-3-kinase subunit alpha. *PIK3R1* = phospoinositide-3-kinase regulatory subunit 1. CNV = copy number variation. MVP = microvascular proliferation. * = $0.10 > p \geq 0.05$. ** = $0.05 > p \geq 0.01$. *** = $p < 0.01$.

**Figure 4: Forest plots of predictive Cox proportional hazards models for *IDH1/2mt* anaplastic astrocytoma patients. A: The clinical/histological model based on clinical and histological variables. Four out of 432 *IDH1/2mt* anaplastic astrocytoma patients were missing histological data and were subsequently omitted from this final model due to incorporation of necrosis and/or microvascular proliferation as a significant factor. B: The combined model based on clinical, molecular and histological variables.** *IDH1/2mt* = isocitrate dehydrogenase 1 and 2 mutant. OS = overall survival. HR = hazard ratio. CI = confidence interval. WHO = World Health Organization. MMSE = mini-mental state examination score. CNS = central nervous system. A_IDH = *IDH1/2mt*-like astrocytomas lower-grade. A_IDH_HG = *IDH1/2mt*-like astrocytomas high-grade. O_IDH = *IDH1/2mt*-like oligodendrogliomas. G-CIMP = Glioma-CpG Island Methylator Phenotype. No-risk = no-risk of progression to G-CIMP-low. Risk = risk of progression to G-CIMP-low. Codel = 1p/19q codeleted. PA-like = pilocytic astrocytoma-like. *PDGFRA* = platelet derived growth factor receptor alpha. *CDKN2A/B* = cyclin dependent kinase inhibitor 2 A and B. *PI3K* = phospoinositide-3-kinase. CNV = copy number variation.

**Figure 5: Recursive partitioning analysis of the clinical, histological and molecular factors for *IDH1/2mt* anaplastic astrocytoma patients with HRs respective to entire cohort. Prognostic subgroups have been colour-coded for outcome: light grey is fair, dark grey is intermediate, and black is poor.** *IDH1/2mt* = isocitrate dehydrogenase 1 and 2 mutant. CNV = copy number variation. *CDKN2A/B* = cyclin dependent kinase inhibitor 2 A and B. HD = homozygous deletion. HR = hazard ratio. WHO = World Health Organization. PS = performance score. CNS = central nervous system. A_IDH = *IDH1/2mt*-like astrocytomas lower-grade. A_IDH_HG = *IDH1/2mt*-like astrocytomas high-grade.

## Main Tables

**Table 1: Baseline clinical characteristics included patients divided by treatment regimen.** a = Fisher's exact test. b = Wilcoxon rank-sum test. *IDH1/2* = isocitrate dehydrogenase 1 and 2. *MGMT* = methylguanine methyltransferase. WHO = World Health Organization.

| | Radiotherapy alone | Radiotherapy with concurrent temozolomide | Radiotherapy with adjuvant temozolomide | Radiotherapy with adjuvant and concurrent temozolomide | p value | All patients |
|---|---|---|---|---|---|---|
| Patients, n | 168 | 162 | 159 | 165 | | 654 |
| Sex, n (%) | | | | | 0.657[a] | |
| Female | 69 (41.1) | 61 (37.7) | 68 (42.8) | 73 (44.2) | | 271 (41.4) |
| Male | 99 (58.9) | 101 (62.3) | 91 (57.2) | 92 (55.8) | | 383 (58.6) |
| Age, years | | | | | 0.196[b] | |
| Median (range) | 42 (19 – 81) | 43 (20 – 77) | 39 (20 – 82) | 40 (18 – 72) | | 41 (18 – 82) |
| *IDH1/2* status, n (%) | | | | | 0.795[a] | |
| Mutant | 107 (63.7) | 110 (67.9) | 108 (67.9) | 115 (69.7) | | 440 (67.3) |
| Wildtype | 57 (33.9) | 49 (30.2) | 49 (30.8) | 49 (29.7) | | 204 (31.2) |
| Missing | 4 (2.4) | 3 (1.9) | 2 (1.3) | 1 (0.6) | | 10 (1.5) |
| *MGMT* promoter, n (%) | | | | | 0.94[a] | |
| Methylated | 116 (69) | 107 (66) | 109 (68.6) | 113 (68.5) | | 445 (68) |
| Unmethylated | 52 (31) | 55 (34) | 50 (31.4) | 52 (31.5) | | 209 (32) |
| WHO performance score, n (%) | | | | | 0.851[a] | |
| 0 | 96 (57.1) | 94 (58) | 92 (57.9) | 102 (61.8) | | 384 (58.7) |

| | | | | | | |
|---|---|---|---|---|---|---|
| >0 | 71 (42.3) | 68 (42) | 66 (41.5) | 63 (38.2) | | 268 (41) |
| Missing | 1 (0.6) | 0 (0) | 1 (0.6) | 0 (0) | | 2 (0.3) |
| Type of surgery, n (%) | | | | | 0.448[a] | |
| Biopsy | 26 (15.5) | 35 (21.6) | 27 (17) | 26 (15.8) | | 114 (17.4) |
| Resection | 142 (84.5) | 127 (78.4) | 132 (83) | 139 (84.2) | | 540 (82.6) |
| Use of corticosteroids, n (%) | | | | | 0.814[a] | |
| Yes | 44 (26.2) | 48 (29.6) | 40 (25.2) | 46 (27.9) | | 178 (27.2) |
| No | 124 (73.8) | 114 (70.4) | 118 (74.2) | 119 (72.1) | | 475 (72.6) |
| Missing | 0 (0) | 0 (0) | 1 (0.6) | 0 (0) | | 1 (0.2) |
| Necrosis and/or microvascular proliferation, n (%) | | | | | 0.178[a] | |
| Present | 30 (17.9) | 26 (16) | 23 (14.5) | 16 (9.7) | | 95 (14.5) |
| Absent | 136 (81) | 133 (82.1) | 135 (84.9) | 145 (87.9) | | 549 (83.9) |
| Missing | 2 (1.2) | 3 (1.9) | 1 (0.6) | 4 (2.4) | | 10 (1.5) |

Figure 1

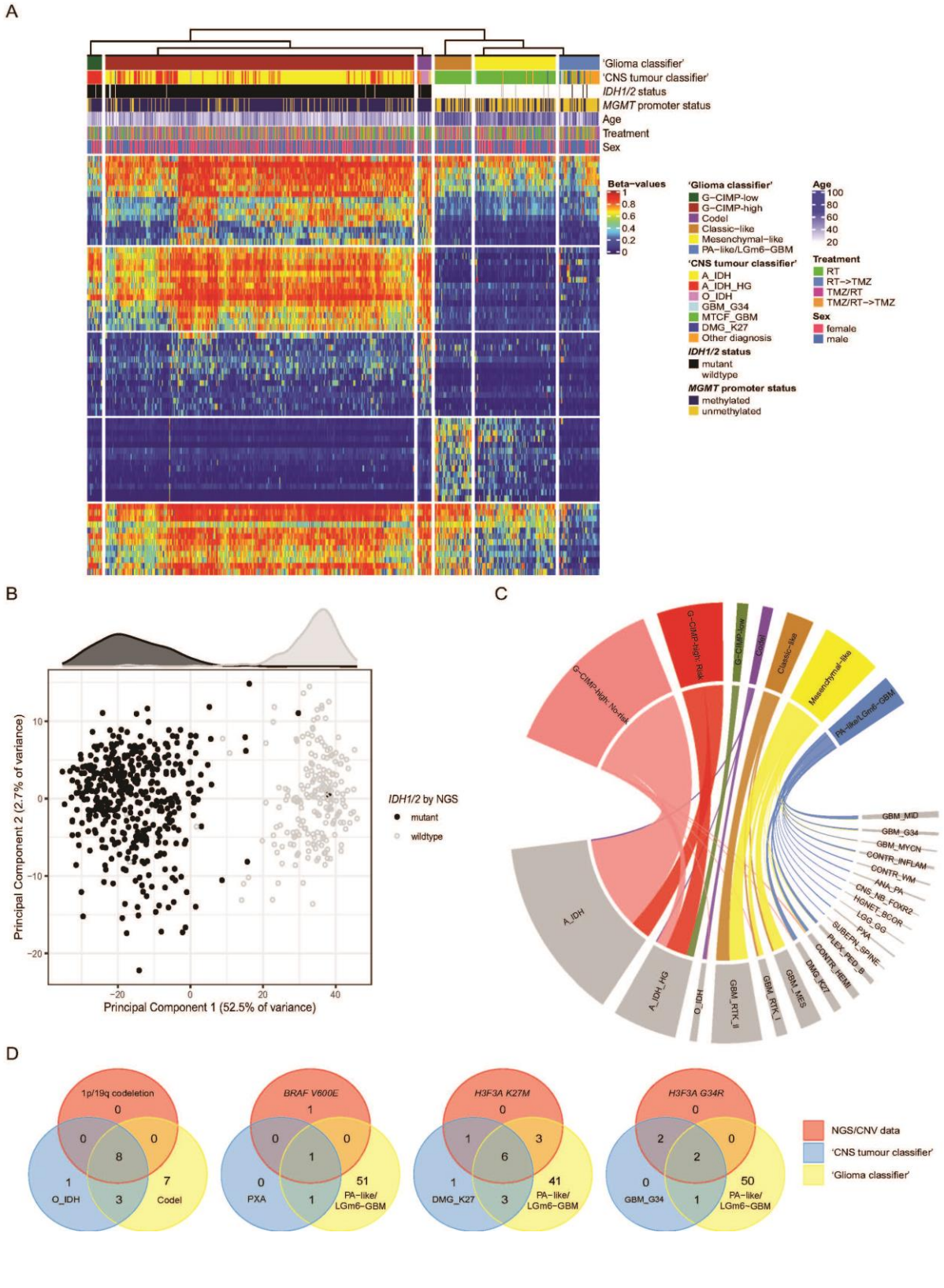

Figure 2

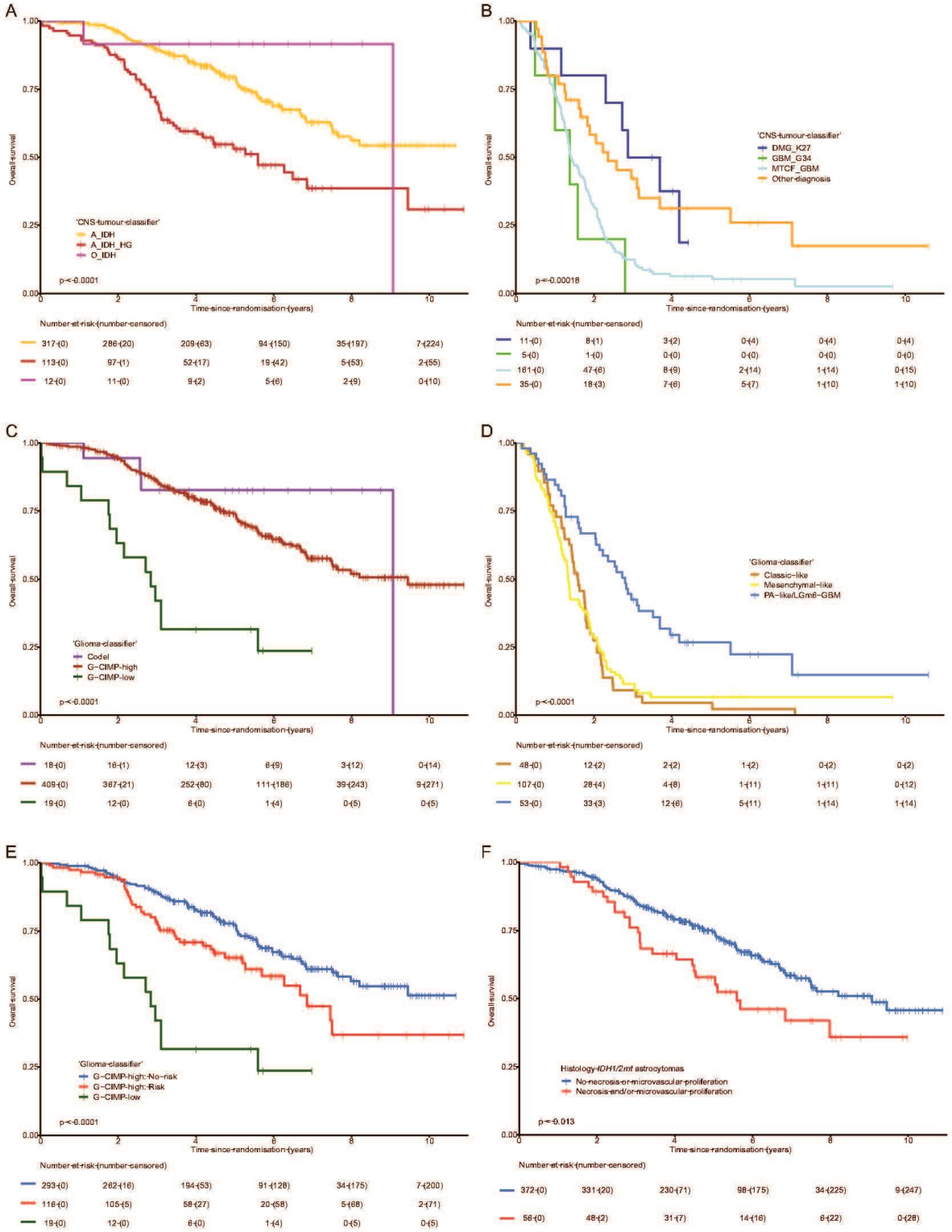

Figure 3

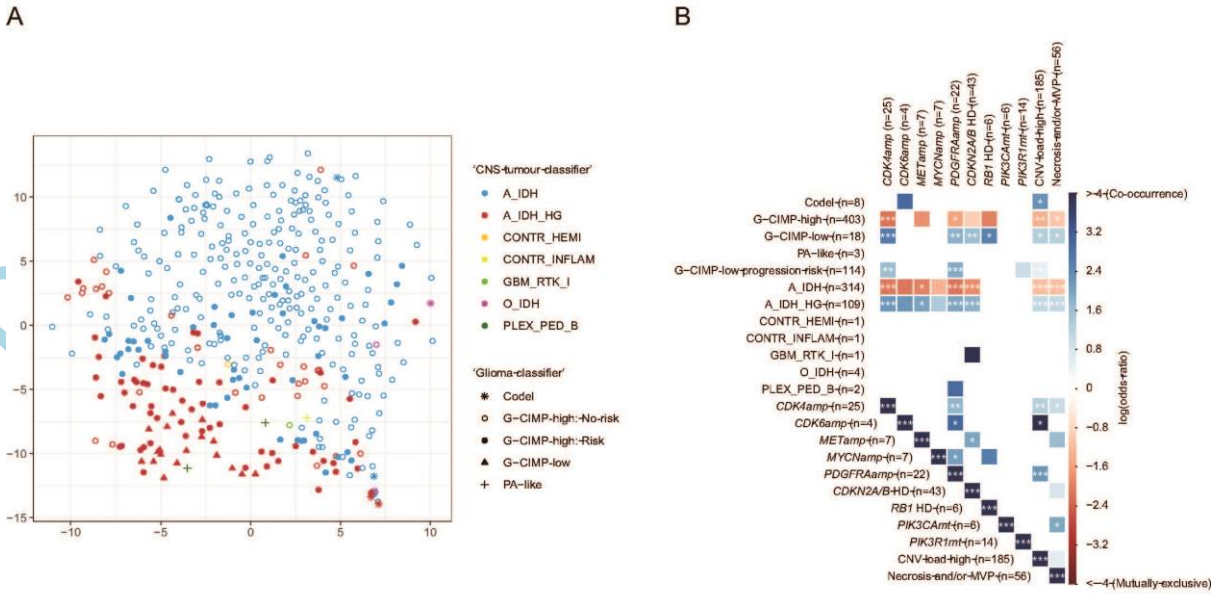

Figure 4

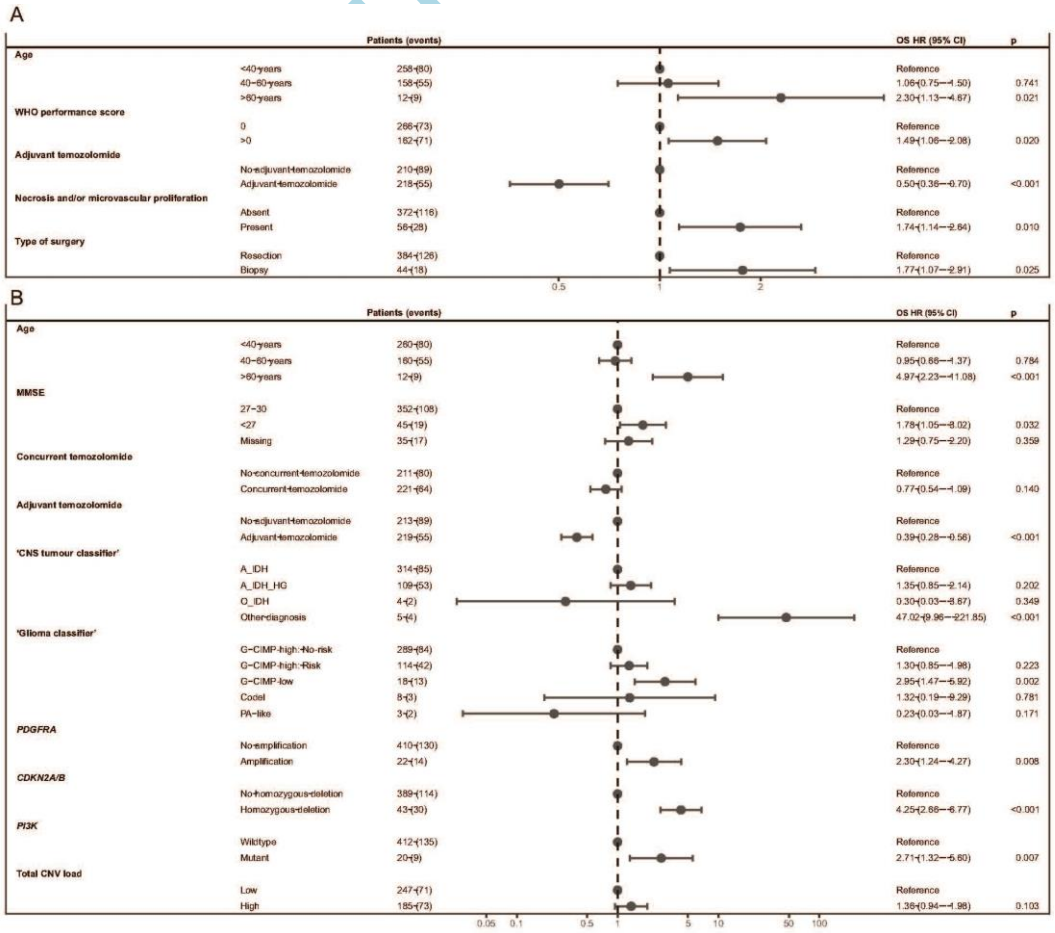

Figure 5