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Functional prediction of response to therapy prior to therapeutic intervention is associated with improved survival in patients with high-grade glioma

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Patients with high-grade glioma (HGG) have an extremely poor prognosis compounded by a lack of advancement in clinical care over the past few decades. Regardless of classification, most newly diagnosed patients receive the same treatment, radiation and temozolomide (RT/TMZ). We developed a functional precision oncology test that prospectively identifies individual patient's response to this treatment regimen. Tumor tissues isolated from patients with newly diagnosed HGG enrolled in 3D PREDICT REGISTRY were evaluated for response to chemotherapeutic agents using the 3D Predict™ Glioma test. Patients receiving RT/TMZ were followed for 2 years. Clinical outcomes including imaging, assessments, and biomarker measurements were compared to patient matched test-predicted therapy response. Median survival between test-predicted temozolomide responders and test-predicted temozolomide non-responders revealed a statistically significant increase in progression-free survival when using the test to predict response across multiple subgroups including HGG (5.8 months), glioblastoma (4.7 months), and *MGMT* unmethylated glioblastoma (4.7 months). Overall survival was also positively separated across the subgroups at 7.6, 5.1, and 6.3 months respectively. The strong correlation of 3D Predict Glioma test results with clinical outcomes demonstrates that this functional test is prognostic in patients treated with RT/TMZ and supports aligning clinical treatment to test-predicted response across varying HGG subgroups.

High grade gliomas (HGG), including astrocytoma, *IDH*-mutant and glioblastoma, *IDH*-wildtype, are a class of aggressive brain cancers with extremely poor prognosis, and minimally effective treatment options[1,2]. Glioblastoma (GBM) is the most commonly occurring type, making up about 50% of all malignant central nervous system tumors[1] and has a worse prognosis than astrocytoma. Following maximal safe surgical resection, almost all newly diagnosed HGG patients undergo the "Stupp" protocol and are treated with radiation and concurrent

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temozolomide (RT/TMZ) followed by adjuvant temozolomide[3,4]. The adoption of this protocol improved median survival in HGG by 2.5 months over radiation treatment alone[5]. Still, almost all patients undergo recurrence, and the 5 year survival rate remains less than 10%, as it has for the past 30 years[6,7].

Biomarkers, such as methylation of the *MGMT* promoter and mutation of *IDH1/2* have been used over the last two decades to classify patients and provide limited evidence as to their likelihood of survival in relation to treatment choice[5,8–13]. Methylation of the *MGMT* promoter is associated with better prognosis and survival with RT/TMZ compared to unmethylated patients[9,14–17]. However, this has not led to a fundamental shift in treatment paradigms for the two groups of patients[4,18]. Additionally, this binary grouping of patients based upon abstraction and reliance on the same treatment regardless of biomarker ignores the fact that there are patients in both groups who do not clinically perform as the group does. This can lead to detrimental effects for those patients, including toxicity, both physical and financial, and the inability to enroll in future clinical trials for which previous treatment may be disqualifying[18–20]. In general, all patients would benefit from a more direct knowledge of their predicted response to standard of care (SOC) prior to treatment to make informed decisions, potentially open the path to clinical trial enrollment, and maximize their time to recurrence.

Current precision medicine provides patients with early stage, personalized direction for treatment choices by linking known genomic mutation(s) to a drug targeting that mutation[21,22]. There is no individualized assurance of response to the targeted agent and many patients do not possess any actionable targets or do not derive clinical benefit from this therapy-matching approach[22]. Personalized treatment options for patients with HGG are especially lacking, due to intratumoral heterogeneity, lack of defined molecular pathways contributing to the disease, multiple potential genetic drivers, the impedance of the blood–brain barrier, and lack of effective targeted therapies[23]. Functional precision medicine can overcome these barriers to personalization as it uses the direct contact of a patient's tumor cells with drugs against which the response is being interrogated. This removes the insufficiency of genetic probability and overcomes the limitation of available drugs for detectable targets. Recent studies in hematologic malignancies have shown the potential to improve clinical outcomes when chemotherapeutic agent selection is aligned with functional results in contrast to genetic signatures[24–26]. Additionally, studies in solid tumors such as GBM and ovarian cancer have shown both the feasibility and predictive power of functional precision medicine approaches measured by clinical correlation and, increasingly, by successful clinical use[27–29]. The scarcity of therapeutic benefit in HGG creates a clinical landscape where functional precision medicine response assessment could have a truly positive clinical impact.

To measure the impact of functional precision medicine on HGG outcomes, we conducted a prospective, observational clinical study in patients with newly diagnosed HGG in which tissue specimens from individual patients were screened to assess therapeutic response to a variety of chemotherapeutic agents using the ex vivo test, 3D Predict™ Glioma. A previous validation dataset demonstrated that test-predicted responders to temozolomide had significantly longer overall survival (OS) compared to test-predicted temozolomide non-responders (5.7 months)[27]. The results presented here expand on this previous dataset and continue to demonstrate a progression-free survival (PFS) and an OS increase for test-predicted responders compared to non-responders across both HGG and GBM specifically. 3D Predict Glioma provides functional results within 7–10 days of tissue receipt, enabling optimization of patient management prior to therapy initiation. Newly diagnosed HGG patients whose tumors do not respond favorably to temozolomide could be preferentially directed to participate in clinical trials or be managed in such a way that might potentially provide greater clinical benefit. These results support the use of 3D Predict Glioma across the spectrum of newly diagnosed HGG patients.

Results

Patient enrollment and characteristics

To examine the ability of 3D Predict™ Glioma to prospectively predict patient response in a clinical setting, 3D PREDICT REGISTRY (NCT03561207) was opened to all patients with a suspected or confirmed diagnosis of HGG, including grade III, IV astrocytoma, *IDH*-mutant and glioblastoma, *IDH*-wildtype. For the purposes of data analysis, HGG was defined as inclusive of astrocytoma and glioblastoma while GBM was defined as exclusively glioblastoma, *IDH*-wildtype. Upon confirmation of diagnosis, 102 patients with HGG were enrolled from March 2018 to May 2022 (Fig. 1a). This included 20 patients included in the previous pilot data analysis[27]. Of those 102 patients, three tissue samples failed prior to testing due to poor sample quality, and 13 samples failed during testing due to failure to meet established quality control metrics, such as cell proliferation, resulting in a test performance success rate of 87% (86/99). The remaining 86 patients were available for clinical correlation with test results and were followed for up to two years. Over the course of clinical follow-up, 10 patients were eliminated from the correlation calculations due to receiving no clinical therapies. Another 14 were eliminated due to significant lack of clinical follow-up making progression calculations impossible. Finally, three were removed from analysis due to progression from non-cancer related events. The remaining 59 patients' data was analyzed for correlation between test results and clinical outcomes.

The distribution of patients by age, sex, and relevant biomarkers was representative of previously published HGG patient population demographics, and univariate analysis showed that the only significant differences in survival were due to histopathology, age, and initial ECOG score (Table 1) ref[1,6,9,11,12,30–32]. Survival of the group overall and of subgroups was consistent with previously published data (Fig. 1b,d, Supplementary Fig. S1). The seven patients diagnosed with astrocytomas, *IDH*-mutant had a median OS that was undefined due to survival throughout the study follow-up period while those in the GBM cohort had a median OS of 11.7 months (Fig. 1b, Supplementary Fig. S1). This is consistent with the better prognosis for patients with astrocytomas[8,9]. While there was no significant difference, the female cohort had a slightly better OS than the male cohort (15.9 months vs. 13.2 months), also reflecting data from the general population (Fig. 1c)[1,30]. Finally, when survival was examined based upon age, the study population continued to follow the general population trend; those 70 and younger

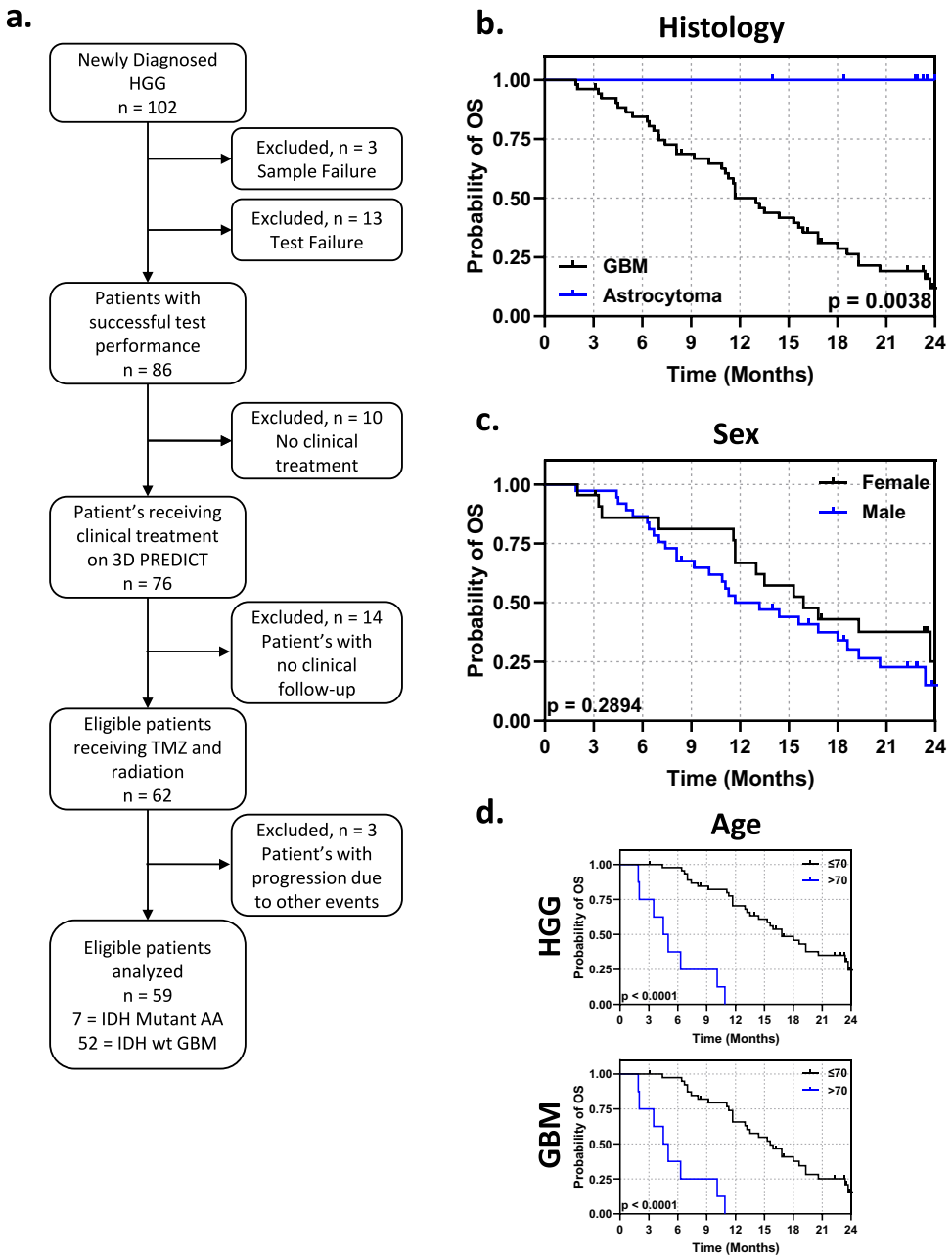


Figure 1. The 3D PREDICT study population reflected the general HGG population and published outcomes for HGG. (**a**) Flow chart describing patient inclusion and exclusion from clinical correlation analysis. (**b–d**) Kaplan–Meier curves of overall survival of the HGG population separated by histopathology (**b**), sex (**c**), and age (**d**).

survived longer than those patients over 70 (16.8 months vs. 4.75 months, $p < 0.0001$)[13,33] (Fig. 1d). This significant difference held true when the GBM only population was examined as well (15.6 months vs. 4.75 months). Stratification by age is important when making treatment decisions as NCCN guidelines provide the option to patients over 70 to receive radiation alone[4]. Taken together this data indicates that the 3D PREDICT REGISTRY study population was representative of real-world newly diagnosed HGG and GBM.

Prospective correlation of clinical outcome and 3D Predict Glioma for temozolomide

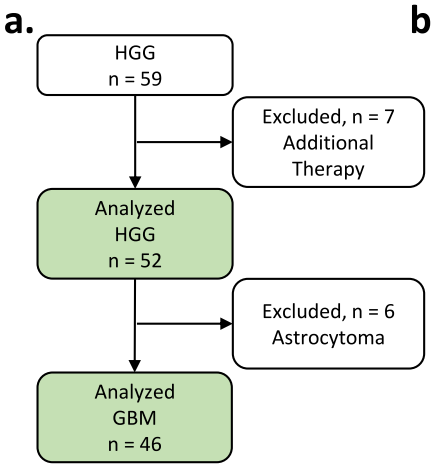
To establish the correlation of 3D Predict Glioma's temozolomide response prediction to clinical outcomes in newly diagnosed patients, all 59 eligible patients with HGG received baseline SOC treatment comprised of surgical debulking followed by radiation and temozolomide (RT/TMZ). Patients were followed for up to 24 months with clinical assessments made by their clinical team and collected to enable correlation with test predictions. Progression was determined by the clinicians based upon imaging and clinical assessments. Initial clinical

Characteristic	Total population			GBM only		
	Patients (n = 59)	p-value (PFS)	p-value (OS)	Patients (n = 52)	p-value (PFS)	p-value (OS)
Age—years						
Median	61			63		
Range	32–86			40–86		
Age—no. (%)						
≤ 70 year	46 (85.2)	0.003	< 0.001	40 (83.3)	0.007	< 0.001
> 70 year	8 (14.8)			8 (16.7)		
Unknown	5			4		
Sex—no. (%)						
Male	37 (62.7)	> 0.9	0.3	33 (63.4)	0.5	0.3
Female	22 (37.3)			19 (36.6)		
Histopathology—no. (%)						
Astrocytoma, IDH-mutant	6 (10.2)	0.002	0.004			
Glioblastoma, IDH-wildtype	53 (89.8)					
IDH mutation status—no. (%)						
Mutated	7 (11.9)	0.002	0.002			
Wild Type	52 (88.1)					
MGMT methylation status—no. (%)						
Methylated	23 (39.6)	0.7	0.6	17 (34.6)	0.3	0.8
Unmethylated	35 (60.4)			32 (65.4)		
Unknown	1			3		
Tumor location—no. (%)						
Right Brain	8 (34.7)	> 0.9	0.7	7 (36.9)	> 0.9	0.9
Left Brain	15 (62.3)			12 (63.1)		
Unknown	36			33		
Tumor acquisition type—no. (%)						
Biopsy	7 (13)	0.3	> 0.9	6 (12.5)	0.5	0.9
Resection	47 (87)			42 (87.5)		
Unknown	5			4		
Residual gross tumor—no. (%)						
Yes	23 (44.2)	0.8	> 0.9	20 (43.4)	> 0.9	0.2
No	29 (55.8)			26 (56.6)		
Unknown	7			6		
ECOG at enrollment—no. (%)						
0	22 (52.4)	0.009	0.005	19 (50)	0.05	0.05
≥ 1	20 (47.6)			19 (50)		
Unknown	17			14		

Table 1. Demographics of patients at clinical correlation, Irrespective of study predicted treatment response.

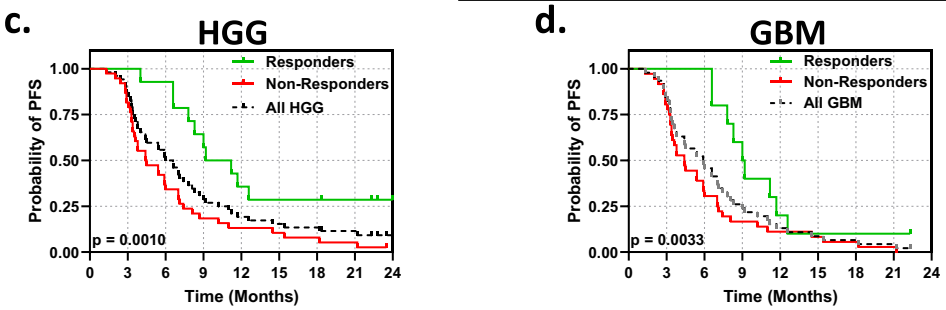
correlation was performed against PFS to remove confounding variables associated with the use of additional treatments following recurrence. Of the 59 analyzable HGG patients, seven received additional therapies prior to recurrence along with temozolomide, including two who received bevacizumab, three who received pembrolizumab, and two that received a cancer vaccine (Fig. 2a). These seven were removed from PFS statistical analysis. Correlation of test-predicted temozolomide responses and clinical response to treatment revealed the ability of the 3D Predict Glioma test to identify those HGG and GBM patients with improved PFS outcomes compared to test-predicted non-responders (Fig. 2b–f). HGG test-predicted responders to temozolomide had a longer median PFS by 5.8 months (p = 0.0010, HR = 0.36 (0.20 to 0.64)) compared to test-predicted non-responders (Fig. 2b,c,f). The increase in PFS amongst test-predicted temozolomide responders compared to test-predicted non-responders remained statistically significant when refined to GBM only as that subgroup of patients (n = 46) also had a longer median PFS by 4.7 months when predicted to respond to temozolomide compared to those predicted to not respond (p = 0.0033, HR = 0.44 (0.24 to 0.81)) (Fig. 2b,d,f). Only 22—27% of patients were predicted to respond to temozolomide in either of the histopathology groups. This may be reflective of a general lack of long-term response to temozolomide seen for most patients with HGG. Notably, in the treatment controlled PFS analysis of GBM patients, 69% (25/36) of those predicted to not respond to temozolomide progressed before the first predicted responder did.

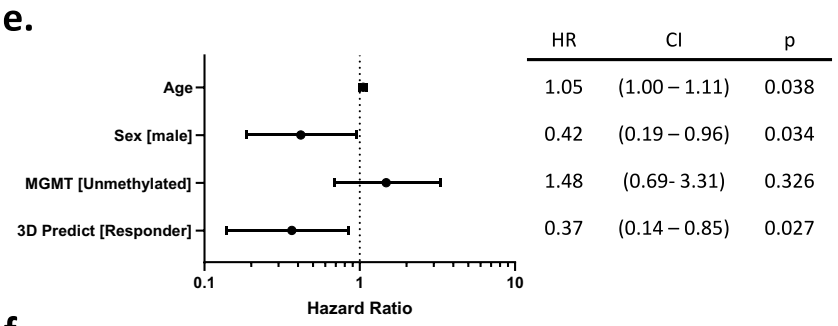
Multivariate analysis of the GBM population (Fig. 2e) found that with each incremental year in age there was a slight but statistically significant increase in the risk of disease progression (p = 0.038, HR = 1.05 (1.00 to 1.11)). Being male also had a significant impact upon PFS (p = 0.034, HR = 0.42 (0.19 to 0.96)) while having



b.

Histopathology and Response	Test-Predicted Responders	Test-Predicted Non-Responders
HGG – no. (%)	**14 (27)**	**38 (73)**
Progression-Free Survival (PFS)		
Median PFS (months)	10.2	4.45
p-value	0.0010	
Hazard Ratio (95% CI)	0.36 (0.20 to 0.64)	
GBM – no. (%)	**10 (22)**	**36 (78)**
Progression-Free Survival (PFS)		
Median PFS (months)	9.1	4.4
p-value	0.0033	
Hazard Ratio (95% CI)	0.44 (0.24 to 0.81)	





	HR	CI	p
Age	1.05	(1.00 – 1.11)	0.038
Sex [male]	0.42	(0.19 – 0.96)	0.034
MGMT [Unmethylated]	1.48	(0.69- 3.31)	0.326
3D Predict [Responder]	0.37	(0.14 – 0.85)	0.027

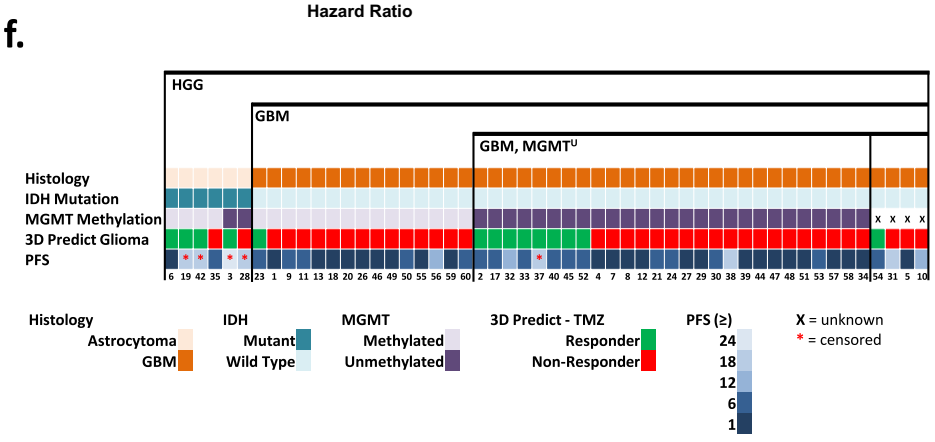


Figure 2. 3D Predict Glioma identified HGG and GBM patients with improved PFS with temozolomide. (**a**) Flow chart describing the population transition from HGG to GBM for PFS analysis. Green boxes highlight the groups described in the subsequent analyses. (**b**) Table summarizing the PFS and corresponding statistics for HGG and GBM patients. (**c,d**) Kaplan–Meier curves of PFS for the HGG (**c**) and GBM (**d**) populations separated as test-predicted responders (green) and test-predicted non-responders (red) to temozolomide. The dashed black line is the patient population unseparated by test prediction. (**e**) Multivariate Cox proportional hazards model for PFS. The dashed vertical line at HR = 1 represents the null effect, where covariates do not influence the risk of progression. (**f**) Individual patient information and drug response.

an unmethylated *MGMT* promoter did not (p = 0.326, HR = 1.48 (0.69 to 3.31)). These results suggest that in this study, age and sex are significant predictors of progression whereas *MGMT* methylation status is not. The subgroup of patients with temozolomide responder status by 3D Predict Glioma demonstrated a statistically significant association with improved PFS (p = 0.027, HR = 0.37 (0.14 to 0.85)).

When examining OS, 3D Predict Glioma predictions also had a trend towards longer survival in predicted responders versus non-responders in the 59 HGG patients and 52 GBM patients (Fig. 3). Test-predicted responders for both histopathologies had an increased median OS compared to test-predicted non-responders (differences of 7.6 and 5.1 months, respectively) although only the HGG population difference was significant (p = 0.0433, HR = 0.63 (0.33 to 1.21)) (Fig. 3b,c,e). The GBM patients in general did worse than the HGG population (Fig. 3b,c), most likely due to the inclusion of astrocytoma patients in the HGG population who would be expected to survive longer. The same difference in time to event between first predicted responder and the predicted non-responders was seen when OS was examined for both HGG and GBM with 42% (20/48) and 49% (20/41) respectively of predicted non-responders dying before the first test-predicted responder (Fig. 3c).

Multivariate analysis of the GBM population (Fig. 3d) found that increasing age is associated with a higher risk of mortality (p = 0.001, HR = 1.09 (1.04 to 1.15)). The risk associated with being male or having an unmethylated MGMT promoter did not reach statistical significance (p = 0.446, HR = 1.33 (0.65 to 2.88) and p = 0.202, HR = 1.74 (0.77 to 4.26), respectively). These results suggest that in this study age is a significant predictor of survival whereas sex and MGMT methylation status are not. Unlike with PFS, the subgroup of patients with temozolomide responder status in 3D Predict Glioma showed a non-significant association with improved survival (p = 0.194, HR = 0.57 (0.23 to 1.29)).

21 GBM patients received additional therapies and/or additional surgeries which could serve to confound the OS statistics (Supplementary Figure S2). When those patients were removed to examine the patients that only received radiation and temozolomide (RT/TMZ), the survival difference remained non-significant and decreased to 4.25 months but the p-value and hazard ratio improved (p = 0.0841 from p = 0.1210 and HR = 0.57 (0.25 to 1.33) from HR = 0.79 (0.39 to 1.6)) (Supplementary Figure S2). The same difference in time to event between first predicted responder and the non-responders was seen with this subgroup as well with 64% (16/25) of predicted non-responders dying before the first test-predicted responder (Supplementary Figure S2). Finally, multivariate analysis of this subgroup did not change from the full OS analysis. The precipitous drop-off in survival in the test-predicted responder group at 12 months, especially when a patient only received RT/TMZ, may be indicative of the need for additional treatments including surgery or further therapy.

Age is consistently recognized as a significant prognostic indicator for HGG, with advancing age strongly associated with shorter survival[13]. NCCN guidelines stratify patients by age with those over 70 given the option of radiation alone regardless of performance or *MGMT* promoter methylation status. Thus, the 3D PREDICT REGISTRY population was interrogated for drug response prediction based upon age stratification (Fig. 4). An interrogated cutoff of 65 years of age was used based upon the median age at diagnosis, 64, and the healthcare implications of the Medicare population. Importantly, within this cohort of the study population, all patients were GBM, *IDH*-wildtype. In the study population over 65 years of age, test-predicted responders to temozolomide had a statistically significant increase in median PFS by 4.6 months (p = 0.0494, HR = 0.47 (0.18 to 1.24)) and a non-significant trend towards an increase in median OS by 6.1 months (p = 0.1336, HR = 0.59 (0.22 to 1.61)) compared to test-predicted non-responders (Fig. 4a–c). This data indicates that 3D Predict Glioma may provide clinicians and their patients with an additional piece of information to enable decisions for patient populations, such as the elderly, that are more prone to suffer treatment related adverse events and are guidelines directed to potentially avoid chemotherapy.

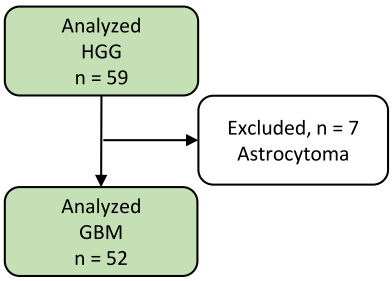
3D Predict Glioma predicts response to temozolomide regardless of MGMT methylation status

Methylation of the promoter region of *MGMT* has been recognized as a predictor of response to temozolomide treatment in glioma patients for approximately 20 years[9,34]. However, it is not a perfect predictor as there are unmethylated patients that still do well on temozolomide and there are methylated patients who do not as evidenced by the survival curves in numerous studies with both short- and long-term survivors in both categories[9,18,34]. We examined the correlation of 3D Predict Glioma results against clinical outcomes in relation to *MGMT* promoter methylation (Fig. 5a). When the HGG and GBM populations were separated only by *MGMT* methylation (Fig. 5b,c), they followed published trends with the methylated patients having a slightly longer median survival than the unmethylated patients (15.3 months versus 13.2 months). The survival curves followed a trend noted in previous studies[9,34] of the survival curves not separating until approximately 12 months. Notably, the differences in survival based on methylation were less pronounced in the GBM only population because the astrocytoma, *IDH*-mutant patients contributed to the longer survivors in the HGG cohort (Fig. 5b,c).

When the test-predicted responders and non-responders were stratified to *MGMT* methylation status, the low number of methylated responders made it difficult to draw any conclusion (Supplementary Figure S3) while the separation in the unmethylated population became evident. Independent examination of the *MGMT* unmethylated GBM population revealed that 3D Predict Glioma was able to identify the unmethylated patients that did relatively well on temozolomide (test-predicted responders) with median PFS (9.1 months) and OS (18 months) similar to the responders in the non-stratified populations (9.1 months and 16.8 months, respectively) (Fig. 5d–f). Approximately 30% of the unmethylated *MGMT* GBM patients were test identified as responders to temozolomide and had a longer median PFS (4.7 months longer, p = 0.003, HR = 0.35 (0.16 to 0.75)) and OS (6.3 months longer, p = 0.0664, HR = 0.65 (0.30 to 1.39)) than the test predicted non-responders (Fig. 5d–f).

The testing methods for *MGMT* methylation vary and the criteria for a positive (methylated) or negative (unmethylated) response also varies with each available test. To examine the role of test type and compare it to 3D Predict Glioma test outcomes, we categorized patient *MGMT* methylation data by test type, examined

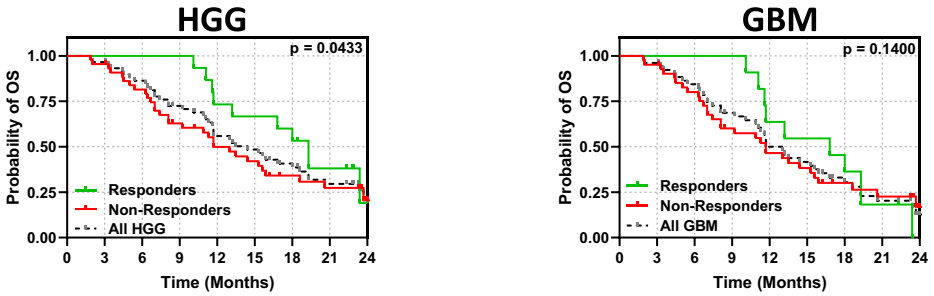
a.



b.

Histopathology and Response	Test-Predicted Responders	Test-Predicted Non-Responders
HGG – no. (%)	**15 (25)**	**44 (75)**
Overall Survival (OS)		
Median OS (months)	19.3	11.7
p-value	0.0433	
Hazard Ratio (95% CI)	0.63 (0.33 to 1.21)	
GBM – no. (%)	**11 (21)**	**41 (79)**
Overall Survival (OS)		
Median OS (months)	16.8	11.7
p-value	0.1400	
Hazard Ratio (95% CI)	0.85 (0.43 to 1.70)	

c.



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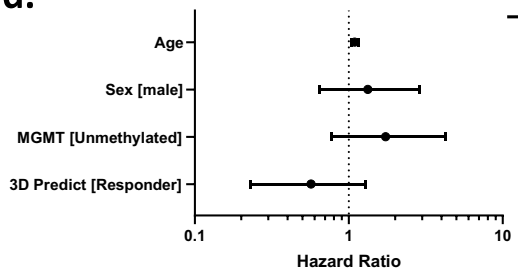


	HR	CI	p
Age	1.09	(1.04 – 1.15)	0.001
Sex [male]	1.33	(0.65 – 2.88)	0.446
MGMT [Unmethylated]	1.74	(0.77 – 4.26)	0.202
3D Predict [Responder]	0.57	(0.23 – 1.29)	0.194

e.

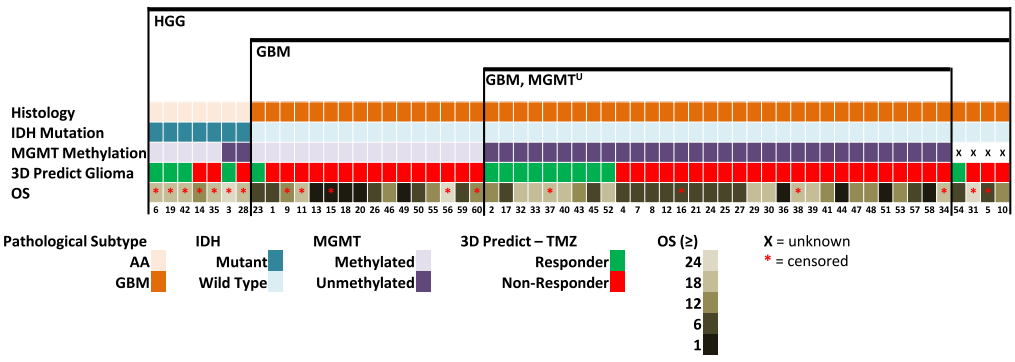


Figure 3. 3D Predict Glioma identified glioma patients with improved OS with temozolomide. (**a**) Flow chart describing the population transition from HGG to GBM for OS analysis. Green boxes highlight the groups described in the subsequent analyses. (**b**) Table summarizing the OS and corresponding statistics for HGG and GBM patients. (**c**) Kaplan–Meier curves of OS for the HGG and GBM populations separated as test-predicted responders (green) and test-predicted non-responders (red) to temozolomide. The dashed black line is the patient population unseparated by test prediction. (**d**) Multivariate Cox proportional hazards model for OS. The dashed vertical line at HR = 1 represents the null effect, where covariates do not influence the risk of progression. (**e**) Individual patient information and drug response.

a.

Histopathology and Response	Test-Predicted Responders	Test-Predicted Non-Responders
GBM, >65 – no. (%)	**4 (22)**	**14 (78)**
Progression-Free Survival (PFS)		
Median PFS (months)	8.4	3.8
p-value	0.0494	
Hazard Ratio (95% CI)	0.47 (0.18 to 1.24)	
Overall Survival (OS)		
Median OS (months)	12.45	6.35
p-value	0.1336	
Hazard Ratio (95% CI)	0.59 (0.22 to 1.61)	

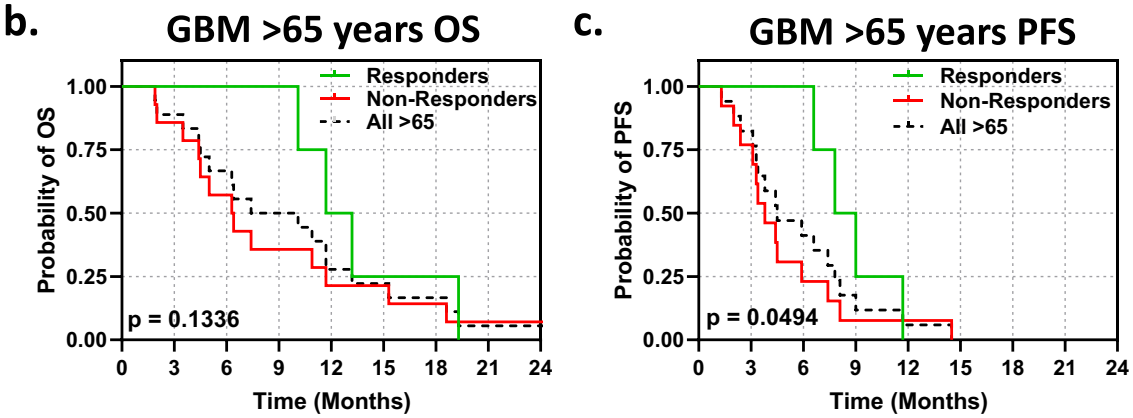
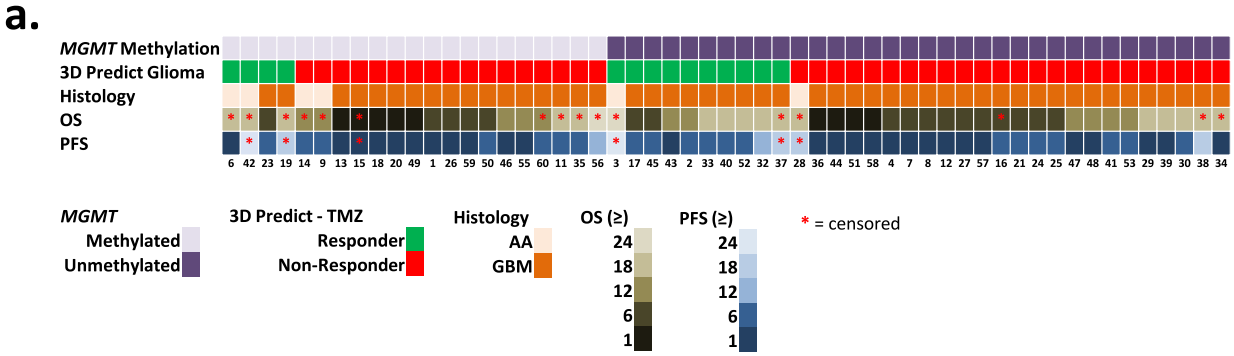


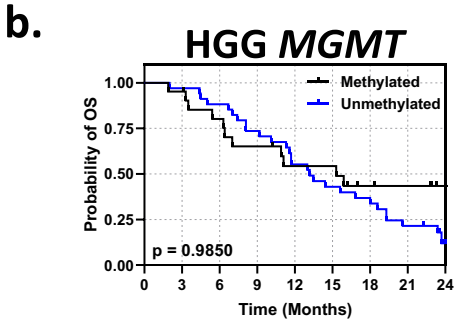
Figure 4. 3D Predict Glioma identified elderly glioma patients with improved PFS and OS with temozolomide. (**a**) Table summarizing the PFS and OS and corresponding statistics for GBM patients greater than 65 years of age. (**b,c**) Kaplan–Meier curves of OS (**b**) and PFS (**c**) for the greater than 65 GBM population separated as test-predicted responders (green) and test-predicted non-responders (red) to temozolomide. The dashed black line is the patient population unseparated by test prediction.

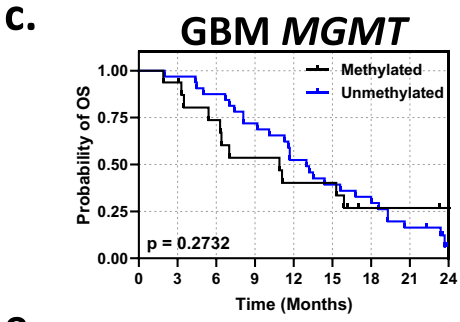
methylation-based survival outcomes in the GBM patients, and compared the survival outcomes for the same patients using 3D Predict Glioma. The majority of study patients (32 of 59 patients) were interrogated for *MGMT* promoter methylation using the same methylation-specific polymerase chain reaction (msPCR) test (Fig. 6a). This was followed in prevalence by pyrosequencing on 12 patients and NGS on six. The msPCR test utilized a real-time methylation specific PCR assay to evaluate 8 CpG sites within the *MGMT* promoter[35]. A positive result does not require methylation at all 8 sites. The pyrosequencing test assessed 4 CpG sites with a positive test requiring all four sites be methylated.

Two patients tested by msPCR and two by pyrosequencing were removed from survival analysis due to an indeterminate result. Interestingly, when the remaining 30 patients tested using msPCR were stratified by methylation status, the msPCR based outcomes did not reflect previously reported *MGMT* methylation data as the unmethylated patients had a longer median OS than the methylated patients (14.4 months versus 6.4 months) (Fig. 6b,c). However, when the 3D Predict Glioma results were used to stratify this same patient population, the predicted responders survived longer than the predicted non-responders (18.05 months versus 11.5 months) (Fig. 6b,d). The split by patient number was approximately equal with 23–26% of patients being predicted to respond to temozolomide whether by methylation or functional response. However, only two of the seven methylated patients were identified as responders by 3D Predict Glioma (data not shown). When the pyrosequencing patients were examined, the difference in survival based upon *MGMT* methylation was not as pronounced but the unmethylated patients still had a longer median OS than the methylated patients (Fig. 6b,e) and 3D Predict Glioma only identified one responder within this ten-patient set who happened to also be an unmethylated patient (Fig. 6b,f).

Since the data suggests the type of *MGMT* methylation test may affect the determination of *MGMT* methylation status, we retrospectively standardized the categorization across all patients by testing those that we had material for with our own clinically available *MGMT* methylation test (Fig. 7). Those performing the *MGMT* methylation testing in our labs were blinded as to the outcome of the tests at the clinical sites. Our msPCR test covers 12 CpG sites (75—86) of the *MGMT* promoter. This overlaps the 8 sites used in the previously discussed

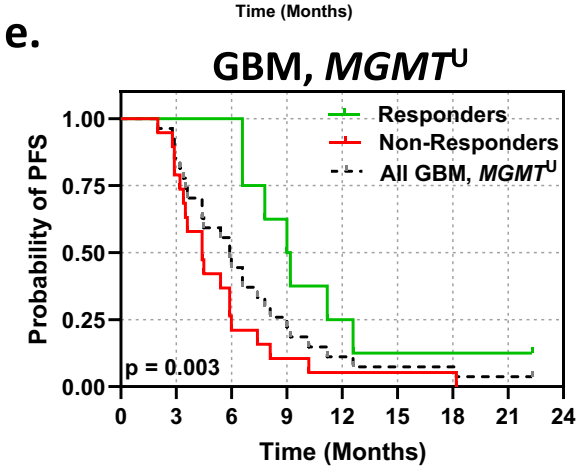






d.

Histopathology and Response	Test-Predicted Responders	Test-Predicted Non-Responders
GBM, *MGMT*^{U} – no. (%)	**8 (30)**	**19 (70)**
Progression-Free Survival (PFS)		
Median PFS (months)	9.1	4.4
p-value	0.003	
Hazard Ratio (95% CI)	0.35 (0.16 to 0.75)	
GBM, *MGMT*^{U} – no. (%)	**9 (28)**	**23 (72)**
Overall Survival (OS)		
Median OS (months)	18	11.7
p-value	0.0664	
Hazard Ratio (95% CI)	0.65 (0.30 to 1.39)	



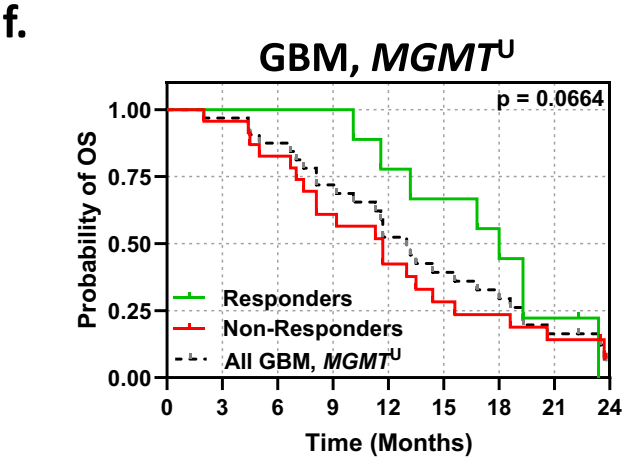
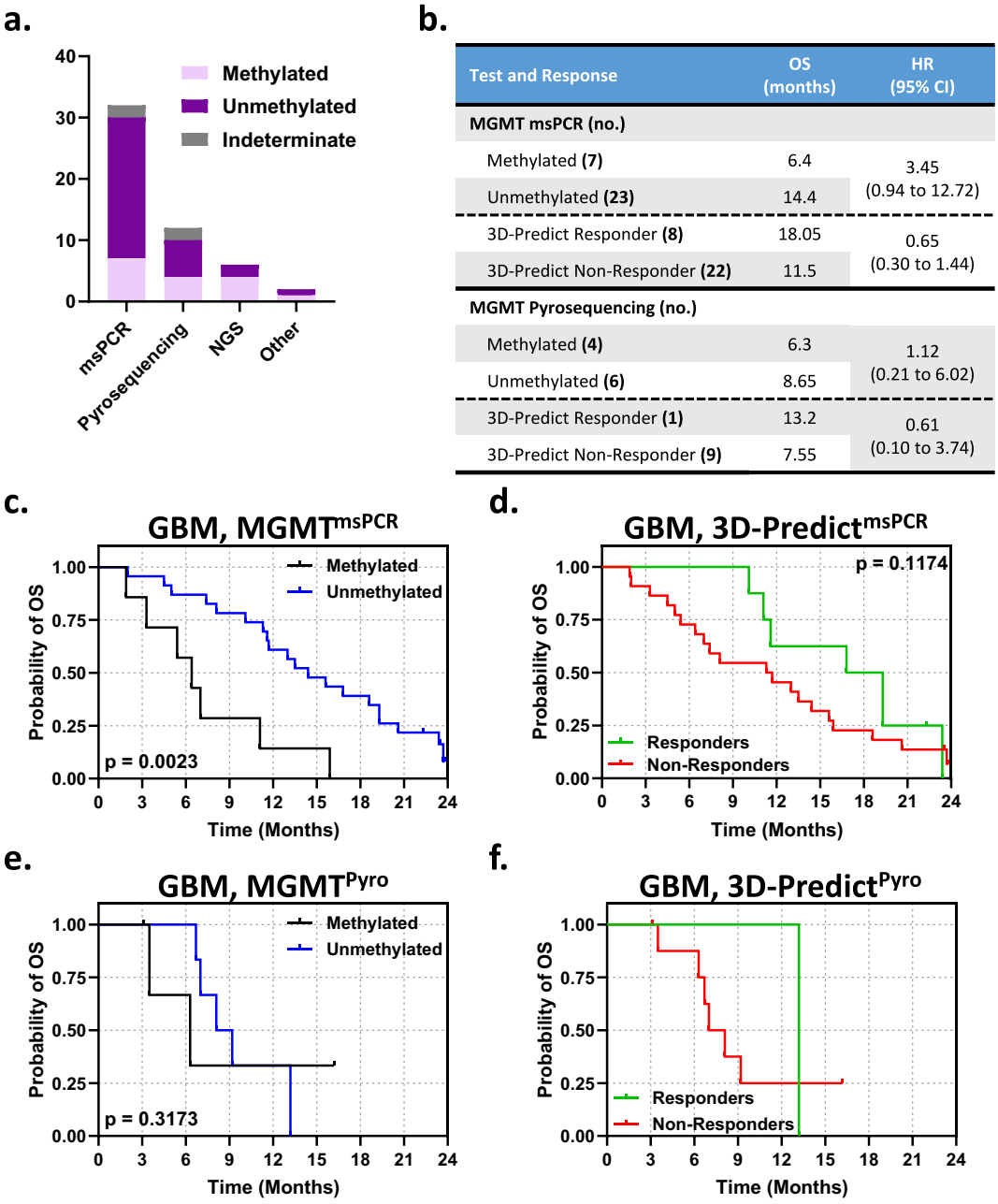


Figure 5. 3D Predict Glioma identified those patients with an unmethylated *MGMT* promoter that still did well with temozolomide treatment. (**a**) Individual patient information and drug response. (**b,c**) Kaplan–Meier survival curves stratified by *MGMT* promoter methylation in the HGG (**b**) and GBM (**c**) populations. (**d**) Table summarizing the PFS and OS and corresponding statistics for GBM patients with an unmethylated *MGMT* promoter (GBM, *MGMT*^{U}). (**e,f**) Kaplan–Meier survival curves for GBM, *MGMT*^{U} patients for PFS (**e**) and OS (**f**) separated by test-predicted responders (green) and test-predicted non-responders (red). The dashed black line is the patient population unseparated by test prediction.

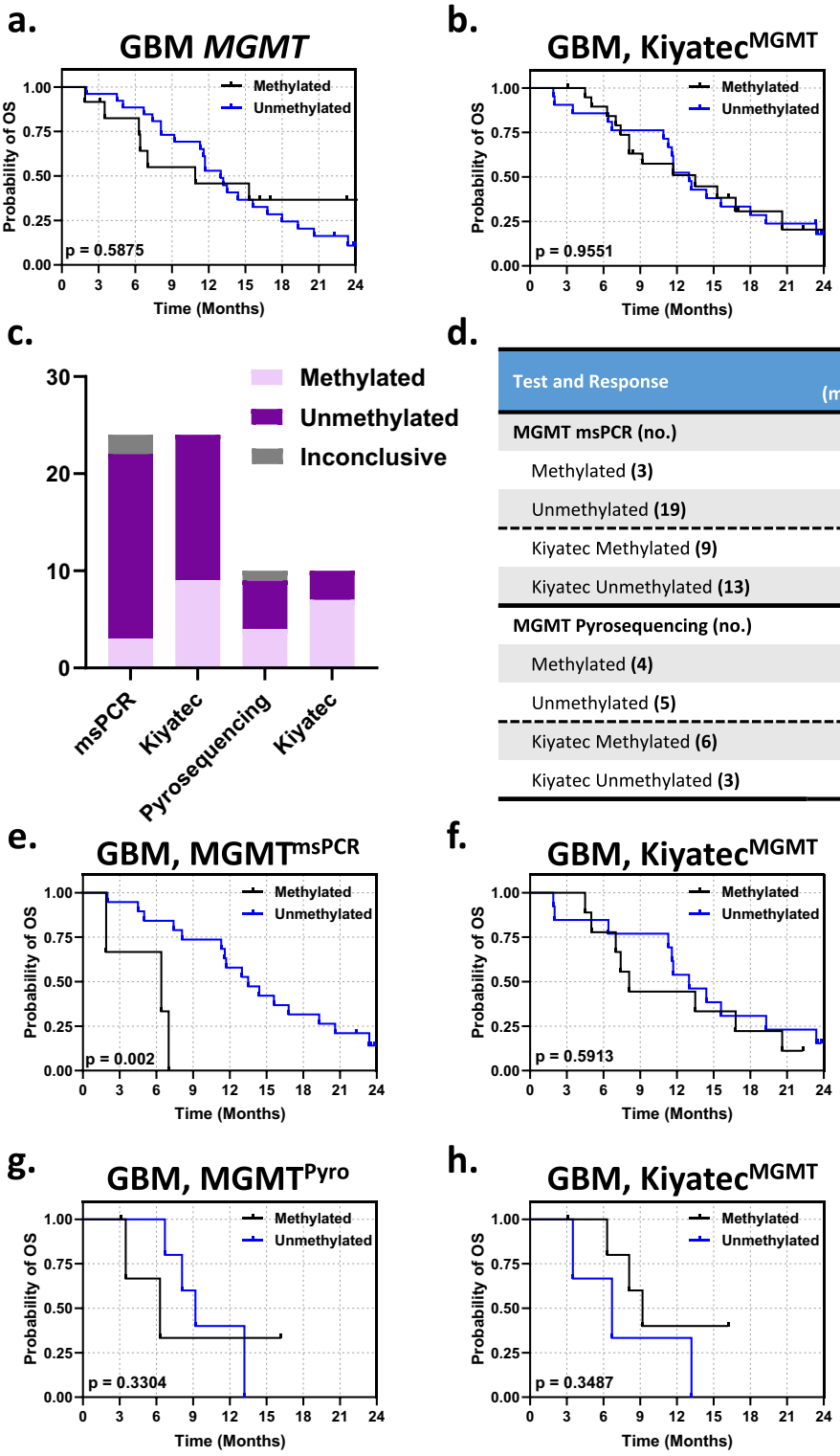
msPCR assay (79—86). As with the other test, a positive outcome is calculated relative to the amplification of an endogenous control gene (Actin B). All 12 sites do not have to be methylated for a positive outcome.



Test and Response	OS (months)	HR (95% CI)
MGMT msPCR (no.)		
Methylated **(7)**	6.4	3.45 (0.94 to 12.72)
Unmethylated **(23)**	14.4	
3D-Predict Responder **(8)**	18.05	0.65 (0.30 to 1.44)
3D-Predict Non-Responder **(22)**	11.5	
MGMT Pyrosequencing (no.)		
Methylated **(4)**	6.3	1.12 (0.21 to 6.02)
Unmethylated **(6)**	8.65	
3D-Predict Responder **(1)**	13.2	0.61 (0.10 to 3.74)
3D-Predict Non-Responder **(9)**	7.55	

Figure 6. 3D Predict Glioma stratified patient response regardless of the *MGMT* promoter methylation test used. (**a**) bar graph indicating the different tests used to clinically determine *MGMT* promoter methylation, including the number of methylated (light purple), unmethylated (dark purple) and inconclusive (gray) patients identified with each test type. (**b**) Table summarizing the OS for each test and categorization of patients along with the associated statistics. (**c**,**e**) Kaplan–Meier survival curves of the populations stratified by methylation state for the msPCR (**c**) and pyrosequencing (**e**) tested populations. (**d**,**f**) Kaplan–Meier survival curves of the populations stratified by 3D Predict Glioma test prediction for the msPCR (**d**) and pyrosequencing (**f**) tested populations.

Importantly, unlike other tests, our lab performs testing upon unfixed, fresh or frozen cells rather than FFPE samples removing some of the issues that can come from fixation, such as over-fragmentation. We were able to test 41 of the 52 GBM samples including three of the four inconclusive samples. When we compared the 38 samples with results from both the mix of clinical tests and our clinical test, the methylated patients identified by our test survived slightly longer (13.5 vs. 10.9 months) while the unmethylated patients survived the same (13 months) (Fig. 7a,b). In general, our test identified more patients as methylated with 12 unmethylated switching to methylated categorization and five methylated switching to unmethylated (Fig. 7c,d). When comparing the msPCR tested cohort to our *MGMT* test we had a concordance of 50% (12/24) and similar outcomes for the methylated and unmethylated unlike the test performed for clinical use which revealed a statistically significant



Test and Response	OS (months)	HR (95% CI)
MGMT msPCR (no.)		
Methylated **(3)**	6.4	5.68 (0.44 to 73.32)
Unmethylated **(19)**	13.5	
Kiyatec Methylated **(9)**	8.1	1.311 (0.51 to 3.35)
Kiyatec Unmethylated **(13)**	13	
MGMT Pyrosequencing (no.)		
Methylated **(4)**	6.3	1.19 (0.21 to 6.85)
Unmethylated **(5)**	9.2	
Kiyatec Methylated **(6)**	9.2	0.48 (0.09 to 2.65)
Kiyatec Unmethylated **(3)**	6.7	

Figure 7. Standardization of *MGMT* promoter methylation testing affects *MGMT* promoter methylation categorization. (**a,b**) Kaplan–Meier survival curves stratified by *MGMT* promoter methylation in the GBM population as a conglomerate of multiple test types (**a**) and tested with Kiyatec's *MGMT* methylation test (**b**). (**c**) Bar graph indicating the different tests used to clinically determine *MGMT* promoter methylation, including the number of methylated (light purple), unmethylated (dark purple) and inconclusive (gray) patients identified with each test type. (**d**) Table summarizing the OS for each test and categorization of patients along with the associated statistics. (**e,g**) Kaplan–Meier survival curves of the populations stratified by methylation state for the msPCR (**e**) and pyrosequencing (**g**) tested populations. (**f,h**) Kaplan–Meier survival curves of the populations stratified by 3D Predict Glioma test prediction for the msPCR (**f**) and pyrosequencing (**h**) tested populations.

survival benefit for the unmethylated cohort (Fig. 7d–f). When comparing to the pyrosequenced patients, we also had a concordance of 50% (5/10) and the methylated patients, as determined by our test, performed better than the unmethylated patients (Fig. 7d,g–h). Taken together, this data supports what others have found, that if *MGMT* methylation is to be used to determine treatment, testing type and categorization requirements should be standardized as they can affect results[14,36,37].

Discussion

In this study, prospectively generated 3D Predict Glioma test results were correlated with clinical outcomes in newly diagnosed HGG patients and subgroups, including GBM, age, and unmethylated *MGMT*. In all groups analyzed, test-predicted responders survived longer than test-predicted non-responders and historical median OS (14.6 months) and PFS (6.7 months)[8]. The inverse was also true with test-predicted non-responders performing worse than test-predicted responders and historical medians. For unmethylated *MGMT* promoter patients, 3D Predict Glioma test results identified those patients that responded even though the biomarker indicated they were unlikely to. Notably, for all GBM populations, at least 50% of test-predicted non-responders either progressed or died before the first test-predicted responder. The data in this analysis, along with previously published data [27], demonstrates the power of 3D Predict Glioma and functional precision medicine to provide patient-specific drug response predictions beyond the current limitations of traditional biomarkers in HGG and GBM.

In 2005, Stupp et al. provided evidence that the addition of temozolomide to radiation therapy for patients with HGG would improve survival outcomes[8]. The survival increase was approximately 2.5 months across a population that included astrocytoma and did not select for *MGMT* methylation or *IDH* mutation, but recurrence rates did not change[5]. This led to the current SOC for all HGG patients of radiation with concomitant temozolomide followed by adjuvant temozolomide. At the same time, the use of *MGMT* methylation as a biomarker to predict response in patients treated with radiation and concomitant temozolomide was published[9]. While these two seminal publications have resulted in treatments that provide a survival increase to some patients, they have not led to the truly individualized personalization of treatment for newly diagnosed patients. 3D Predict Glioma is truly personal medicine on an individual level and the survival improvements in all studied groups of patients in this analysis far exceed the 2.5 months that today's SOC is based on. Importantly, for clinical utility, the use of this test in SOC to inform decision making does not affect current surgical resection guidance and can work in conjunction with *MGMT* methylation, NGS, and other already utilized tests to provide even better-informed patient care.

While our observational study had several strengths, the small sample size and observational format did result in weaknesses. The assay format avoided some of the pitfalls of other studies by not requiring subculturing or expansion of cells prior to testing [29,38–41] which can lead to the elimination of immune cells and has been shown to potentially result in changes to *MGMT* methylation. This also allowed test results to be returned prior to the initiation of treatment which will allow clinicians to change treatment paradigms without disrupting standard procedures. The use of extended dose response curves rather than categorization based upon a single dose of drug, when combined with a significant sample size, may eventually inform upon clinical dosing or treatment length. The reliance on fresh tissue is both a weakness and a strength. Fresh tissue provides the most robust representation of the patient's tumor, free of cross-linkers or other preservation techniques, but requires special handling and logistics. It additionally adds variables to the resulting readout such as the amount of actual tumor tissue, the heterogeneity of the cell types, and the inclusion of necrotic tissue which can be good or bad for the readout. Finally, the ability to abstract test results determined with treatment naïve tissue at new diagnosis to 2nd, 3rd, or 4th line treatment has not been established.

This registry study was prospective and reflected the real-world practice for HGG resulting in ex vivo test results that were unbiased to clinical outcome. Similar to other studies, it did limit the study population to those patients that could receive surgery and were also able to undergo radiation and temozolomide treatment prior to progression. The small sample size did result in low power, and while the median survival rates were improved for test-predicted responders, significant differences in long-term survival were not observed. This has been seen in numerous other studies attempting to predict therapy response and may reflect an inability of temozolomide to result in a long-term and lasting response for most HGG patients beyond approximately 2 years [5,9,42,43]. Importantly, the time to first event was greatly extended in the test-predicted responders compared with the test-predicted non-responders with 50% or more test-predicted non-responders experiencing their first event before the first test-predicted responder did. The test provides a way to identify those patients more likely to see an extended response to a treatment that has a median survival of only approximately 14.6 months and provide early warning for those patients who will not respond to it so decisions can be made earlier. Without changes in the treatment paradigm, significant differences in survival times will not be possible no matter how well 3D Predict Glioma performs.

The small sample size may also contribute to the fact that the survival of the patients in our study did not align with previously reported outcomes based upon *MGMT* promoter methylation. Interestingly, when we examined the *MGMT* promoter methylation survival curves in relation to the test type used, we observed that the msPCR test did not stratify patients as expected based upon previously published data. When a different msPCR test, covering more CpG sites was used across all clinical samples, the survival data was similar between the methylated and unmethylated patients. When the different testing methodologies were individually compared to the Kiyatec *MGMT* methylation test, the Kiyatec *MGMT* methylation test yielded results more similar to previously published data although the differences may still be due to the small sample size. Further studies examining the relation of different *MGMT* promoter CpG sites and their methylation to MGMT protein expression may help explain the outcome. The addition of 3D Predict Glioma readouts to *MGMT* promoter methylation, *IDH*

mutation, and other clinical tests such as NGS should lead to a better informed, more personalized treatment plan for each patient.

Patients with HGG have a very short survival window that has not significantly improved in the last two decades. They are subjected to radiation and therapy that can have a serious impact upon their quality of life. Additionally, the initiation of chemotherapy often precludes them from inclusion in clinical trials of new agents. Thus, it is critical that the best treatment strategy is determined as early as possible in each patient's journey. Given reasonable evidence of non-response to standard chemotherapy and results from other standard clinical tests, the lack of other therapeutic options, and in alignment with guidelines, the best treatment strategy for some individuals within current subgroups such as the elderly and unmethylated *MGMT* patients may be enrollment in a clinical trial or treatment with radiotherapy alone. Currently practiced precision medicine bases therapy selection decisions upon probability of response within a group, while decisions using functional precision medicine are based upon individual evidence of actual tissue response measured in a lab. For HGG, personalized precision medicine approaches to date have not delivered the clinical impact that was hoped for. 3D Predict Glioma test results regarding each individual patients' predicted response or non-response to SOC adds actionable data to current patient management. Functional response profiling can be a tool in the armamentarium of information a physician has to manage patients with HGG in the newly diagnosed setting by providing timely information on temozolomide susceptibility and increasing the personalization of their treatment. 3D Predict Glioma has the potential to transform HGG clinical care by providing treatment information spanning different subgroups of patients along the entire spectrum of disease.

Methods

Study design

Patients with known or suspected newly diagnosed HGG were enrolled in a multicenter, non-randomized, observational clinical study entitled "3D-PREDICT REGISTRY: 3D Prediction of Patient-Specific Response Using Ex Vivo Interrogation of Live Cells from Tumors" (acronym 3D PREDICT; ClinicalTrials.gov ID NCT03561207) from March 2018 to May 2022. The primary outcome of the study was to correlate clinical outcomes to 3D Predict Glioma test-predicted results. To reflect the new World Health Organization classification, patients were defined as astrocytoma, *IDH*-mutant and glioblastoma, *IDH*-wildtype for analysis purposes[44].

Clinical study participants

Central, or site-specific Institutional Review Board (IRB) approval was obtained for each participating site, including WIRB-Copernicus Group (WCG) (20,190,021), Roswell Park IRB (CR00005809), Prisma Health (formerly Greenville Health System) (PRO00075655), OHSU IRB (STUDY00019921), and UCSF IRB (19-27864). All enrolled patients provided written informed consent. Patients previously analyzed as part of the pilot study[27] were included in this data analysis. Eligible patients were 18 years or older with known or suspected astrocytoma, *IDH*-mutant or glioblastoma, *IDH*-wildtype. Patients were not excluded based upon performance status or older age. Newly diagnosed patients underwent maximal safe surgical resection or biopsy as standard of care to provide fresh, live tissue for testing using 3D Predict Glioma. Tissue was collected according to IRB regulations and guidelines and results of the test were provided to the treating physician, and/or patient, based upon predefined requirements. All newly diagnosed HGG patients received SOC therapy after debulking (radiation therapy plus concurrent temozolomide followed by adjuvant temozolomide) per NCCN Guidelines. When allowed by IRB, test results were generated and received by the clinician within 10 business days of the patient's surgical resection enabling the clinician to see the results prior to the initiation of therapy.

Data collection and clinical outcomes

Clinical data were collected at approximately 3-month intervals during follow up visits. PFS and OS were calculated from the time of surgical resection (tissue sampling) to either progression or death, respectively. Progression was defined by radiographic imaging, and/or clinician judgement at each participating institution to reflect the real-world use of the test. *IDH* mutation and *MGMT* methylation status were obtained during routine evaluation of the patient by the participating clinical site.

3D Predict Glioma test performance

Patient specimens were evaluated for response to temozolomide and up to 11 other compounds using 3D Predict™ Glioma. Methods, along with the analytical and clinical validation of the test were previously published[27]. Briefly, fresh, live tumor tissue from patients acquired during surgery was dissociated and cells were plated to form multicellular spheroids consisting of all cell types present in the tumor tissue. Spheroids were exposed to compound specific concentration curves followed by viability assessment at prespecified time points. For temozolomide response assessments, response/non-response classification values were previously established[27]. For other agents on the panel, IC50 thresholds were used to determine status as responder, non-responder, or moderate responder as previously described[27].

MGMT promoter methylation testing

When enough cells were available, *MGMT* promoter methylation was assayed using Kiyatec's in-house, validated *MGMT* promoter methylation test to compare with and standardize the *MGMT* promoter methylation categorization coming from individual clinical sites. Briefly, DNA was isolated using the QIAamp DNA isolation kit (Qiagen) from either frozen cell pellets or cryopreserved cells previously isolated from the same patient samples as used for 3D Predict Glioma testing. The isolated DNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit from Zymo Research and *MGMT* promoter methylation was measured using the

MGMT Methylation Detection Kit from EntroGen using the manufacturer's instructions for all kits. Testing results were not returned to the clinicians or the patients.

Statistical analysis

Demographics and baseline clinical data was summarized using descriptive statistics and analyzed for significance using univariate analysis. Kaplan–Meier curves were used to compare drug response categories using the Mantel–Haenszel test (two-sided p-value, level of significance, $p < 0.05$). For time-to-event endpoints, OS was calculated starting with the initial sample acquisition (the first surgery) and ending with death or loss to follow-up reports. The PFS rate was measured from the date of sample acquisition until the first report of disease progression or death in the medical records of the subject. The statistical analysis was performed by GraphPad Prism version 10.0.2, R software version 4.2.1, and RStudio version 2022.02.0. Survival Curves were developed with both GraphPad Prism and the R packages Survival and Survminer. The base R Statistics package was used to calculate frequency and mean values and the results of the Log Rank test. Medrio eClinical EDC (Electronic Data Capture) software (Medrio Inc., San Francisco, CA, USA) was used for data collection and quality control.

The influence of patient demographics, 3D Predict Glioma test response to temozolomide, and molecular features on survival outcomes were quantified using multivariate Cox proportional hazards model. Both OS and PFS models were adjusted for age, sex, 3D Predict result, and *MGMT* methylation status. Hazard ratios and 95% confidence intervals were calculated to estimate the relative risk associated with each covariate. To visualize the relationships between these covariates and survival outcomes, forest plots were generated. Each plot depicts the point estimate of the hazard ratio and its confidence interval against a reference line denoting no effect. The statistical significance of each covariate was assessed using p-values, with alpha level of 0.05.

Data availability

Data generated and collected in this study are not publicly available due to patient privacy requirements but are available upon reasonable request from the corresponding author.

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Author contributions

A.L.: drafted the manuscript and performed statistical analysis. A.R., L.L., A.A., R.F., J.E., C.K., N.R., A.M., B.Z., N.B., J.L., S.J.H., M.Z., A.L.C., and A.F.: contributed materials, provided clinical expertise, reviewed data, and reviewed the manuscript. K.M.: performed experiments and analyzed data. M.R.: performed experiments, analyzed data, and planned and supervised the study. J.T., K.T.: collected and organized the data. P.A.: collected and organized data and performed statistical analysis. L.H.: collected and organized data and planned and supervised the study. M.G.: edited the manuscript. T.M.D: performed statistical analysis, planned and supervised the study, and drafted the manuscript.

Competing interests

A.L., K.M., M.R., J.T., K.T., P.A., L.H., M.G., and T.M.D. are all current or former employees of Kiyatec, Inc. Both J.E. and N.B. received advisory board fees from Kiyatec, Inc. No other disclosures were reported for the other authors.

Additional information

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