

1 **Glioma Through the Looking GLASS: the Glioma**  
2 **Longitudinal Analysis consortium, molecular evolution of**  
3 **diffuse gliomas**

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5 The GLASS consortium\*

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134 **Abstract**

135 Adult diffuse glioma are a diverse group of intracranial neoplasms  
136 associated with a disproportional large number of productive life years  
137 lost, thus imposing a highly emotional and significant financial burden on  
138 society. Patient death is the result of an aggressive course of disease  
139 following diagnosis. The Cancer Genome Atlas and similar projects have  
140 provided a comprehensive understanding of the somatic alterations and  
141 molecular subtypes of glioma at diagnosis. However, gliomas undergo  
142 significant molecular evolution during the malignant transformation. We  
143 review current knowledge on genomic, epigenomic and transcriptomic  
144 abnormalities before and after disease recurrence. We outline an effort  
145 to systemically catalogue the longitudinal changes in gliomas, the  
146 Glioma Longitudinal Analysis Consortium. The GLASS initiative will  
147 provide essential insights into the evolution of glioma towards a lethal  
148 phenotype with the potential to reveal targetable vulnerabilities, and  
149 ultimately, improved outcomes for a patient population in need.

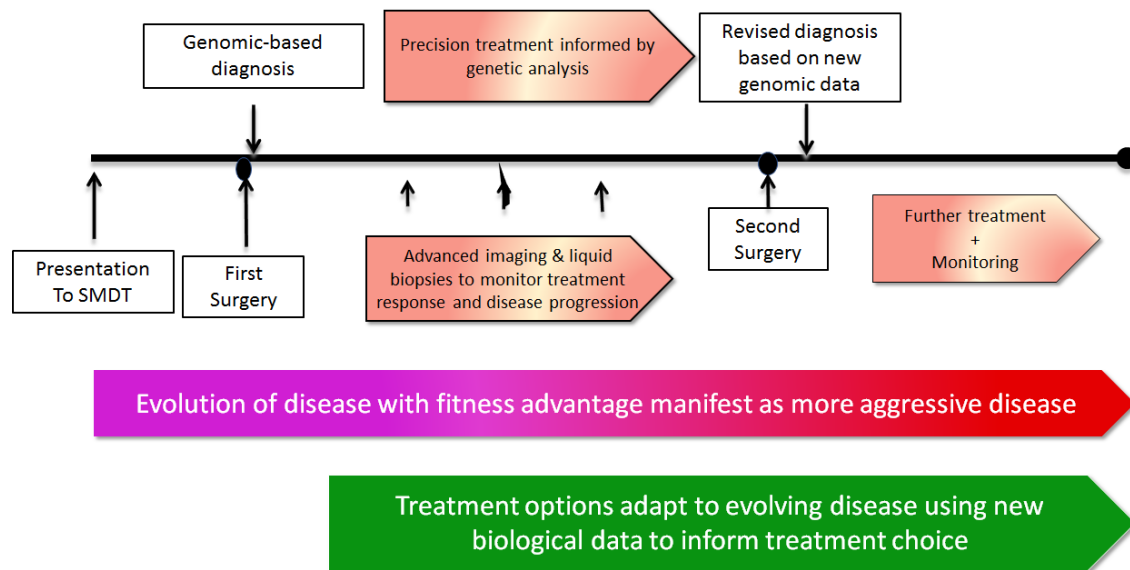
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## 151 **1. Introduction**

152 Diffuse gliomas are the most frequent malignant primary brain tumors in adults.<sup>1</sup>  
153 Almost all relapse despite intense treatment with surgery, radiation, and  
154 chemotherapy. The most common and most aggressive gliomas, glioblastoma  
155 (GBM), are IDH-wildtype and classified as World Health Organization (WHO) grade  
156 IV. They are characterized by a median overall survival that has remained static at  
157 around 15 months for decades even in selected clinical trial populations.<sup>2-5</sup> Patients  
158 with lower-grade (WHO grades II and III) IDH mutated gliomas have a more  
159 favorable prognosis but their tumors are also lethal, as they too recur and become  
160 resistant to therapy.<sup>1</sup> The standard of care for infiltrative/ diffuse gliomas is maximal  
161 safe resection, followed by chemoradiation.<sup>6</sup> Patients are then monitored for disease  
162 progression by imaging at regular intervals following surgery. Evaluation of disease  
163 progression is commonly guided by specific criteria (e.g. Response Assessment in  
164 Neuro-Oncology (RANO)),<sup>7</sup> which rely on visual evaluation of contrast enhancement  
165 and the non-enhancing hyperintense area on T2-weighted imaging. Radiologic  
166 features sometimes do not distinguish between true tumor progression and its  
167 imaging mimicker, pseudo-progression, and disease progression cannot be reliably  
168 established based on imaging alone. Inaccurate assessment can result in premature  
169 withdrawal from a specific treatment or to continue an ineffective therapy. A further  
170 challenge in particular in the monitoring of lower-grade gliomas is the prediction of  
171 malignant transformation without another surgical intervention leading to a new  
172 histological analysis. At present, this is based on clinical progression and rather  
173 imprecise imaging signs such as contrast enhancement in a previously non-  
174 enhancing lesion or increase in size of measurable abnormalities, that are better  
175 indicators of existing imaging features rather than impending malignant  
176 transformation.

177         Molecular characterization of gliomas in recent years has advanced our  
178 understanding of their genesis and has identified abnormalities that allow a better  
179 classification and may be therapeutically targetable<sup>8</sup>. Through efforts by the Cancer  
180 Genome Atlas (TCGA),<sup>9-14</sup> the genomes of over 1,100 grades II–IV gliomas have  
181 been characterized in detail, with other groups contributing further important results  
182 and datasets.<sup>15-20</sup> This wealth of information has provided a detailed molecular  
183 portrait of primary glioma. The atlas, by design, focused on untreated tumors. The

184 next frontier in glioma genomics is to understand recurrent disease. This is  
185 important, as patients generally die from tumor regrowth after therapy that becomes  
186 increasingly more resistant. Recent reports on pilot sets of paired tumors obtained  
187 before and after therapy show that there are many differences between the primary  
188 neoplasm at diagnosis and the recurrent tumor.<sup>21</sup> Malignant progression of gliomas,  
189 similar to other cancers, is the result of an evolutionary process that involves  
190 reiterative cycles of clonal expansion, genetic diversification, and clonal selection  
191 under microenvironmental pressures, including overcoming antitumor immune  
192 responses.<sup>22</sup> The presence of multiple cell populations with an array of different  
193 mutations is at least partly responsible for rapid induction of intrinsic resistance to  
194 therapy in gliomas.<sup>23</sup> Adaptive epigenetic and phenotypic responses are equally  
195 important. The emerging understanding of this dynamic evolution of the glioma  
196 genome has major implications for cancer biology research and potential  
197 development of effective therapy. However, like the molecular landscape of primary  
198 tumors, a full understanding of the dynamic evolution of gliomas there can only be  
199 achieved through (a) profiling of sufficiently large patient cohorts to achieve statistical  
200 significance and (b) standardization across biospecimen processing and data  
201 platforms. The Glioma Longitudinal AnalySiS (GLASS) consortium has been initiated  
202 to generate a comprehensive molecular and radiological portrait from paired primary  
203 and recurrent adult gliomas, including clinical patient data, to enable discovery of  
204 vulnerabilities that render the tumor sensitive to therapeutic intervention (Figure 1).  
205  
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207

208 **Figure 1. Usual course of glioma management.** GLASS would improve the assessment of gliomas,  
 209 particularly prediction of malignant transformation, treatment monitoring, and assessment of tumor  
 210 alterations non-invasively with imaging and/or liquid biopsies. SMDT: Specialist multidisciplinary team;  
 211 CSF: Cerebrospinal fluid; RT: Radiotherapy; TMZ: Alkylating antineoplastic agent Temozolomide;  
 212 1p19q: Short arm of chromosome 1 (1p) and long arm of chromosome 19 (19q); IDH: Isocitrate  
 213 dehydrogenase; MGMT: O-6-methylguanine-DNA methyltransferase; TERT: Telomerase reverse  
 214 transcriptase.

215

## 216 **2. Molecular profiling offers new possibilities for diagnosis and** 217 **therapy of gliomas**

### 218 **2.1 Clinical classification of adult diffuse glioma**

219 For over a century, microscopic evaluation provided the gold standard for diagnosis  
 220 of diffuse gliomas, and assessment of prognosis and therapeutic management of  
 221 patients were based on histopathologic diagnosis.<sup>24</sup> Over the past two decades, it  
 222 has become clear that combination of histopathology with specific molecular  
 223 characteristics of gliomas provides a more robust and objective basis for clinical  
 224 stratification.<sup>8,11,13,17,19,25-30</sup> Three large clinically relevant subgroups of diffuse  
 225 gliomas in adults can be defined on the basis of firstly, presence or absence of  
 226 mutations in the isocitrate dehydrogenase (*IDH*) 1 gene or *IDH2* gene and secondly,  
 227 within the category of IDH mutated glioma, either complete 1p/19q codeletion  
 228 (combined loss of the short arm of chromosome 1 and the long arm of chromosome  
 229 19) or *TP53* mutation and *ATRX* loss.<sup>11,13,17,19,25,27-30</sup> Most WHO grade II and III  
 230 diffuse astrocytomas and oligodendrogliomas are IDH mutant, and about a third of  
 231 these contain 1p/19q codeletion. In contrast, approximately 95% of glioblastomas



232 are IDH-wildtype.<sup>14</sup> IDH mutations are centered on codon 132 of *IDH1* and 172 of  
233 *IDH2*. About 90% of IDH-mutant gliomas are R132H *IDH1* mutant.<sup>31,32</sup> The protein  
234 resulting from this mutation can be recognized by a highly specific and sensitive  
235 antibody available for clinical practice<sup>31</sup>, or through targeted sequencing. Remaining  
236 IDH-mutant cases may carry alternate R132 alleles such as R132C or variants in  
237 *IDH2*. Expert consensus on how these molecular data should be implemented in  
238 routine clinical practice<sup>26</sup> led to revision in 2016 of the WHO 2007 classification of  
239 CNS tumors.<sup>8</sup> These revisions indeed integrate histopathological and molecular data  
240 <sup>8</sup> and for the first time, this scheme provides integrated data for diagnosis, prognostic  
241 grading, and guidance of therapeutic decisions.<sup>33,34</sup> In the revised classification,  
242 mutations in genes such as *TP53*, and *ATRX* can also be added to support or refine  
243 the diagnosis but are not mandatory. However, even this greatly improved  
244 classification system is predicated on primary, untreated disease; it is still unclear  
245 how these molecular markers impact the biology and prognosis of post-therapy,  
246 recurrent glioma. The promoter DNA methylation status of the gene *MGMT* is  
247 predictive of response to temozolomide therapy in primary tumors and this status is  
248 thought to be mostly stable between primary and recurrent disease<sup>35</sup>. The value of  
249 re-testing *MGMT* status after disease progression is debatable and a methylated  
250 *MGMT* promoter continues to predict treatment response at this stage.

251

## 252 **2.2 Intratumoral heterogeneity in primary gliomas**

253 Cancer results from a single normal cell that has acquired molecular alterations  
254 providing it with a proliferative advantage. In glioma, the most frequent somatic  
255 abnormalities are thought to be founding events. This included the mutations in the  
256 IDH genes and mutations in the promoter of telomerase reverse transcriptase  
257 (*TERT*) gene, especially characteristic of IDH-wildtype GBM and IDH-mutant,  
258 1p/19q-codeleted gliomas.<sup>25</sup> Major aneuploidy, such as the 1p/19q co-deletion but  
259 also whole chromosome 7 gain and chromosome 10 loss which is common in IDH-  
260 wildtype glioma, are also thought to be glioma originating alterations.<sup>36-38</sup> The  
261 divergence in somatic abnormality profiles between the three major glioma subtypes,  
262 IDH-wildtype, IDH mutant co-deleted, IDH mutant non-codeleted, converges with  
263 different patient age at diagnosis distributions, which strongly suggests that the three  
264 groups represent distinct gliomagenic biologies.

265 Gliomas display significant intertumoral and intratumoral heterogeneity:  
266 cancer cells from the same tumor cell of origin may contain a wide range of genetic  
267 and epigenetic states.<sup>39-41</sup> Intratumoral heterogeneity confounds diagnosis,  
268 challenges the design of effective therapies, and is a determinant of tumor  
269 resistance.<sup>42</sup> Molecular heterogeneity in GBM has been characterized with multiple  
270 approaches. For example, fluorescent in-situ hybridization (FISH) analysis of the  
271 most commonly amplified receptor tyrosine kinases (RTK) in GBM (*EGFR*, *PDGFRA*  
272 and *MET*) revealed a mosaic of tumor subclones marked by different RTK  
273 amplifications in 2–3% of GBM,<sup>43-45</sup> possibly indicating cooperation between cell  
274 populations. Single-cell sequencing demonstrated comparable non-overlapping  
275 subclonal GBM cell populations marked by different *EGFR* truncation variants,  
276 suggesting convergent evolution of *EGFR* mutations.<sup>46</sup> Genomic profiling of spatially  
277 distinct tumor sectors has revealed partial overlap in mutation content in multiple  
278 samples from IDH-mutant lower-grade glioma<sup>19,38,47,48</sup> and IDH-wildtype  
279 GBMs.<sup>36,37,49-51</sup> Mutations/DNA copy number alterations in important glioma driver  
280 genes such as *TP53* and *PTEN* have been found to be subclonal, i.e. not present in  
281 all cells from the same tumor, suggesting they were acquired after tumor initiation.  
282 These unexpected discoveries show the many options tumor cells have to  
283 circumvent anti-tumorigenic hurdles such as senescence and geno7c instability. The  
284 possibility of extrachromosomal oncogene amplification adds an additional layer of  
285 complexity, allowing tumors to rapidly increase intratumoral heterogeneity in  
286 response to a microenvironment sparse in resources.<sup>52-57</sup>

287 Mutation retention rates may be correlated with the geographical distance of  
288 samples in the tumor,<sup>51</sup> and by extension, the level of heterogeneity between  
289 different lesions of multifocal GBM is greater than between different areas of the  
290 same GBM.<sup>51,58,59</sup> Spatial heterogeneity determined by genetic alterations is  
291 reflected in the epigenetic patterns of different tumor sections examined by  
292 combined analysis of DNA methylation and genetic abnormalities.<sup>47,50</sup> These  
293 accumulating data suggest that intratumoral heterogeneity is encoded through a  
294 genomic-epigenomic codependent relationship,<sup>47</sup> in which epigenetic changes may  
295 modulate mutational susceptibility in proximal cells, and specific mutations dictate  
296 aberrant epigenetic patterns.<sup>47,60,61</sup> Although gene expression signatures can be  
297 used to subclassify GBMs, the predominant subtype often varies from region to  
298 region within a given tumor.<sup>37,50</sup> This relative instability may be in part due to the

299 variable levels of tumor-associated non-neoplastic cells that can be found in different  
300 parts of the tumor.<sup>62,63</sup> Single-cell RNA sequencing of GBM cells confirmed that  
301 multiple subtype classifications can be detected in all tumors, often with one subtype  
302 dominating the others.<sup>51,63-65</sup> Transcriptomics and genomics converge at the single-  
303 cell level with the observation of mosaic expression of RTK, extending previous  
304 observations of mosaic RTK amplification in a small subset of GBM to being a more  
305 frequent disease characteristic.<sup>64,65</sup> Single-cell RNA sequencing further has shown  
306 that all gliomas contain cellular hierarchies along an axis of undifferentiated  
307 progenitors to more differentiated cell populations, reminiscent of the hematopoietic  
308 stem cell hierarchy. The balance shifts towards proliferating progenitors in IDH-  
309 wildtype glioma reflecting the clinically more aggressive disease course.<sup>66-68</sup> Such  
310 developmental and functional hierarchies are associated with dynamic neural stem  
311 cell expression patterns in which stem or progenitor cells may function as units of  
312 evolutionary selection.<sup>69</sup>

313

### 314 **2.3 Longitudinal DNA profiling in pre-treatment and post-treatment** 315 **tumors**

316 One of the earliest reports on the effects of therapy on the tumor genomic landscape  
317 analyzed a 23-patient cohort of IDH-mutant lower-grade glioma treated with  
318 temozolomide chemotherapy.<sup>70</sup> A subset of the recurrent tumors acquired hundreds  
319 of new mutations that bore a characteristic signature of temozolomide-induced  
320 mutagenesis, suggesting that treatment pressure from an alkylating agent induced  
321 the growth of tumor cells with new mutations (therapy-induced acquired  
322 resistance).<sup>38</sup> These hypermutated tumors may be sensitive to immune checkpoint  
323 inhibitors,<sup>23</sup> including programmed death-1 (PD-1) inhibitors<sup>71</sup> and poly-adenosine  
324 diphosphate ribose polymerase (PARP) inhibitors (PARPi).<sup>72</sup> However, clinical trial  
325 data supporting these hypotheses have yet to emerge. Another study used whole-  
326 genome and multisector exome sequencing of 23 predominantly IDH-wildtype GBM  
327 and matched recurrent tumors.<sup>36</sup> The study showed that some GBM recurrences  
328 bore ancestral p53 driver mutations detectable in the primary GBM counterparts  
329 (intrinsic resistance), while other recurrences were driven by branched subclonal  
330 divergent mutations not present in the parental primary GBM. This may reflect  
331 treatment-induced resistance through DNA mutagenesis and a distinct evolutionary

332 process.<sup>36</sup> As in the study of IDH-mutant lower-grade glioma, a subset of the disease  
333 recurrences were characterized by an accumulation of mutations in association with  
334 temozolomide treatment. Notably, this effect was limited to cases with *MGMT*  
335 promoter methylation. *MGMT* is a gene in the DNA repair pathway, and mutations of  
336 other pathway members, such as *MSH2* and *MSH6*, have been nominated as drivers  
337 of the hypermutation process.<sup>73</sup> The spatiotemporal evolutionary trajectory in paired  
338 gliomas between initial diagnosis and relapse was further portrayed via integrative  
339 genomic and radiologic analyses. Whole-exome sequencing of 38 primary and  
340 corresponding recurrent tumors revealed two prevalent patterns of tumor evolution.  
341 Linear evolution, in which a recurrent tumor is genetically similar to the initial tumor,  
342 was predominantly observed in a subset of recurrent tumors that relapsed adjacent  
343 to the primary tumor site. Branched evolution was more common in recurrences at  
344 distant sites, which were marked by a substantial genetic divergence in their  
345 mutational profile from the initial tumor, with key driver alterations differing in more  
346 than 30% of the cases, demonstrating branched evolution. Geographically separated  
347 multifocal tumors and/or long-term recurrent tumors were seeded by distinct clones,  
348 as predicted by an evolution model defined as multiverse, i.e. driven by multiple  
349 subclonal cell populations.<sup>51</sup>

350 Comprehensive genomic analysis of the processes regulating tumor evolution  
351 necessitates serial profiling of pre-treatment and post-treatment tumors. Patients  
352 receiving medical care may move to a different medical center in the interval  
353 between initial diagnosis and recurrence, which creates significant challenges for the  
354 serial collection of tumor tissue. In an effort to elucidate the diverse evolutionary  
355 dynamics by which gliomas are initiated and recur, the clonal evolution of GBM  
356 under therapy was assessed from an aggregated analysis of datasets generated by  
357 multiple institutions.<sup>74</sup> Systematic review of the exome sequences from 93 patients  
358 revealed highly branched evolutionary patterns involving a Darwinian process of  
359 clonal replacement in which a subset of clones with selective advantage during a  
360 standard treatment regimen renders the tumor susceptible to malignant progression.  
361 Mathematical modeling delineated the sequential order of somatic mutational events  
362 that constitute GBM genome architecture, identifying mutations in *IDH1*, *PIK3CA*,  
363 and *ATRX* as early events of tumor progression, whereas *PTEN*, *NF1*, and *EGFR*  
364 alterations were predicted to occur at a relatively later stage of the evolution.<sup>51</sup>  
365 Similar observations have been reported from comprehensive studies of low-grade

366 gliomas, demonstrating that the mutations in *IDH1*, *TP53*, and *ATRX* were frequently  
367 acquired and retained throughout tumor progression from primary to relapse<sup>19,48</sup>.  
368 Additionally, these studies have demonstrated crucial insights into oncogenic  
369 pathways that drive malignant progression of low-grade gliomas with mutations in  
370 *IDH1*, suggesting convergence on aberrantly activated MYC, RB and RTK-RAS-  
371 PI3K signaling pathways. Longitudinal profiling of paired samples continues to reveal  
372 deeper insights into the genomic background of treatment-induced  
373 hypermutagenesis and its potential increased aggressive clinical behavior and  
374 relevance in targeted therapy and immunotherapy.<sup>19,48,75,76</sup> The implications of these  
375 data and how these insights can be integrated into clinical practice require further  
376 evaluation. Collectively, longitudinal genomic profiling will be essential in  
377 implementing clinical application towards patient-tailored treatment regimen.  
378

## 379 **2.4 Transcriptional changes during glioma progression**

380 Unsupervised transcriptome analysis of GBM converged on four expression  
381 subtypes, referred to as classical, mesenchymal, neural, and proneural, which are  
382 associated with specific genomic abnormalities.<sup>14,15,18,77</sup> The proneural and  
383 mesenchymal subtypes have been most consistently confirmed in the literature,  
384 whereas the neural type may simply represent GBM containing a relatively high  
385 amount of admixed non-neoplastic neurons.<sup>63,78</sup> Transcriptional subtyping of the  
386 relatively homogeneous IDH-mutant and 1p/19q-codeleted groups have been less  
387 emphasized in the literature, as these cases usually carry a proneural signature.<sup>12,14</sup>  
388 While expression subtype classification is a widely used research tool, it has not  
389 been shown to correlate with clinical outcome, and has not been incorporated in the  
390 recent WHO CNS tumor classification update. Much is still unknown about the  
391 drivers of transcriptional subclasses in GBM, their plasticity and how they evolve  
392 under therapy. A switch from proneural to mesenchymal expression has been  
393 observed upon disease recurrence and proposed as a source of treatment  
394 resistance in GBM relapse,<sup>18,79-81</sup> but the relevance of this phenomenon in glioma  
395 progression remains ambiguous, particularly considering a. the increased fraction of  
396 microglial/macrophage cells in mesenchymal GBM that confound subtype  
397 characterization<sup>62,63</sup> and b. glioma neurospheres derived from mesenchymal GBM  
398 are frequently classified as proneural.<sup>79</sup> Deriving an expression subtype classification

399 on the basis of glioma-intrinsic genes has maintained the proneural, classical, and  
400 mesenchymal classes<sup>63</sup>. Determining subtypes in a cohort of 91 IDH-wildtype GBM  
401 showed subtype switching following therapy and disease relapse in 45% of the  
402 cohort<sup>63</sup>. These patterns converged with changes in the tumor microenvironment,  
403 corroborating observations from single-cell transcriptomics that every GBM  
404 comprises different subtype mixtures but also revealing that *NF1* loss results in  
405 macrophage/microglia recruitment. The ability of genomic abnormalities to regulate  
406 the tumor microenvironment suggest cooperation and shows how tumors act as a  
407 system or an organ, rather than an aggregation of individual aberrant cells.

408

## 409 **2.5 Epigenetic changes during glioma progression**

410 DNA methylation profiling of gliomas has prognostic value independent of the age of  
411 the patient and the tumors pathologic grade.<sup>11</sup> Although clonal selection under  
412 therapy of genetic mutations nominates mutations as drivers of therapy resistance,  
413 strong evidence also suggests that evolutionary selection acting on the epigenome,  
414 in the absence of genetic changes, affords plasticity of cells to resist therapy.<sup>11,47</sup> For  
415 example, recurrent IDH-mutant gliomas profiled for mutations and DNA methylation  
416 independently evolved deregulation of their cell cycle programs, through genetic  
417 mutations or epigenetic mechanisms.<sup>47</sup> Epigenetic convergence on genetically  
418 deregulated biological processes demonstrates that epigenetic abnormalities provide  
419 cell subpopulations with fitness advantages that could undermine therapy.

420 Nearly all IDH-mutant gliomas exhibit a characteristic CpG island  
421 hypermethylator phenotype (G-CIMP).<sup>12</sup> Possible hypotheses for this relationship  
422 are as follows (i) DNA hypermethylation induces silencing of key extracellular matrix  
423 and cell migration gene promoters,<sup>12</sup> (ii) DNA methylation mediates alteration of  
424 chromosome topography, leading to oncogene upregulation<sup>82,83</sup> (iii) histone  
425 methylation-related changes in gene expression, (iv) DNA hypermethylation  
426 associated with mutant IDH may play a role in creating an immunosuppressed  
427 microenvironment.<sup>84</sup>

428 G-CIMP tumors can be further parsed into subsets with reduced genome-wide  
429 DNA methylation levels.<sup>11</sup> While almost all IDH-mutant tumors are G-CIMP at  
430 diagnosis, a longitudinal analysis showed that 34% of cases exhibited demethylation  
431 towards G-CIMP-intermediate or G-CIMP-low DNA methylation at recurrence.<sup>85</sup>



432 Substantial epigenetic heterogeneity between tumor samples from the same patient  
433 collected at subsequent surgeries was also observed in a cohort of 112 primary  
434 GBM patients<sup>86</sup>. Characteristic trends in DNA methylation between primary and  
435 relapsed GBM included a prominent demethylation of Wnt signaling gene promoters,  
436 which was associated with worse patient outcome. Moreover, patients whose  
437 primary tumors harbored higher levels of DNA methylation erosion showed longer  
438 progression-free survival and a trend towards longer overall survival<sup>86</sup>. This study  
439 also explored associations between changes in DNA methylation and magnetic  
440 resonance imaging (MRI) and digital pathology data, highlighting the connectedness  
441 of the various levels of molecular, cellular and phenotypic heterogeneity in GBM.  
442 Analysis of larger cohorts is needed to determine the association between genomic  
443 and epigenetic deregulation.

444

## 445 **2.6 Imaging and (epi)genomics**

446 MRI is a crucial part of standard diagnostic work-up and follow-up of brain tumor  
447 patients. It is noninvasive, and owing to the lack of radiation exposure, repeat  
448 imaging is not harmful. Conventional MR tumor imaging includes precontrast and  
449 post-contrast T1-weighted (T1w) and T2-weighted (T2w)/T2w fluid-attenuated  
450 inversion recovery (T2-FLAIR) imaging to assess tumor location, size, and certain  
451 macrostructural features.<sup>87</sup> Newer techniques such as perfusion imaging provide a  
452 measure of tumor vascularization in terms of relative cerebral blood volume, which  
453 correlates with tumor grade.<sup>88,89</sup> Relative cerebral blood volume reflects biological  
454 behavior of tumors, which might relate to molecular profiles.

455 In the rapidly growing field of research called radiogenomics,<sup>90</sup> a rich set of  
456 quantitative imaging features are linked with genomic profiles. It has recently been  
457 applied in the context of high-grade glioma.<sup>90-92</sup> Given the major differences in DNA  
458 characteristics, gene expression profiles, and DNA methylation profiles, a priority of  
459 radiogenomics research on glioblastoma is to identify MR imaging based biomarkers  
460 of molecularly defined lower-grade glioma subtypes such as IDH-mutant versus  
461 wildtype and 1p/19q codeleted versus non-codeleted. Noninvasive phenotypical  
462 assessment has several clear advantages. First, it provides an early test to stratify  
463 IDH-mutant, 1p/19q non-codeleted glioma tumors, identifying those patients who are  
464 candidates for the most aggressive therapeutical strategies and those for whom a

465 more conservative approach may be preferred. Providing reliable prognostic  
466 information through MR imaging can improve patients' quality of life by postponing,  
467 and potentially obviating the need for surgery for a subset of patients.<sup>93</sup> Second, it  
468 would be a means of selecting and tracking patients for personalized treatment  
469 regimens in clinical trials.<sup>94</sup> Third, a detailed global assessment of spatial and  
470 longitudinal heterogeneity of gliomas becomes feasible.<sup>95</sup>

471

### 472 **3. Barriers to progress**

473 The major obstacle to glioma patients currently is a lack of effective treatments, yet  
474 we have little understanding of why treatments fail. These failures are likely related  
475 to dynamic tumor evolution where treatment-resistant glioma cells are favored over  
476 treatment-sensitive cells. As a result, therapy has profound effects on tumor  
477 composition by activating intrinsic and adaptive resistance mechanisms, most clearly  
478 reflected by the temozolomide induced hypermutator phenotype<sup>70</sup>. Such processes  
479 may be directly induced by the therapy itself, or they are the result of survival and  
480 clonal expansion of tumor cells with genetic, epigenetic and/or regulatory alterations  
481 that confer drug resistance. Moreover, tumor cells may attenuate the immune  
482 response, locally and systemically, to prevent immunological recognition and  
483 clearance. All of these processes result in molecular characteristics of the recurrent  
484 tumor that differ in significant ways from those found in the primary tumor<sup>36,38</sup>.

485 To improve the outcomes of patients with gliomas, we need to explore new  
486 therapeutic approaches based on a thorough understanding of treatment-induced  
487 molecular and genetic diversity that leads to resistance. The TCGA glioma effort and  
488 similar initiatives elsewhere have established comprehensive portraits of the  
489 interpatient variability of untreated glioma genomes. Single cell sequencing and  
490 barcoding experiments have demonstrated functional hierarchies providing important  
491 insights into characteristics of the most relevant cells to target<sup>66-68</sup>. We are  
492 increasingly able to infer the life history of glioma, from tumor-initiating events such  
493 as *IDH1* mutation to tumor-promoting events such as RTK alterations. A detailed  
494 understanding of the biological diversity within every tumor following clinical  
495 presentation and disease progression is needed if we are to successfully understand  
496 how treatment affects glioma progression, a needed step towards integration of  
497 precision therapeutics into clinical decision making. These considerations also



498 highlight the danger in considering treatment options for patients with recurrent  
499 tumors solely on the basis of the molecular analysis of their treatment-naïve tumors.  
500 This is particularly important in the setting of clinical research, which often recruits  
501 patients with recurrent GBM to evaluate drugs developed on the basis of mechanistic  
502 data obtained on treatment-naïve tumors.

503 Studying heterogeneity and spatiotemporal evolution of cancer in general, and  
504 particularly in brain cancer, is challenging. Many tumor samples, and therefore large-  
505 scale collaboration, are needed to achieve meaningful comprehensive results. For  
506 example, to identify 80% of all somatic alterations occurring in at least 3% of the  
507 patient population, a cohort of 500 samples would be needed.<sup>96</sup> It is crucial to recruit  
508 sufficient numbers to validate findings and to capture low-frequency alterations.  
509 Individual research groups typically do not have the resources to use a multiplatform  
510 analysis of their samples, owing to cost or availability of expertise. Existing  
511 longitudinal datasets that have been published consist of a mixture of different  
512 modalities, ranging from only exomes<sup>38</sup> or DNA methylation profiles<sup>47,86</sup> to a  
513 combination of exome sequencing, RNA sequencing, and DNA copy number  
514 profiling,<sup>36,63</sup> thwarting meta-analyses based on cross-publication comparisons. The  
515 value of establishing a comprehensive multiplatform reference dataset quickly has  
516 been demonstrated by the success of TCGA glioma projects, which have led to a  
517 fundamental reclassification of gliomas by the WHO<sup>8</sup> and are highly cited.<sup>9,12-14</sup>  
518 Similarly, a consortium would be the most effective approach assembling the large  
519 cohorts of primary and recurrent pairs needed to identify somatic alterations enriched  
520 after disease progression. Systematizing and standardizing what we do and how we  
521 do it will be essential for affecting paradigmatic change to clinical practice in neuro-  
522 oncology. This philosophy is at the core of the international GLASS consortium.

523

#### 524 **4. The Glioma Longitudinal AnalySiS (GLASS) consortium**

525 The rapidly growing body of knowledge in molecular and genomic data is refining  
526 clinical diagnosis and prognostication (exemplified by the revised fourth edition of the  
527 WHO classification of CNS tumors published in 2016<sup>8</sup>), but has not resulted in  
528 improvements clinical outcomes, particularly in the more aggressive gliomas. This is  
529 evidenced by numerous trials that have failed to reach their primary endpoint.<sup>97,3,98</sup>

530 These successes and failures highlight the need for large-scale collaborations that to

531 help us understand the impact of treatment on evolutionary dynamics and, most  
532 importantly, why treatments fails?

533         The recognition of the need is why we initiated the GLASS consortium: to  
534 achieve power from the use of large numbers of paired samples from all contributors.  
535 GLASS aims to perform comprehensive molecular profiling of matched primary and  
536 recurrent glioma specimens from an unprecedented 1500 patients, 500 in each of  
537 the three major glioma molecular subtypes: 1. IDH-wildtype; 2. IDH-mutant; and 3.  
538 IDHmutant with 1p/19q codeletion. The consortium at the time of writing includes  
539 investigators from 32 academic hospitals, universities, and research institutes from  
540 12 countries, (see list of participants on the GLASS website, [http://www.glass-](http://www.glass-consortium.org)  
541 [consortium.org](http://www.glass-consortium.org)). By analogy with the International Cancer Genomics Consortium <sup>99</sup>  
542 GLASS is structured into country-specific franchises (GLASS-NL, GLASS-AT,  
543 GLASS-AU, GLASS-Korea, etc.) led by local investigators who are invested in the  
544 team's overall goal of assembling a meaningful sample cohort of pretreatment and  
545 post-treatment samples for each glioma grade and type, while taking advantage of  
546 country-specific opportunities. This enables each GLASS branch to have unique  
547 features that allow deeper analysis of subcohorts, that is, with additional imaging  
548 annotation, parallel characterization of drug response through xenografting of tumor  
549 samples, specific focus on a glioma subtype, etc., thereby making them competitive  
550 and enabling them to address non-overlapping aspects of the phenotypic diversity  
551 seen in the clinic. Country-specific branches will be coordinated to connect with the  
552 larger analyses, to drive specific research topics for both. There are no explicit  
553 restrictions on publishing, that is, each group is invited to publish their substudies  
554 independently. The overall goal is to establish a reference data set by pooling  
555 samples and aggregate data from all multiplatform analyses, countries and  
556 substudies, and to make datasets comparable through coordinated sample and data  
557 processing guidelines. Country franchises are centrally connected through a number  
558 of committees, each overseeing different aspects of the analysis (Figure 2).  
559



**Figure 2. Overview of GLASS committees.** Details on committee mandates are provided in section 4.

560

#### 561 **4.1 Biospecimens and characterization platforms**

562 Biospecimens from gliomas are often snap frozen or conserved formalin fixed,  
563 paraffin embedded (FFPE). For genomic and transcriptomic analyses, snap frozen  
564 material is preferred, while historically FFPE is the common approach to tissue  
565 preservation. Methods for generating sequencing data from FFPE material are  
566 increasingly improving, with 5–20% of samples failing quality controls. Given that  
567 samples from multiple timepoints are required for inclusion into GLASS, patients for  
568 whom only FFPE material is available are twice as likely to not yield sufficient high  
569 quality DNA. RNA extracted from glioma tissue is often highly degraded resulting in  
570 higher attrition rates<sup>100</sup>, but high quality RNA sequencing data from FFPE samples  
571 has been reported<sup>101</sup>. For DNA methylation profiling of FFPE material, a recent  
572 study focusing on primary glioblastoma reported a high success rate using the  
573 reduced representation bisulfite sequencing assay<sup>86</sup>. While we require the  
574 availability of a matching germline sample (often but not always from blood) for  
575 inclusion of DNA sequencing data into GLASS, cases without a germline match may  
576 be candidates for transcriptome and DNA methylation analysis. Ideally, we aim to  
577 generate DNA, RNA and epigenomic sequencing data from every patient. Single-cell

578 analysis methods require fresh tissue from which individual cells can be dissociated;  
579 they are currently outside the scope of GLASS, but may be considered in the future  
580 as the project evolves or as part of specific subprojects. Similarly subsets of the  
581 GLASS cohort will be compared longitudinally by spatial correlation using multisector  
582 analysis (3–6 samples per tumor) to understand whether any differences between  
583 paired tumor samples are the result of intratumoral heterogeneity or longitudinal  
584 heterogeneity. Where available, these will be correlated with conventional and novel  
585 MR imaging to explore spatiotemporal heterogeneity noninvasively. We aim to take  
586 current radiogenomic approaches further, not only to establish features of genetic  
587 characteristics at first diagnosis, but also in relation to molecular alterations over time  
588 (including under pressure of standard therapy).

589       Comprehensive genomic sequencing is needed to identify patterns of disease  
590 evolution as well as key mutations and chromosomal alterations that confer  
591 resistance to standard radiation, temozolomide, and novel clinical trial therapies.  
592 Sequencing paradigms and their costs are rapidly evolving, and each method  
593 provides different but often complementary information. There is no consensus on  
594 optimal methods. With the accessibility of \$1000 per biospecimen whole genome  
595 sequencing (WGS), the costs of WGS and whole exome sequencing (WES) have  
596 become comparable. WES has better sensitivity in detecting mutations in coding  
597 regions, but does not interrogate noncoding regions of the genome, structural  
598 variants, or noncoding copy number variants. The comprehensive nature of WGS  
599 enables analysis of evolution and clonality at higher resolution. WGS and WES  
600 combined may provide the optimal window on the breadth, depth, and allelic fraction  
601 of somatic events. However, where limitations in tissue or resources mandate a  
602 choice of one or the other, the decision will depend on the purpose of the (sub)  
603 project.

604

## 605 **4.2 GLASS committees**

606 GLASS has established different committees with the expertise to coordinate the  
607 various aspects of the consortium. They include pathology, clinical annotation, data  
608 infrastructure, ethics and publication, and funding committees.

609 **4.2.1 The GLASS pathology committee** maintains centralized classification and  
610 tissue processing. The committee has set up a panel of specific inclusion criteria for

611 tissue samples. A prerequisite for inclusion is that the patient has given informed  
612 consent to donate tissue to research and that an adequate blood sample or any  
613 other adequate source of genomic DNA is available. Both FFPE and snap-frozen  
614 tissue samples will be included in the GLASS study and evaluated by the committee.  
615 The area of viable tumor tissue should be at least 50 mm<sup>2</sup> and the tumor cell  
616 percentage should be higher than 50%. Hematoxylin and eosin slides of each  
617 sample will be digitized by an automated slide scanner and the images will be stored  
618 on a central server in order to be accessible by all members of the GLASS expert  
619 pathology committee. An anonymized pathology report of both the original and the  
620 recurrent tumor has to be submitted for review to the committee with information on  
621 microscopic (including immunohistochemical) findings and, if performed, results of  
622 molecular analyses, as well as the integrated diagnosis. On the basis of this  
623 information, the GLASS expert pathology committee will formulate a (tentative)  
624 review diagnosis and thereby select patient samples that can be used for further  
625 study by the GLASS consortium. Corresponding whole-slide images of all patient  
626 samples that are included in GLASS will then be made available as a digital resource  
627 for further image analysis<sup>102,103</sup>.

628 **4.2.2 The GLASS clinical annotation committee** maintains standardized data  
629 processing, data management and data sharing. The currently available large-scale  
630 datasets suffer from relatively weak clinical annotation. Consequently, linkage of  
631 genotype with clinical and morphological phenotype remains to be fully exploited in  
632 primary and recurrent settings. The GLASS clinical annotation committee will  
633 address this by standardizing clinical and imaging data collection for prospective  
634 studies and oversee aggregation of the clinical and imaging data from patients  
635 whose profiles are already included in the composite dataset. Insight into  
636 mechanisms of response and resistance and exposed therapeutic vulnerabilities will  
637 be fed into current and future clinical trial designs by GLASS investigators and trials  
638 designed collaboratively in academia and industry or across both. This strategy will  
639 require an integrated bioinformatics interface across the molecular and clinical  
640 research. The necessary data processing infrastructure will be developed by the  
641 GLASS consortium and distributed among its franchises, to ensure compatibility,  
642 comparability, and reproducibility. Each individual franchise will make clinical,  
643 imaging, and molecular data accessible in a comprehensible way by integrating  
644 clinical, imaging, and molecular parameters to explore correlation with relapse data.

645 Currently, radiology and imaging are part of the clinical annotation committee. By  
646 mapping imaging features in a voxel-wise manner and correlating these spatially with  
647 molecular alterations obtained from different parts of the tumor we aim to assess the  
648 entire tumor and to determine intratumoral heterogeneity.

649 **4.2.3 The GLASS data infrastructure committee** maintains standardized data  
650 processing, data management and data sharing. A characteristic of the GLASS  
651 consortium is that data will be generated at multiple institutions distributed over  
652 multiple countries. As the regulations pertaining to ethical use of sequencing  
653 datasets are continuously evolving, GLASS will follow the example set by ICGC to  
654 perform decentralized data analysis to avoid cross-border exchange of patient-  
655 sensitive raw sequencing data. The GLASS data infrastructure committee has  
656 developed Docker software images that can be shared by participating institutions to  
657 ensure analysis uniformity. Like a shipping container, a Docker image packages one  
658 or more software tools to establish a workflow resembling an executable application.  
659 Comparable to platform-independent Java software, the ready-to-run Docker images  
660 are independent of the local computational environment. The GLASS participants  
661 run the Docker image locally, which initializes a per-sample-per-analysis Docker  
662 container, resulting in data analysis using an identical software environment and run  
663 parameters. Docker images are available to process exome sequencing data, which  
664 includes alignment, quality control, mutation calling, and DNA copy number  
665 estimation. Comparable Docker images are ready for processing of whole-genome  
666 sequencing and transcriptome sequencing data. Docker images are available for  
667 download through <http://docker.glass-consortium.org> (*RV: currently pending*).

668 The data infrastructure committee will also coordinate mechanisms for  
669 dissemination of results, as to widely share datasets with the community. We may  
670 explore mechanisms such as the Genomic Data Commons, or similar, in order to  
671 align our efforts with other molecular profiling studies.

672 **4.2.4 The GLASS ethics and publications committee** was created to identify and  
673 address critical ethical, legal and social questions faced by researchers and patients  
674 participating in the GLASS program. The guidelines established by this group will  
675 continue to inform future policies that ensure effective and fair use of cancer  
676 genomic information coupled with relevant clinical annotations. All participating sites  
677 in the program have institutional ethics approval for data protection and the use of  
678 tissue and/or DNA and clinical and where applicable imaging data from patients that



679 have given written informed consent. Data will be made available rapidly after  
680 generation for community research use. Publication guidelines will follow that  
681 established by the TCGA policy for authorship and publication.

## 682 **5. Final remarks and perspectives**

683 Survival and quality of life for patients with diffuse gliomas remains dismal with  
684 standard treatments. Diffuse glioma is a fatal disease with an enormous societal  
685 burden as a result of short survival following high-grade disease and the relatively  
686 young age at diagnosis of lower-grade disease. This not only affects patients in the  
687 prime of their life, but also puts enormous burden on their immediate entourage, as  
688 they need extensive supportive care and navigation through a complicated medical  
689 landscape, and difficulties with medical costs and insurance. While cures of diffuse  
690 gliomas remain elusive, our patients demand better therapies and, with no  
691 substantive impact of molecular medicine, in practice treatments remain a 'one size  
692 fits all'. The GLASS Consortium will enable the improvement of clinical outcomes by  
693 establishing a broadly useful reference data set that will provide pivotal new insights  
694 into mechanisms used by gliomas to defy therapeutic challenges. For example,  
695 hypermutation following temozolomide treatment occurs in up to 15% of glioma, but  
696 too few samples have been profiled to understand what is driving this process or to  
697 identify biomarkers predictive of a TMZ associated hypermutator phenotype. GLASS  
698 will have the power to identify molecular markers indicating evolution from newly  
699 diagnosed to highly aggressive therapy-resistant malignancy, in addition to  
700 molecular aberrations that occur under pressure from different therapeutic  
701 modalities. It will also allow for the identification of currently undiscovered molecular  
702 targets for resistance-prevention agents that might be co-administered with classical  
703 therapies. Systematic correlation of the molecular information with clinical, imaging,  
704 and pathology data will help improve interpretation of prognostic findings in the  
705 course of the disease.

706 Finally, and importantly, GLASS is an opportunity for interchange of  
707 knowledge among an international group of collaborators to ultimately build smarter  
708 clinical trials and develop therapies that will extend survival and improve the quality  
709 of life of people with diffuse gliomas. GLASS is well positioned to demonstrate the  
710 value of well-coordinated collaborative efforts. To that extent, new investigators are  
711 invited to join to the Consortium, where major criteria for participation are the ability

712 to offer datasets of longitudinally profiled glioma patients or the availability of suitable  
713 tissue samples.

714 In summary, we hope that through the GLASS Consortium, we continue the  
715 immeasurable success of The Cancer Genome Atlas while increasing the focus on  
716 making a difference to patients and their families. Therapy resistance is what kills  
717 patients, and GLASS will inform on how to avoid it.

718

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736

## 737 **Conflicts of interest**

738



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