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Targeted next-generation sequencing of adult gliomas for retrospective prognostic evaluation and up-front diagnostics

Petersen, J. K.; Boldt, H. B.; Sørensen, M. D.; Blach, S.; Dahlrot, R. H.; Hansen, S.; Burton, M.; Thomassen, M.; Kruse, T.; Poulsen, F. R.; Andreasen, L.; Hager, H.; Ulhøi, B. P.; Lukacova, S.; Reifenberger, G.; Kristensen, B. W.

Published in:

Neuropathology and Applied Neurobiology

DOI:

10.1111/nan.12645

Publication date:

2021

Document version:

Accepted manuscript

Citation for published version (APA):

Petersen, J. K., Boldt, H. B., Sørensen, M. D., Blach, S., Dahlrot, R. H., Hansen, S., Burton, M., Thomassen, M., Kruse, T., Poulsen, F. R., Andreasen, L., Hager, H., Ulhøi, B. P., Lukacova, S., Reifenberger, G., & Kristensen, B. W. (2021). Targeted next-generation sequencing of adult gliomas for retrospective prognostic evaluation and up-front diagnostics. *Neuropathology and Applied Neurobiology*, 47(1), 108-126. <https://doi.org/10.1111/nan.12645>

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6

7 Article type : Original Article

8

9

10 **Article type:** original article

11 **Title:** Targeted next-generation sequencing of adult gliomas for retrospective prognostic evaluation and up-front
12 diagnostics

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/NAN.12645](https://doi.org/10.1111/NAN.12645)

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48 **Running title:**

49 Gene panel NGS of gliomas for prognostic evaluation and up-front diagnostics

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52 **Keywords (max 8 words):**

53 Gliomas, targeted next-generation sequencing (NGS), cIMPACT-NOW, reclassification, mutational profiles, prognostic
54 evaluation

55

56 **Number of words in manuscript: 5395**

57 **Number of figures (main): 5**

58 **Number of tables (main): 4**

59 **Number of figures (supplements): 4**

60 **Number of tables (supplements): 9**

61 **Abstract (250 words)**

62 **AIMS** We aimed to reclassify a population-based cohort of 529 adult glioma patients to evaluate the prognostic impact
63 of the 2016 World Health Organization (WHO) central nervous system tumour classification. Moreover, we evaluated
64 the feasibility of gene panel next generation sequencing (NGS) in daily diagnostics of 225 prospective glioma patients.

65 **METHODS** The retrospective cohort was reclassified according to WHO 2016 criteria by immunohistochemistry for
66 IDH-R132H, fluorescence *in situ* hybridization for 1p/19q codeletion and gene panel NGS. All tumours of the prospective
67 cohort were subjected to NGS analysis up-front.

68 **RESULTS** The entire population-based cohort was successfully reclassified according to WHO 2016 criteria. NGS
69 results were obtained for 98% of the prospective patients. Survival analyses in the population-based cohort confirmed
70 three major prognostic subgroups, i.e. isocitrate dehydrogenase (IDH)-mutant and 1p/19q-codeleted oligodendrogliomas,
71 IDH-mutant astrocytomas and IDH-wildtype glioblastomas. The distinction between WHO grade II and III was
72 prognostic in patients with IDH-mutant astrocytoma. The survival of patients with IDH-wildtype diffuse astrocytomas
73 carrying *TERT* promoter mutation and/or *EGFR* amplification overlapped with the poor survival of IDH-wildtype
74 glioblastoma patients.

75 **CONCLUSIONS** Gene panel NGS proved feasible in daily diagnostics. In addition, our study confirms the prognostic
76 role of glioma classification according to WHO 2016 in a large population-based cohort. Molecular features of
77 glioblastoma in an IDH-wildtype diffuse glioma were linked to poor survival corresponding to IDH-wildtype
78 glioblastoma patients. The distinction between WHO grade II and III retained prognostic significance in patients with
79 IDH-mutant diffuse astrocytic gliomas.

80

81 **List of Abbreviations**

82 +7/-10 - combined whole chromosomal imbalances on chromosome 7 and 10

83 ATRX - alpha-thalassemia/mental retardation X-linked syndrome

84 cIMPACT-NOW - The Consortium to Inform Molecular and Practical Approaches to CNS Tumour Taxonomy

85 CNS – central nervous system

86 CNV – copy number variation

87 FFPE – formalin-fixed paraffin-embedded

88 FISH –fluorescence *in situ* hybridization

89 IDH – isocitrate dehydrogenase

90 IHC – immunohistochemistry

91 NGS – next-generation sequencing

92 OS – overall survival

93 P53 - tumour protein p53

94 WHO – World Health Organization

95 **Introduction**

96

97 Since the introduction of integrated “histomolecular” classification into central nervous system (CNS) tumour diagnostics
98 in 2016 [1], the diagnosis of adult diffuse gliomas has been based on the combination of histopathologic appearance and
99 three key defining molecular alterations, namely mutations in the isocitrate dehydrogenase genes 1 and 2 (*IDH1* and
100 *IDH2*), whole-arm codeletion of chromosomal arms 1p and 19q, as well as the histone 3 K27M missense mutation. With
101 these novel and more precise diagnoses, the glioma research field faces difficulties of reinterpreting results of prior
102 prognostic studies in which tumour diagnoses were only based on histological classification. Large population-based
103 survival analyses of patients with gliomas stratified according to WHO 2016 integrated diagnoses to further support the
104 prognostic consequences of the WHO 2016 classification are still missing. Moreover, the role of conventional histological
105 grading within the WHO 2016 defined glioma entities, in particular in IDH-mutant astrocytomas, is still unclear and a
106 matter of ongoing debate [2].

107
108 New clinically relevant molecular markers continue to emerge and targeted next-generation sequencing (NGS) has
109 become a promising approach in daily diagnostics allowing screening for genetic alterations in several diagnostic,
110 prognostic, and predictive genes in a single analysis with a short turn-around time and at reasonable costs. Several studies
111 have been published on NGS panels used for detection of genetic alterations in brain tumours [1, 3-13], but most studies
112 are based on retrospective analyses. Moreover, most neuropathologic laboratories still use single gene analyses for routine
113 detection of diagnostically important molecular biomarkers. The composition of the investigated NGS panels vary from
114 small customized 20-130 gene panels [1, 3-5, 7-9] designed to cover the most common alterations in gliomas, to large
115 commercially available comprehensive cancer gene panels including more than 250 genes [6, 10-12]. These studies
116 reported NGS panels as an accurate and sensitive technique for detection of defining molecular alterations matching
117 established methods such as immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH).

118
119 Recently, 20 of the most common genes harbouring molecular alterations in diffuse gliomas have been selected for a
120 customized targeted glioma-tailored gene panel (glioma panel) by Zacher and colleagues, covering mutations in *ATRX*,
121 *BRAF*, *CDKN2A*, *CDKN2B*, *CDKN2C*, *CIC*, *EGFR*, *FUBP1*, *H3F3A*, *IDH1*, *IDH2*, *NF1*, *NF2*, *NRAS*, *PIK3CA*, *PIK3R1*,
122 *PTEN*, *RBI*, *TERT* and *TP53* [14]. This glioma panel was designed for diagnostic use in a clinico-pathological setting
123 where quick cancer diagnostics is required and since mid-2016, it has been implemented in our institution and used up-
124 front on all brain tumours in daily diagnostics.

125
126 The aims of this study thus were twofold: (1) To use IHC, FISH and the 20-gene glioma NGS panel to reclassify the
127 tumours of a well-annotated population-based cohort including 529 adult glioma patients in order to obtain more precise
128 insights in the prognostic impact of the WHO 2016 CNS tumour classification; (2) To evaluate the use of the 20-gene
129 glioma panel NGS in up-front daily diagnostics incorporating some of the novel diagnostic mutations by performing
130 prospective analyses of 225 glioma patients. The genetic alterations detected in both studies were combined and
131 mutational frequencies and profiles in the distinct entities of diffuse gliomas were explored.

132 **Materials and Methods**

133 **Patient population and glioma specimens**

134 The retrospective population-based cohort comprised archived human glioma tissue (formalin-fixed paraffin-embedded
135 (FFPE)) of 529 adult patients (age 18 years and over) with diffuse gliomas from two regions in Denmark: Southern
136 Denmark and Central Denmark (Table 1). All tumours were originally classified according to the 2007 WHO CNS tumour
137 classification [15] by neuropathologists from the Dept. of Pathology, Odense University Hospital, and the Dept. of
138 Pathology, Aarhus University Hospital. Histologic grading was performed according to the WHO 2007 criteria which
139 have been retained in the WHO 2016 classification [1]. All glioma tissue samples were obtained in routine clinical practice
140 when the patients underwent initial surgery at the Dept. of Neurosurgery, Odense University Hospital between 1991-2014
141 or at the Dept. of Neurosurgery, Aarhus University Hospital between 2005-2009.

142 The prospective cohort comprised 225 adult patients with diffuse gliomas diagnosed according to WHO 2016 criteria as
143 part of routine clinical practice when the patients underwent initial surgery at the Dept. of Neurosurgery, Odense
144 University Hospital between February 2016 and August 2018 (Supporting Information Table S1). The patients from both
145 cohorts had not received any treatment, except glucocorticoids, prior to initial surgery.

146 **Molecular testing using immunohistochemistry (IHC), fluorescent *in situ* hybridization (FISH), DNA 147 methylation profiling and glioma panel next generation sequencing (NGS)**

148 An algorithm including IHC, FISH and targeted NGS results was used to reclassify the 529 diffuse gliomas in the
149 retrospective cohort according to the WHO 2016 classification (Supporting Information Fig. S1). All 225 diffuse gliomas
150 in the prospective cohort underwent targeted NGS sequencing, and results were used in the integrated diagnostic work-
151 up together with results from IHC for IDH1-R132H, nuclear expression of alpha-thalassemia/mental retardation X-linked
152 syndrome (ATRX) and tumour protein (p53) (Supporting Information Fig. S2). IHC staining as well as detection of
153 1p/19q-codeletion by FISH were performed as previously reported [16]. Twenty-six of the glioma specimens were
154 submitted for DNA methylation profiling using Illumina Infinium Methylation EPIC BeadChip array analysis (Illumina,
155 San Diego, USA) as previously described [17].

156 To investigate the prognostic impact of recent cIMPACT-NOW recommendations for diffuse astrocytic gliomas in the
157 retrospective cohort, IDH-wildtype were stratified into tumours with or without molecular features of glioblastomas,
158 based on identification of *TERT* promoter mutation and/or *EGFR* amplification according to the cIMPACT-NOW update
159 3 recommendation [18]. Diffuse astrocytic gliomas, IDH-mutant were stratified with or without *CDKN2A/B* homozygous
160 deletion according to the cIMPACT-NOW update 5 recommendation [19].

161
162 In total, we performed gene panel NGS on 345 diffuse gliomas, including 120 gliomas of the retrospective cohort and
163 225 gliomas of the prospective cohort using the Ion AmpliSeq CNS Next Generation Sequencing Panel v1 (CNSv1-NGS)
164 as a glioma-targeted custom-designed gene panel [14]. Library preparation for gene panel sequencing was carried out
165 according to the manufacturer's protocol and has been published elsewhere [14]. NGS data were analysed for sequence
166 variants using Ion Reporter (v5.4 through v5.10). BAM alignment files were visualized using Golden Helix
167 GenomeBrowse 2.1.0 (Golden Helix, Bozeman, MT, USA). The following filter steps were used: (1) UCSC Common
168 SNPs filter, (2) removal of intronic variants except for splice sites, (3) read depth greater or equal 40, (4) minimum allele

169 frequency of 10%, and (5) at least 10% reads from each strand. Filtered variants were individually evaluated using
170 available public databases to identify pathogenic alterations.

171

172 Copy number variation (CNV) of *EGFR* were identified by manual interpretation of sequencing data as focal high-level
173 amplification of the gene. Single-amplicon sample coverage relative to bi-allelic controls of specific *EGFR* exons was
174 furthermore used to systematically define a lower cut off to distinguish low-level amplification from gain of chromosome
175 7. CNV of *CDKN2A/B/C* deletions and homozygous deletions of *CDKN2A/B* were identified using Ion Reporter™ 5.12
176 Software CNV analysis (Life Technologies). The software estimates CNVs based on an algorithm (Hidden Markov
177 Model) build on a baseline of 49 tonsil control samples and 6 glioma tumour samples with no known CNVs in any region
178 covered by the 20-gene glioma panel. The algorithm uses normalized read coverage across amplicons to predict the copy
179 number or ploidy states.

180

181 **Test of inter-laboratory variation and robustness of the glioma panel**

182 NGS on 14 selected samples was performed both at the Dept. of Pathology Odense and Dept. of Pathology, Vejle Hospital,
183 Denmark to test inter-laboratory variation and robustness of the glioma panel. NGS was also performed on 4 selected
184 samples for validation purposes at the Institute of Neuropathology, Heinrich Heine University, Düsseldorf [14].

185 **Statistical analyses**

186 Unsupervised hierarchical clustering was performed by constructing two binary matrices applied to the NGS data obtained
187 from the 225 prospective samples and the other on the 120 retrospective samples in which columns are patients and rows
188 are the 23 selected mutations and/or chromosomal abnormalities. Each cell in these matrices was scored 0 or 1, based on
189 the absence or presence of the particular aberration defined above. Next, the relationships between samples within the
190 two matrices were calculated using the Simple matching coefficient of Sokal & Michener [20] followed by dendrogram
191 construction using average clustering. The binary matrices and dendrograms were visualized by two heatmaps. All
192 calculations were performed using the open source R-environment (R version 3.5.1, (<http://cran.r-project.org/>)). The R-
193 package *ade4* [21-23] and *ComplexHeatmap* [24] were used for calculating the Simple matching coefficient of Sokal &
194 Michener and for heatmap visualization, respectively.

195

196 Survival analyses were carried out for the population-based retrospective patient cohort. Relevant clinical characteristics
197 of this cohort stratified according to the WHO 2007 or WHO 2016 classification systems are listed in Tables 1 and 2,
198 respectively, which provide information on patient age, clinical performance status, initial postsurgical treatment and
199 patient survival. Overall survival (OS) was defined as time from primary surgery until death from any cause or date of
200 censoring in November 2018. Kaplan-Meier survival curves were used for exploring differences in overall survival
201 between WHO diagnoses using the 2007 and 2016 WHO CNS tumour classification and the recent cIMPACT-NOW
202 recommendations for the different glioma subgroups. Log-rank tests were used for univariate comparisons, and Cox
203 proportional hazards model were used to evaluate hazards ratios (HRs) to determine the prognostic impact of the two
204 WHO classifications. Application of multivariable regression analysis was not performed due to insufficient numbers of
205 patients per variable per group as well as establishment of therapeutic heterogeneous groups as a result of the WHO 2016

206 reclassification (Tables 1 and 2). Analyses related to patient characteristics and overall survival were carried out using
207 STATAIC 15 (StataCorp LP) and Prism (GraphPad Software Inc., San Diego, CA, USA). Sensitivity, specificity and the
208 concordance rates between IHC and NGS results for IDH1-R132H, ATRX and TP53 were calculated using standard
209 statistical methods. Differences in mutational frequencies were investigated using Fisher's exact test. Significance was
210 defined at p -values < 0.05 .

211 **Results**

212 **Reclassification of the retrospective cohort according to the 2016 WHO CNS tumour classification**

213 The combined analysis of IHC, FISH and NGS data resulted in successful reclassification of all 529 retrospectively
214 investigated diffuse gliomas according to the WHO 2016 classification of CNS tumours [1] (Fig. 1A) (Supporting
215 Information, Table S2). Patient characteristics after reclassification are summarized in Table 2. The reclassification
216 resulted in a marked decrease in the number of patients with WHO grade III oligodendroglial tumours (from 63 to 37
217 patients), an increase in the number of patients with glioblastomas (from 327 to 383 patients) and a reclassification of all
218 oligoastrocytomas. Only smaller changes in patient numbers after reclassification were seen for WHO grade II and III
219 astrocytic gliomas (from 113 to 107 patients) (Fig. 1B). Of note, at revision according to 2016 WHO classification, 19
220 anaplastic astrocytomas and one anaplastic oligodendroglioma were reclassified as IDH-wildtype glioblastomas based on
221 morphological identification of focal necrosis and/or glomeruloid vascular proliferation and lack of IDH mutation and
222 1p/19q-codeletion.

223 The stratification of WHO grade II/III IDH-wildtype astrocytic gliomas according to cIMPACT-NOW recommendation
224 3 resulted in about half (17/39) of the tumours in the retrospective cohort being categorized as diffuse astrocytic gliomas,
225 IDH-wildtype with molecular features of glioblastoma, WHO grade IV. Further, stratification of WHO grade II/III IDH-
226 mutant astrocytic gliomas according to cIMPACT-NOW recommendation 5 identified *CDKN2A/B* homozygous deletions
227 in 17% (2/12) of the tumours in the retrospective cohort.

228 **Concordance between IHC (IDH1-R132H, ATRX, p53) and NGS results**

229 Correlative analyses between immunohistochemical findings and NGS results were based on the combined retro- and
230 prospective glioma cases that were subjected to NGS. In total, IHC data were available for IDH1-R132H, ATRX and p53
231 for 345, 299 and 296 of the 345 sequenced gliomas, respectively. IHC analysis of IDH1-R132H was well correlated with
232 targeted NGS analysis showing high sensitivity (95%) and specificity (100%) for this mutation (Supporting information
233 Table S3). Forty-two WHO grade II or III gliomas were positive for IDH1-R132H by IHC analysis and all harboured an
234 IDH1-R132H mutation by sequencing. Three-hundred and one gliomas were negative for IDH1-R132H by IHC and for
235 2 gliomas staining results were inconclusive. NGS of these 303 gliomas identified 23 additional *IDH1* or *IDH2* mutations
236 (*IDH1*; R132H: n=4, R132G: n=4, R132C: n=4, R132S: n=2, R132L: n=1, *IDH2*; R172K: n=3, R172S: n=2, R172M:
237 n=1, R172W: n=1, R140W: n=1). Nuclear expression of ATRX staining as demonstrated by IHC was strongly correlated
238 with identification of no ATRX mutation (sensitivity 98%), but loss of expression was less tightly correlated with
239 identification of ATRX mutations (specificity 76%) (Supporting Information Table S4). Both low sensitivity and
240 specificity was found when comparing IHC and NGS results for p53 (sensitivity 75%, specificity 63%) (Supporting
241 Information Table S5).

242 **Hierarchical cluster analysis and identification of distinct mutational frequencies of molecular subgroups of**
243 **diffuse gliomas.**

244 Separate unsupervised hierarchical cluster analyses were performed on the NGS data (including 1p/19q-codeletion data
245 from FISH/850k) obtained from both cohorts (Fig. 2 and 3). In both analyses, three separate molecular subgroups
246 dominated by distinct mutational patterns were identified: (1) IDH-mutant astrocytic gliomas with frequent mutations in
247 *TP53* and *ATRX* (Fig. 2, 3 and 4A, green bars); (2) IDH-mutant and 1p/19q-codeleted oligodendrogliomas with frequent
248 mutations in *TERT* promoter, *CIC* and *FUBP1* (Fig. 2, 3 and 4A, yellow and orange bars) and (3) IDH-wildtype astrocytic
249 gliomas/glioblastomas with frequent mutations in *TERT*, *PTEN*, *NF1*, *TP53* and *EGFR* with increasing mutational
250 frequencies concomitantly to increasing WHO grade (Fig. 2, 3 and 4A, blue bars).

251
252 The stratification of WHO grade II/III astrocytic gliomas in accordance to the cIMPACT-NOW recommendations
253 mutational profiles of diffuse astrocytic gliomas, IDH-wildtype, with molecular features of glioblastomas (*TERT*
254 promoter mutation and/or *EGFR* amplification), WHO grade IV, harboured higher, but not significantly different
255 frequencies of mutations in *PTEN* (p=0.14) and *EGFR* (p=0.35), and lower, but also not significantly different frequencies
256 of mutations in *TP53* (p=0.33), *NF1* (p=0.18) and *ATRX* (p=0.15) when compared with IDH-wildtype diffuse astrocytic
257 gliomas without these molecular features (Fig.4B, blue and red). Diffuse astrocytic gliomas, IDH-mutant, with
258 *CDKN2A/B* homozygous deletion showed higher, but not significantly different frequencies of mutations in *TP53*
259 (p=0.99) and *NF1* (p=0.27) and lower, but also not significantly different frequencies of mutations in *ATRX* (p=0.3).

260

261 **Prognostic impact of integrated diagnostics**

262 Comparison of OS data from patients with WHO grade II diffuse astrocytomas before (WHO 2007) and after
263 reclassification (WHO 2016), showed that patients with IDH-wildtype diffuse astrocytomas (WHO 2016) had
264 significantly shorter OS than patients with diffuse astrocytoma diagnosed according to WHO 2007 (HR 2.74; 95% CI:
265 1.36-4.49; p=0.003, Fig. 5A and Table 3) as well as patients with IDH-mutant diffuse astrocytomas (HR 3.68; 95% CI:
266 1.94-6.97; p=0.000, Fig. 5A and Table 4). OS of patients with IDH-mutant diffuse astrocytomas (WHO 2016) showed a
267 trend towards longer OS when compared to patients with diffuse astrocytomas according to WHO 2007, but this
268 difference was not statistically significant (Fig. 5A and Table 3). Patients with IDH-mutant anaplastic astrocytomas
269 (WHO 2016) showed significantly longer OS compared to patients diagnosed with anaplastic astrocytomas (WHO 2007)
270 (HR 0.44; 95% CI: 0.26-0.75; p=0.002, Fig. 5B and Table 3). In contrast, patients with IDH-wildtype anaplastic
271 astrocytomas (WHO 2016) showed a trend towards shorter OS when compared to patients with anaplastic astrocytomas
272 classified according to WHO 2007, although this difference did not reach statistical significance (Fig. 5B and Table 3).
273 Similar associations were found in the WHO grade IV glioblastoma group (Fig. 5C and Table 3). Comparison of OS of
274 patients with WHO grade II versus III oligodendroglial tumours (WHO 2007) showed shorter survival of patients with
275 anaplastic tumours of WHO grade III (Fig. 4D). For patients with IDH-mutant and 1p/19q-codeleted oligodendroglial
276 tumours, this survival difference was smaller, but a trend towards shorter OS remained for patients with WHO grade III
277 anaplastic oligodendrogloma (HR 2.17; CI: 95% 0.88-5.31; p=0.091). For the oligodendroglial tumours, patients with
278 IDH-mutant and 1p/19q-codeleted anaplastic oligodendrogliomas of WHO grade III showed significantly longer OS

279 when compared to patients with anaplastic oligodendrogliomas classified according WHO 2007 (HR 0.44; 95% CI: 0.21-
280 0.92; p=0.029, Fig. 5D and Table 3).

281

282 An overall comparison (Fig. 5E and Table 4) of WHO 2016 diagnoses confirmed a clear prognostic value of IDH mutation
283 in the different glioma subgroups. Importantly, we also found that WHO grading of astrocytic gliomas had a significant
284 effect on OS, including patients with IDH-mutant astrocytomas (WHO grade II vs. III: HR 2.90; 95% CI: 1.61-5.20;
285 p<0.001, WHO grade II vs. IV: HR 4.75; CI: 95% 2.30-9.77; p<0.001) and patients with IDH-wildtype astrocytomas
286 (WHO grade II vs. III: HR 2.67; CI: 95% 1.33-5.38; p=0.006, WHO grade II vs. IV: HR 2.51; CI: 95% 1.48-4.27;
287 p=0.001). Comparing patients with astrocytomas across IDH mutation status and WHO grade showed a more favourable
288 outcome of patients with WHO grade IV IDH-mutant glioblastoma in comparison to patients with WHO grade III IDH-
289 wildtype anaplastic astrocytomas (HR 0.39; CI: 95% 0.18-0.84; p=0.015), but no statistical difference in outcome between
290 WHO grade IV IDH-mutant glioblastoma patients and WHO grade II IDH-wildtype diffuse astrocytoma patients, due to
291 contamination of the IDH-wildtype groups by prognostically unfavourable tumours with molecular features of
292 glioblastoma. Finally, we investigated the prognostic role of novel molecular alterations in WHO grade II/III diffuse
293 astrocytic gliomas. Patients with IDH-wildtype astrocytomas with molecular features of glioblastomas, WHO grade IV
294 [18] (n=17) and IDH-wildtype glioblastomas WHO grade IV (n=370) showed no survival difference (HR; 0.85 CI: 95%
295 0.51-1.44; p=0.55) (Fig. 5F), whereas comparison of IDH-mutant astrocytomas with (n=2) and without (n=10)
296 *CDKN2A/B* deletions indicated (only twelve tumours included, low statistical power) prognostically unfavourable
297 outcome in patients carrying the molecular alteration (Supporting Information, Fig.S3).

298 **Feasibility, robustness and detection of potential actionable therapeutic targets with the glioma panel in daily** 299 **routine diagnostics**

300 Integrated histomolecular diagnoses were established for all 225 gliomas prospectively investigated in the daily diagnostic
301 setting. The patient demographics and results of important findings are summarized in Table S6. The feasibility of the
302 glioma panel in a daily clinico-pathological setting was assessed by the number of samples found with reduced quality
303 parameters compared to the number of total sequenced samples in the prospective cohort. Tissue samples to be
304 characterized molecularly included all types from a routine diagnostic setting with varying tissue quality and amounts.
305 We found that 2.2% (5/225) of the samples had reduced quality parameters based on low RNase_P and DNA
306 concentrations. Retrospectively, these samples were evaluated and only one sample was found with abundant necrosis
307 that could explain the reduced quality parameters. Despite reduced quality parameters, we were able to detect mutations
308 in all five gliomas.

309 Potentially actionable molecular targets, defined as targetable alterations by FDA-approved drugs, were detected in 67%
310 (110/163) of the glioblastomas in the prospective cohort. Some cases had more than one targeted with the genetic
311 alterations being considered as targetable when there is either an approved or investigational therapy available (Supporting
312 Information Table S7). Additionally, the robustness of the glioma panel was evaluated across two external laboratories
313 with an overall good inter-laboratory reproducibility (Supporting Information Table S8).

314 **Discussion**

315 The molecular reclassification of a large population-based cohort of glioma patients revealed considerable changes in the
316 glioma diagnoses between the WHO classifications of 2007 and 2016, with the main shifts being in the number of WHO
317 grade III oligodendroglial tumours and WHO grade IV glioblastomas, and to a lesser extent shifts in the numbers of WHO
318 grade II and III astrocytomas. For example, only 11 of 43 tumours histologically classified as anaplastic
319 oligodendrogliomas were molecularly confirmed to be IDH-mutant and 1p/19q-codeleted anaplastic oligodendrogliomas.
320 The majority of the remaining WHO 2007 anaplastic oligodendrogliomas were reclassified as either IDH-wildtype or
321 IDH-mutant astrocytic gliomas/glioblastomas according to WHO 2016, which of course bears major consequences on
322 post-surgical therapy and prognosis [25]. Recent studies by Iuchi *et al.* [26], Orhirjav *et al.* [27] and Brito *et al.* [28] also
323 reported oligodendroglial tumours and WHO grade II/III astrocytomas as the main targets of reclassification, while the
324 French nationwide POLA cohort (cohort of high-grade glioma with an oligodendroglial component) [29] showed most
325 frequent classification changes among astrocytomas and glioblastomas, but less common re-classification of
326 oligodendroglial tumours. The differences between these studies are largely related to the distribution of glioma subgroups
327 in the individual cohorts and variable stringency in the histological criteria used for oligodendroglia classification [30].
328 Our results thus reflect the main effects of the WHO 2016 classification in a population-based cohort of diffuse glioma
329 patients.

330
331 One of the major benefits of applying the NGS panel sequencing in routine glioma diagnostics concerns detection of a
332 spectrum of genetic alterations with prioritized, diagnostic impact including rare mutations that cannot be investigated by
333 standard IHC panels. For example, non-canonical *IDH1* or *IDH2* mutations are not detectable by IHC with the antibody
334 against IDH1-R132H [31]. In our series of WHO grade II and III gliomas, an additional 8% (23/303) of *IDH1* or *IDH2*
335 mutations were detected by NGS and this led to a change in diagnosis in up to 8% of the cases. Additional important
336 findings provided by NGS were *TERT* promoter mutation, *EGFR* amplification and *CDKN2A/B* homozygous deletion,
337 which have become diagnostically relevant in WHO grade II/III astrocytic gliomas. In our series of WHO grade II and
338 III gliomas, 30% (22/59) of IDH-wildtype gliomas carried a *TERT* mutation and/or *EGFR* amplification and 20% of IDH-
339 mutant gliomas showed *CDKN2A/B* homozygous deletion. This results in change of diagnosis according to cIMPACT-
340 NOW recommendations. *H3F3A* K27M and *BRAF* V600E were also detected by gene panel NGS in our study. In the
341 retrospective cohort, NGS revealed one *H3F3A* K27M mutation and one *BRAF* V600E, which upon review led to
342 reclassification of two anaplastic astrocytomas as a diffuse midline glioma, H3-K27M-mutant and an anaplastic
343 pleomorphic xanthoastrocytoma, respectively. Even though both *H3F3A* K27M and *BRAF* V600E mutations now can be
344 reliably detected by immunohistochemistry [32, 33], targeted NGS facilitate identification of a broad spectrum of
345 mutations up-front, which is useful in the differential diagnosis of diffuse gliomas.

346
347 As a result of our setup using both conventional testing with IHC analysis (IDH-R132H, p53, ATRX) and glioma gene
348 panel NGS, we were able to compare results from both assays. In line with previous studies [1, 3, 6, 26] we found that
349 IDH1 R132H IHC analysis was highly concordant with results of NGS, thus further confirming the reliability of this
350 antibody in the diagnostic setting (Supporting information, Table 3). IHC and mutational status of *ATRX* also was found
351 to be well-correlated with a 95% concordance rate, but loss of *ATRX* expression was not consistent with *ATRX* mutation
352 in 19% of the gliomas. These findings are similar to results found in other studies and have been related to technical

353 limitations or complex mutational and expressional patterns [14, 34] that need to be further investigated. In contrast,
354 assessment of p53 expression by IHC and detection of *TP53* mutations were not well correlated (concordance rate of
355 67%), consistent with the wide range of concordance rates shown previously in gliomas [35]. The poor correlation may
356 be explained by imperfect cut-off criteria, rare truncating mutations without nuclear p53 accumulation, and non-tumour
357 cells showing p53 expression [31]. IHC analysis results of p53 should therefore be interpreted with caution in the
358 differential diagnostics.

359
360 The mutational profiles identified for each molecular subgroup of gliomas in this study as well as the frequencies of
361 mutations for each subgroup were highly comparable with results obtained in large-scale sequencing studies like The
362 Cancer Genome Atlas (TCGA) project [36, 37] as well as targeted NGS panel studies [1, 3, 5, 7-9], thereby supporting
363 that targeted NGS is a robust technology with high sensitivity and inter-laboratory reproducibility.

364
365 Unfortunately, it emerged that the amplicon-based glioma panel NGS was not able to detect mutational frequencies for
366 the challenging *TERT* promoter at high sensitivity on all analysed tumour samples due to low read depth around the two
367 mutational hot spots ("C228T" and "C250T"). As a consequence for example, in IDH-mutant and 1p/19q-codeleted
368 oligodendroglial tumours, we thus observed a frequency of *TERT* promoter mutations of 61% in WHO grade II tumours
369 and 70% in WHO grade III tumours, whereas the reported frequency in studies using deep sequencing and frozen tissue
370 is > 95% [36, 38]. Only few targeted NGS panels include the *TERT* promoter and different authors report challenges to
371 some degree with the detection of mutations in the *TERT* promoter [5, 7, 9, 14]. Challenges related to low or variable read
372 depth from amplicons in GC-rich regions (e.g. the *TERT* promoter) as well as large amplicons (e.g. the coverage for *RBI*
373 was 90%) are known limitations of the IonTorrent AmpliSeq panels [39]. Capture-based sequencing is an alternative and
374 commonly used method, shown to be superior to amplicon-based sequencing by providing higher uniformity of coverage
375 depth and higher sensitivity for variant calling [40]. Novel molecular methods, such as droplet digital PCR and qPCR-
376 based allele specific assays are promising new techniques for fast and sensitive *TERT* promoter mutation detection [41,
377 42], which indeed is needed in molecular classification of diffuse gliomas.

378
379 A major aim of this study was to molecular reclassify a large retrospective cohort of diffuse glioma patients to get further
380 insights into the prognostic associations of integrated diagnostics of adult gliomas according to the 2016 WHO
381 classification. Comparison of survival data from patients with gliomas classified according to WHO 2007 versus WHO
382 2016 diagnoses revealed that addition of molecular biomarkers significantly alters OS associated with several entities.

383
384 Diffuse astrocytomas (WHO grade II) in our retrospective cohort were roughly divided into 70% IDH-mutant and 30%
385 IDH-wildtype tumours consistent with prior studies [43, 44]. Patients with IDH-wildtype diffuse astrocytomas showed
386 significantly shorter OS compared to patients with IDH-mutant diffuse astrocytomas. Similarly, patients with IDH-mutant
387 anaplastic astrocytomas showed a significantly longer OS when compared to patients with the respective IDH-wildtype
388 entity. Taken together, these findings support previous studies that the majority of IDH-wildtype diffuse and anaplastic
389 astrocytomas in adults in fact resemble histologically underdiagnosed IDH-wildtype glioblastomas [45]. The cIMPACT-
390 NOW consortium recently published a third update introducing *EGFR* amplification, *TERT* promoter mutation, and/or
391 combined whole chromosomal imbalances on 7 and 10 (+7/-10) as minimal molecular diagnostic criteria for a new tumour

392 category termed diffuse astrocytic glioma, IDH-wildtype, with molecular features of glioblastoma, WHO grade IV [18].
393 In line with this new recommendation, and results published very recently by Tesileanu *et al.* [46], we found that OS of
394 patients with IDH-wildtype diffuse or anaplastic astrocytomas carrying *TERT* promoter mutation and/or *EGFR*
395 amplification completely overlapped with OS of patients with IDH-wildtype glioblastomas. This finding would argue in
396 favour of considering the traditional histological features of malignancy, i.e., necrosis and/or microvascular proliferation,
397 and/or the presence of molecular alterations as defined by cIMPACT-NOW [18] in a revised definition of glioblastoma,
398 IDH-wildtype, WHO grade IV.

399
400 As expected, patients with IDH-mutant and 1p/19q-codeleted anaplastic oligodendroglioma showed significantly longer
401 OS when compared to patients with only histologically defined anaplastic oligodendrogliomas, which were found to be
402 significantly contaminated by IDH-wildtype glioblastomas. In patients with WHO grade II oligodendrogliomas, OS was
403 only slightly better for the group with IDH-mutant and 1p/19q-codeleted oligodendrogliomas, which to a larger extent
404 overlapped with the WHO 2007 classified oligodendroglioma WHO grade II group.

405
406 Taken together, the comparative survival analyses obtained in this study indicate that part of the results from prognostic
407 association studies prior to WHO 2016 may still have value, but that prognostic results based on cohorts classified only
408 by histology according to prior WHO criteria may not be comparable anymore, in particular for patients with diffuse
409 gliomas of WHO grade II or III.

410
411 In agreement with recent molecular reclassification studies, we showed a distinct prognostic segregation of glioma
412 subtypes with the 2016 WHO classification as well as a prognostic significance of IDH mutation over histological tumour
413 grade [26-29, 47]. Interestingly, however, was the significant prognostic difference found in OS between WHO grade II
414 and III IDH-mutant astrocytoma patients. Previous retrospective studies [48, 49] have shown only modest effect of
415 histological grade on OS in IDH-mutant astrocytomas. However, Shirahata *et al.* [50] reported a significant difference in
416 OS between WHO grade II and III IDH-mutant astrocytomas, although the difference decreased after further molecular
417 stratification based on *CDKN2A* homozygous deletion. Because there were only a few retrospective IDH-mutant
418 astrocytomas with sequencing data (n=12), we were not able to investigate whether the difference in OS in our series
419 were caused by uneven distribution of *CDKN2A/B* homozygous deletion. Similarly, and in line with our data, Cimino *et*
420 *al.* [51] and Yang *et al.* [52] reported on a prognostic role of WHO grade in IDH-mutant astrocytoma patients.

421
422 We have used glioma gene panel NGS up-front in daily diagnostics since mid-2016 where the panel has been part of the
423 routine diagnostics workflow at our institution. Successful prospective NGS analyses were obtained for 98% of the
424 gliomas in the prospective cohort. Since the panel was implemented in 2016, our practice and workflows have been
425 stepwise optimized to shorten the turn-around time. Initially, we had a glioma NGS panel run once per week, later on
426 twice per week and now in our current setup, we run the glioma panel daily together with other cancer panels (lung, colon)
427 providing a sufficient sample load to fill up the chips used in each run. With this setup, our turn-around time from time
428 of arrival of the sample until the final integrated pathology report is at best 7 working days (Supporting Information, Fig.
429 S4). The procedure comprises different steps and delays can occur due logistic challenges between the steps. For a few

430 cases, where the NGS must be repeated, due to e.g. poor tissue quality, the turn-around time can increase up to around 2
431 weeks.

432

433 Panel sequencing is a relatively expensive diagnostic method in comparison with conventional methods like IHC. The
434 costs of a single IHC section with a mutation-specific antibody are approximately 20 € with personnel time included and
435 since we usually perform around 6 immunostains per tumour, the overall costs per patient sample are close to 120 €.
436 Overall costs per patient sample using the 20-gene glioma panel are close to 600 € (Supporting Information Table S9).
437 Even though the costs and turn-around times for IHC are only a fraction of those of panel sequencing, we consider panel
438 sequencing as cost-effective, especially if the approach is focussed on those gliomas patients not suspected to have a
439 glioblastoma and being 55 years or older. When used up-front, the information on the broad spectrum of mutations in
440 multiple genes is available in time and therefore NGS panels can be a more cost-effective way to reach an accurate
441 molecular diagnosis as costs of additional testing are reduced. Further, the costs of treatment are significantly higher than
442 the costs of NGS testing. Allocation of patients to the right treatment early on avoid unnecessary treatment costs and lead
443 to more efficient use of healthcare resources. These economic aspects must be taken in to consideration, when justifying
444 the use of NGS panels.

445

446 From a 2020 diagnostic perspective, the glioma panel we have been using too date has some limitations. Detection of 1p
447 and 19q loss was not reliable due to limitations in the NGS panel design. Detection of CNVs in addition to 1p/19q-
448 codeletion, such as +7/-10 and *EGFR* amplification, has become of increasing importance especially after these
449 biomarkers have been included in a recent recommendation by cIMPACT-NOW [18]. To improve identification of CNVs
450 in general, incorporation of highly polymorphic SNPs (single-nucleotide polymorphisms) across relevant chromosomes
451 in the panel would significantly improve the assessment of combined losses on 1p and 19q, combined +7/-10 as well as
452 deletions of *CDKN2A/B*, with the latter becoming diagnostically relevant for prognostic assessment of IDH-mutant
453 astrocytic gliomas [50]. The principle of SNP incorporation into a targeted gene panel enables a single test for routine
454 brain tumour diagnostics in a molecular pathology laboratory and offers an attractive alternative to methods such as FISH
455 and immunohistochemistry [53].

456

457 In conclusion, we report that glioma gene panel NGS is a robust approach for detection of diagnostically relevant genetic
458 alterations in gliomas in the daily routine diagnostic setting. Using NGS in combination with IHC and FISH for
459 reclassification allowed for the comparison of prognostic associations in glioma subgroups defined by histological criteria
460 according to the WHO 2007 classification or by integrated histomolecular criteria according to the WHO 2016
461 classification and recent cIMPACT-NOW updates. For the classification of IDH-wildtype diffuse astrocytic gliomas, we
462 provide further support for a role of *TERT* promoter mutation and *EGFR* amplification as molecular markers for
463 aggressive tumours corresponding prognostically to IDH-wildtype glioblastoma. We additionally found that distinction
464 of WHO grade II and III astrocytic tumours retained prognostic significance in the group of IDH-mutant astrocytoma
465 patients. The future value of NGS panels in daily brain tumour diagnostics will require continued adjustment to novel
466 diagnostic criteria so that the need of complementary single gene-based methods can be reduced.

467 **Acknowledgments**

468 We thankfully acknowledge the excellent laboratory work performed by technician Helle Wohlleben and the technical
469 assistance from technician Lykke Korsgaard in the Chromosome laboratory as well as personnel in the PCR laboratory,
470 at the Department of Pathology, Odense University Hospital.

471
472 The project was supported by Region of Southern Denmark, University of Southern Denmark, Kathrine and Vigo
473 Skovgaard's Foundation, Sven Hansen and Wife Ina Hansen's Foundation, Georg Bjørkner's Foundation, Frode V.
474 Nyegaard and Wife's Foundation, M. Brogaard and Wife's Memorial Foundation, Hans and Nora Buchard's Foundation,
475 Agnethe Løvgreen's Foundation, Eva and Henry Frænkels Memorial Foundation, The A.P. Møller and Wife Chastine
476 Mc-Kinney Møller's Foundation, A.J. Andersen and Wife's Foundation and MD. Else Poulsen's Memorial Foundation.

477 **Ethical approval**

478 The study was approved by the Regional Committee on Health Research Ethics for Southern Denmark (Project-ID S-
479 20150148) as well as the Danish Data Protection Agency (file number: 16/11065). The use of tissue was not prohibited
480 by any patient according to the Danish Tissue Application Register.

481 **Data Sharing**

482 All data underlying the findings reported in the manuscript have been submitted as part of the article.

483 **Disclosure**

484 The authors declare no conflicts of interest.

485 **Conflict of Interest**

486 The authors declare that they have no conflict of interest. Prof. Guido Reifenberger, member of the NAN editorial
487 committee, has not been involved in the review process of this manuscript.

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777

778 **Figure legends**

779

780 **Figure 1**

781 Results of molecular reclassification of 529 adult diffuse gliomas from the retrospective cohort according to the WHO
782 2016 CNS tumour classification. A) The diagram shows the diagnostic change between histological diagnosis (WHO
783 2007, left side) and integrated histomolecular diagnosis (WHO 2016, right side). B) Summary of the diagnostic changes
784 in the glioma groups.

785

786 **Figure 2**

787 Unsupervised hierarchical cluster analysis performed on NGS data from 120 gliomas from the retrospective cohort.
788 1p/19q-codeletion status (FISH/850k) is included in the analysis. The analysis revealed three separate molecular
789 subgroups in line with the WHO 2016 Classification of CNS tumours; IDH-mutant astrocytic tumours, IDH-mutant and
790 1p/19q-codeleted oligodendroglial tumours and IDH-wildtype astrocytic tumours. Presence and absence of a mutation
791 were coloured black and grey, respectively. The WHO 2007 and WHO 2016 diagnoses are shown and coloured in the
792 column annotation above the heatmaps indicating the shift between the former histology-based classification and the
793 current integrated histomolecular classification. Army green bar indicate failed detection of *TERT* in some samples due

794 to low coverage. * indicate *EGFR* mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR*
795 deletion variant, **** indicate homozygous deletion of *CDKN2A/B*.

796

797

798 Figure 3

799 Unsupervised hierarchical cluster analysis performed on NGS data from 225 gliomas from the prospective cohort. 1p/19q-
800 codeletion status (FISH/850k) is included in the analysis. The analysis revealed three separate molecular subgroups in
801 line with the WHO 2016 Classification of CNS tumours; IDH-mutant astrocytic tumours, IDH-mutant and 1p/19q-
802 codeleted oligodendroglial tumours and IDH-wildtype astrocytic tumours. Presence and absence of a mutation were
803 coloured black and grey, respectively. The WHO 2016 diagnoses are shown and coloured in the column annotation above
804 the heatmaps. Army green bar indicate failed detection of *TERT* in some samples due to low coverage. * indicate *EGFR*
805 mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR* deletion variant, **** indicate
806 homozygous deletion of *CDKN2A/B*.

807

808

809 Figure 4

810 A summary of genetic alterations identified in 345 gliomas analysed (combined retro- and prospective data) with the
811 glioma panel.

812 A) Frequencies of gene mutations and CNVs across the glioma entities of the 2016 WHO classification. The different
813 entities have distinct mutational profiles.

814

815 B) Frequencies of gene mutations and CNVs found in WHO grade II/III astrocytic gliomas. These molecular alterations
816 are of diagnostic importance as described in the cIMPACT-NOW recommendation 3 (*TERT* promoter mutation, *EGFR*
817 amplification, combined whole chromosome 7 gain and whole chromosome 10 loss) and the cIMPACT-NOW 5
818 recommendation (*CDKN2A/B* homozygous deletion).

819

820 * indicate *EGFR* mutations, ** indicate high copy number *EGFR* amplification, *** indicate *EGFR* deletion variant, ****
821 indicate homozygous deletion of *CDKN2A/B*.

822

823

824 Figure 5

825 Kaplan-Meier overall survival curves showing the association between glioma subtypes classified in accordance to the
826 current 2016 CNS tumour classification and the former WHO 2007 CNS tumour classification. Log-rank p-values are
827 coloured, indicating comparison of astrocytic tumours: green: IDH-mutant, WHO 2016 vs. WHO 2007, blue: IDH-
828 wildtype, WHO 2016 vs. WHO 2007, grey: IDH-mutant, WHO 2016 vs. IDH-wildtype, WHO 2016. For the
829 oligodendroglial tumours yellow p-value indicate comparison between WHO grade II tumours (WHO 2016 vs. WHO
830 2007) and orange p-value indicate comparison between WHO grade III tumours (WHO 2016 vs. WHO 2007). E) Overall
831 comparison of WHO 2016 diagnoses. F) Comparison of diffuse astrocytic gliomas, IDH-wildtype, with molecular
832 features of glioblastomas, WHO grade IV and IDH-wildtype glioblastomas, WHO grade IV.

833

834 Figure S1

835 Flow diagram on molecular testing approach used on the retrospective cohort

836 All gliomas in the retrospective cohort were initially stained by IHC with antibodies against IDH1-R132H and in most
837 cases also ATRX and p53. To identify less common *IDH1* and *IDH2* mutations not detectable by IHC, targeted NGS
838 analysis was performed when the IDH1-R132H staining was negative. Status on *TERT* and *EGFR* amplification was
839 investigated in the remaining WHO grade II and III IDH-wildtype astrocytomas for stratification according to cIMPACT-
840 NOW recommendation 3. All WHO grade II and III IDH-mutant gliomas with retained nuclear ATRX expression were
841 further tested for 1p/19q-codeletion by FISH (or 850k DNA methylation arrays) to refine the classification of gliomas
842 into IDH-mutant astrocytomas or IDH-mutant and 1p/19q-codeleted oligodendrogliomas. IDH-mutant gliomas without
843 1p/19q-codeletion were tested for *CDKN2A/B* homozygous deletions for stratification according to cIMPACT-NOW
844 recommendation 5. For glioblastomas, IDH status was investigated by IHC and not followed by targeted NGS analysis.
845 NGS was not performed on WHO grade IV gliomas as the majority of included patients with IDH-wildtype glioblastoma
846 (86%, 280/327) were older than 55 years at the time of diagnosis. Thereby, we followed the proposed age cut-off given
847 by the WHO 2016 classification for IDH molecular testing [54].

848

849 Figure S2

850 Flow diagram on molecular diagnostic approach used on the prospective cohort (2016-2018)

851 All gliomas in the prospective cohort underwent NGS panel sequencing and results were used in the integrated diagnostic
852 work-up together with results from IHC including IDH1-R132H, ATRX, p53 and 1p/19q-codeletion status (FISH or
853 850k). Stratification according to cIMPACT-NOW recommendation 3 and 5 was done to obtain mutational profiles and
854 not used for final diagnostic classification in the study period.

855

856 Figure S3

857 Kaplan-Meier overall survival curve of WHO grade II/III IDH-mutant astrocytomas with and without *CDKN2A/B*
858 homozygous deletion.

859

860 Figure S4

861 Flow diagram of NGS workflow and the 7-day turn-around time.

862 Turn-around time for the used NGS setup from time of arrival of the sample until the final integrated pathology report is

863 7 working days.

Table 1 Patient characteristics – retrospective cohort

	DA		AA		GBM		OA		AOA		OD		AOD	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Patients	60		53		327		11		15		20		43	
Age in years (mean, range, ±SD)	43.6 (18.2-78.5) ±15.45		57.4 (20.35-83.9) ±16.17		62.8 (27.1-89.0) ±11.60		53.6 (27.0-74.8) ±17.20		60.0 (31.9-82.3) ±15.34		46.3 (26.2-75.2) ±11.72		57.3 (25.8-79.1) ±12.13	
Gender														
Male	39	65	33	62.2	201	61.5	10	90.9	6	40	11	55.0	27	62.8
Female	21	35	20	37.8	126	38.5	1	9.1	9	60	9	45.0	16	37.2
Performance status														
0-1	30	88.2	24	68.6	222	70.6	10	90.9	12	80.0	16	88.9	36	83.7
2-4	4	11.8	11	31.4	93	29.4	1	9.1	3	20.0	2	11.1	7	16.3
Unknown	26		18		12		0		0		2		0	
Status														
Alive	15	25	0	0	6	1.8	5	45.4	0	0	10	50.0	7	16.3
Dead	45	75	53	100	321	98.2	6	54.6	15	100	10	50.0	36	83.7
Treatment														
None	35	92.1	5	13.9	35	10.8	9	81.8	3	20	17	85.0	6	14
Stupp protocol	0	0	4	11.1	208	64.1	0	0	2	13.3	0	0	5	11.6
Radiotherapy, 59 Gy/- chemotherapy	2	5.3	21	58.3	23	7.1	2	18.2	9	60	3	15.0	27	62.8
Radiotherapy, 34 Gy +/- chemotherapy	1	2.6	6	16.7	51	15.8	0	0	0	0	0	0	4	9.3
Chemotherapy alone	0	0	0	0	8	2.2	0	0	1	6.7	0	0	1	2.3
Unknown	22		17		2		0		0		0		0	
Survival (median, months)	65.9		13.9		11.97		72.0		17.0		138.8		12.4	

DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade III.

Table 2 Patient characteristics after reclassification of the retrospective cohort in accordance to the 2016 WHO CNS classification.

	DA IDH mutant		DA IDH wildtype		AA IDH mutant		AA IDH wildtype		GBM IDH mutant		GBM IDH wildtype		OD IDH mutant		AOD IDH mutant	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Patients	46		16		22		23		13		370		22		15	
Age in years (mean, range, ±SD)	37.3 (18.2-69.2) ±11.45		57.1 (23.6-78.5) ±14.36		42.5 (20.4-66.2) ±12.74		59.6 (31.8-83.9) ±14.47		50.8 (27.2-79.1) ±17.30		63.4 (25.8-89.0) ±10.83		49.1 (26.2-74.8) ±13.26		56.4 (33.9-72.7) ±13.25	
Gender																
Male	28	60.9	11	68.8	14	63.6	12	52.2	5	38.5	229	61.9	15	68.2	11	73.3
Female	18	39.1	5	31.3	8	36.4	11	47.8	8	61.5	141	38.1	7	31.8	4	26.7
Performance status																
0-1	28	93.3	5	71.4	17	94.4	8	44.4	7	63.6	255	72.2	17	85.0	14	93.3
2-4	2	6.7	2	28.6	1	5.6	10	55.6	4	36.4	98	27.8	3	15.0	1	6.7
Unknown	16		9		4		5		2		17		2		0	
Status																
Alive	16	34.8	1	6.3	2	9.1	0	0.0	1	7.7	6	1.6	11	50.0	6	40.0
Dead	30	65.2	15	93.8	20	90.9	23	100.0	12	92.3	364	98.4	11	50.0	9	60.0
Treatment																
None	30	93.8	6	75	1	5.6	6	33.3	0	0	42	11.6	21	95.5	3	20.0
Stupp protocol	0	0	0	0	2	11.1	0	0	8	72.7	206	56.9	0	0	2	13.3
Radiotherapy, 59 Gy/- chemotherapy	2	6.2	1	12.5	14	77.8	9	50.0	0	0	53	14.7	1	4.5	8	53.3
Radiotherapy, 34 Gy +/- chemotherapy	0	0	1	12.5	0	0	3	16.7	3	27.3	54	14.9	0	0	1	6.7
Chemotherapy alone	0	0	0	0	1	5.6	0	0	0	0	7	1.9	0	0	1	6.7
Unknown	14		8		4		5		2		8		0		0	
Survival (median, months)	83.7		19.4		37.1		10.0		26.0		11.4		138.8		86.7	

DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III. Data on the two patients with PXA and DMG are not shown.

Table 3 Hazard ratio of overall survival between different glioma subgroups based on WHO 2007 and WHO 2016 classifications.

Reference WHO 2007	DA			AA			GBM			OD			AOD		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
WHO 16															
DA IDH mutant	0.74	(0.47-1.17)	0.199												
DA IDH wildtype	2.47	(1.36-4.49)	0.003												
AA IDH mutant				0.44	(0.26-0.75)	0.002									
AA IDH wildtype				1.59	(0.96-2.64)	0.073									
GBM IDH mutant							0.55	(0.31-0.98)	0.043						
GBM IDH wildtype							1.1	(0.92-1.25)	0.355						
OD IDH mutant, 1p/19q-codelet.										0.94	(0.40-2.22)	0.893			
AOD IDH mutant, 1p/19q-codelet.													0.44	(0.21-0.92)	0.029

WHO 2007: DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade.

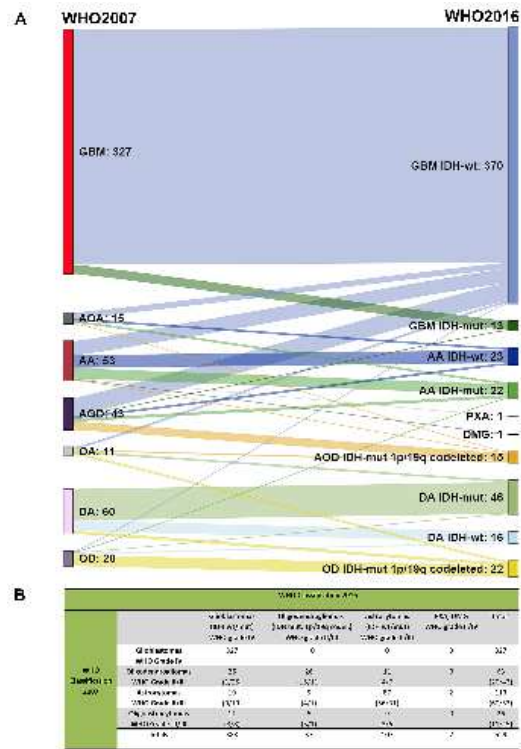
WHO 2016: DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III.

Table 4 Hazard ratio of overall survival between different glioma subgroups based on WHO 2016 classification.

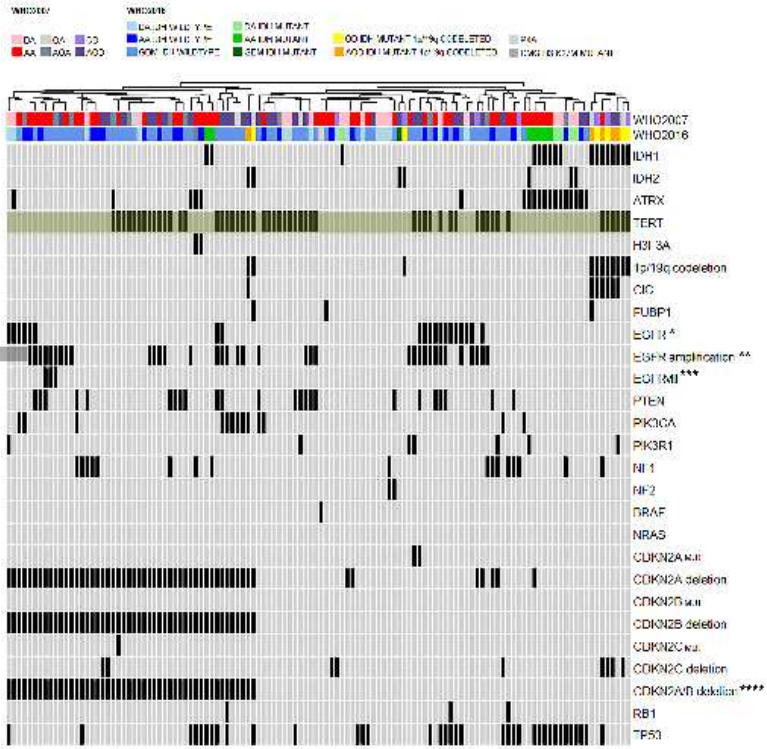
Reference WHO 2016	DA IDH mutant			DA IDH wildtype			AA IDH mutant			AA IDH wildtype			GBM IDH mutant			OD IDH mutant, 1p/19q-codeleted		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
WHO 16																		
DA IDH mutant	1.00																	
DA IDH wildtype	3.68	(1.94-6.97)	0.000	1.00														
AA IDH mutant	2.90	(1.61-5.20)	<0.001	0.68	(0.35-1.35)	0.276	1.00											
AA IDH wildtype	18.5	(7.90-43.50)	<0.001	2.17	(1.08-4.35)	0.028	4.58	2.24-9.33	<0.001	1.00								
GBM IDH mutant	4.75	(2.30-9.77)	<0.001	0.93	(0.43-1.99)	0.844	1.58	0.77-3.26	0.215	0.39	0.18-0.84	0.015	1.00					
GBM IDH wildtype	7.10	(4.72-10.70)	<0.001	1.92	(1.14-3.25)	0.015	2.89	1.82-4.57	<0.001	0.81	0.53-1.23	0.318	1.93	1.08-3.44	0.026			
OD IDH mutant, 1p/19q-codelet.																1.00		
AOD IDH mutant, 1p/19q-codelet.																2.17	0.88-5.31	0.091

WHO 2007: DA = diffuse astrocytoma, WHO grade II, AA = anaplastic astrocytoma, WHO grade III, GBM = glioblastoma, WHO grade IV, OA = oligoastrocytoma, WHO grade II, AOA = anaplastic oligoastrocytoma, WHO grade III, OD = oligodendroglioma WHO grade II, AOD = anaplastic oligodendroglioma, WHO grade.

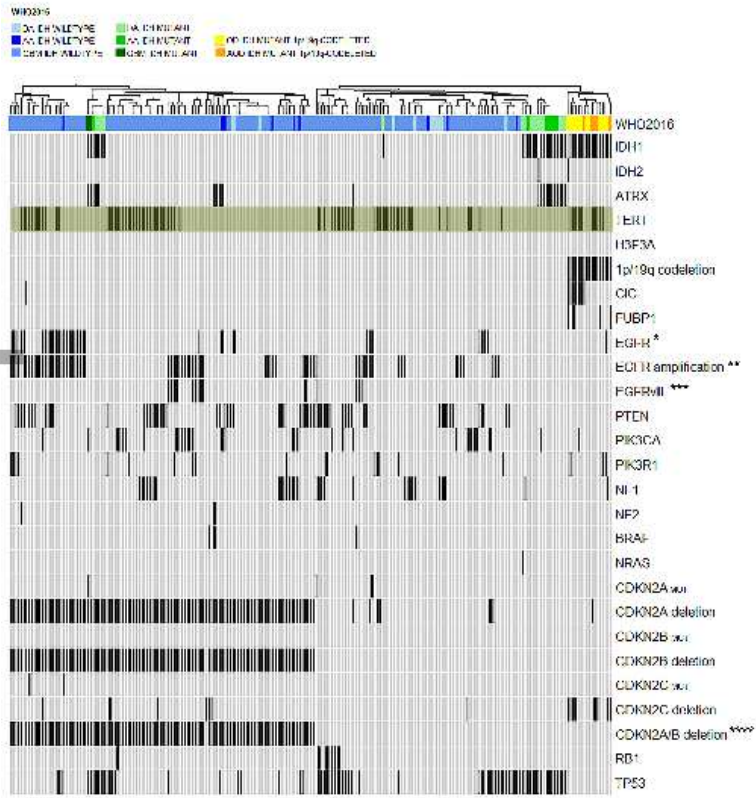
WHO 2016: DA IDH wildtype = diffuse astrocytoma, IDH-wildtype, WHO grade II, AA IDH wildtype = anaplastic astrocytoma, IDH-wildtype, WHO grade III, GBM IDH wildtype = glioblastoma, IDH-wildtype, WHO grade IV, DA IDH mutant = diffuse astrocytoma, IDH-mutant, WHO grade II, AA IDH mutant = anaplastic astrocytoma, IDH-mutant, WHO grade III, GBM IDH mutant = glioblastoma, IDH-mutant, WHO grade IV, OD IDH mutant 1p/19q-codeleted = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II, AOD IDH mutant 1p/19q-codeleted = anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade III.



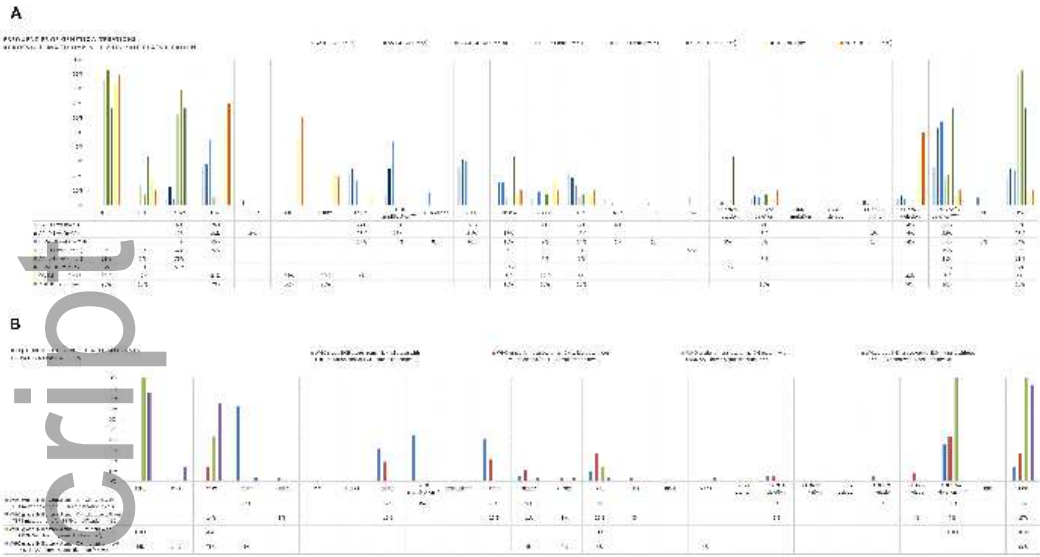
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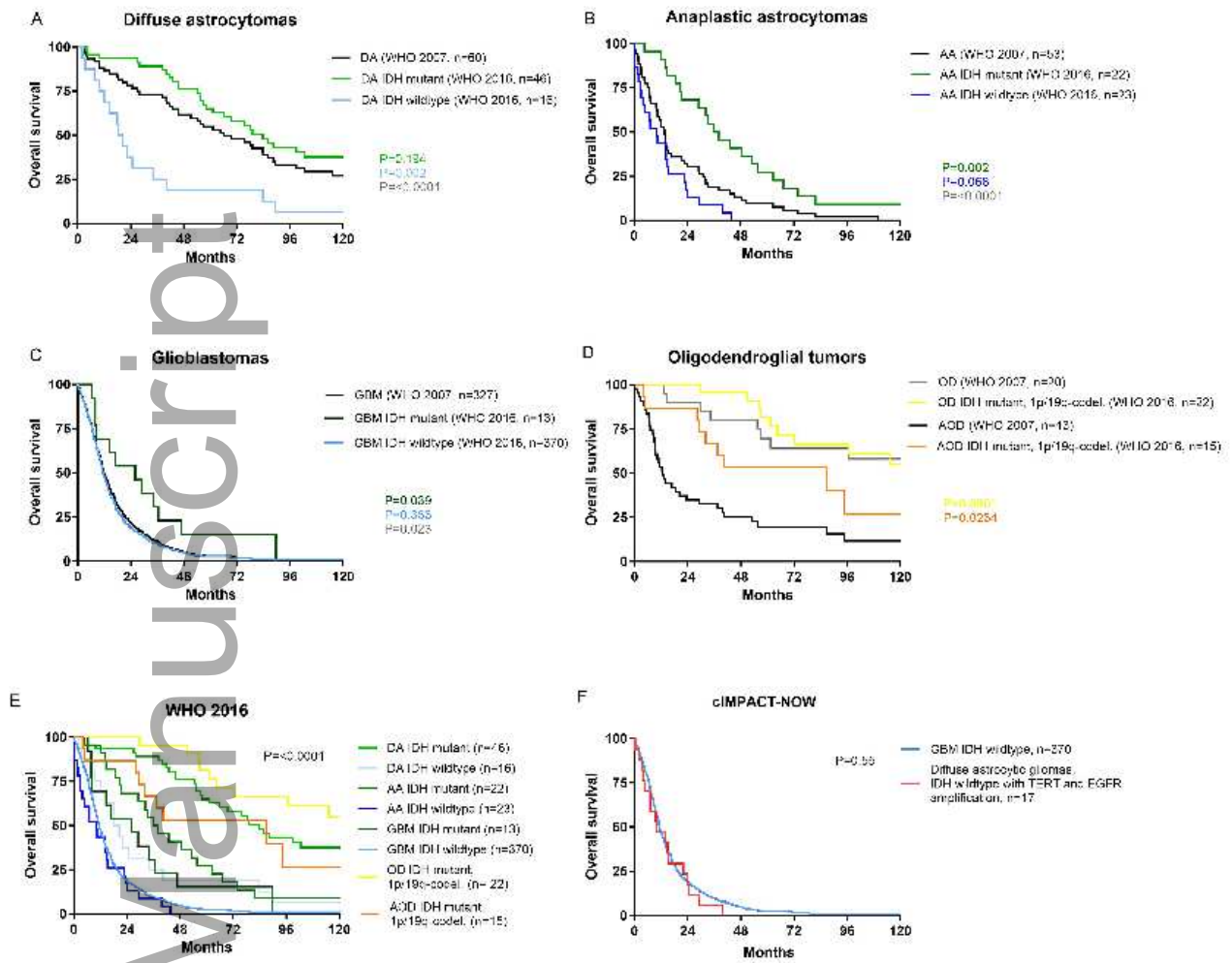
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