RESEARCH ARTICLE: CLINICAL TRIAL

Efficacy of nivolumab in pediatric cancers with high mutation burden and mismatch-repair deficiency

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STATEMENT OF TRANSLATIONAL RELEVANCE

This trial represents the first prospective assessment of the utility of immune checkpoint blockade in pediatric cancers with increased mutation burden and/or mismatch repair. The best overall response rate of 50% and remarkable prolonged overall survival particularly in patients with relapsed high-grade glioma demonstrates a clear role for immune checkpoint inhibition (ICI) in this rare pediatric population and lays a foundation for incorporating ICI in the upfront treatment of these patients.

ABSTRACT

Purpose: Checkpoint-inhibitors have limited efficacy for children with unselected solid and brain tumors. We report the first prospective pediatric trial (NCT02992964) using nivolumab exclusively for refractory non-hematological cancers harboring tumor mutation burden (TMB) ≥5 mutations/megabase (mut/Mb) and/or mismatch-repair deficiency (MMRD).

Patients and methods: Twenty patients were screened, and ten ultimately included in the response cohort of whom nine had TMB >10mut/Mb (three initially eligible based on MMRD) and one patient had TMB between 5-10 mut/Mb.

Results: Delayed immune responses contributed to best overall response of 50%, improving on initial objective responses (20%) and leading to 2-year overall survival (OS) of 50% (95% CI; 27, 93). Four children, including three with refractory malignant gliomas are in complete remission at a median follow-up of 37-months (range: 32.4-60), culminating in 2-year OS of 43% (95% CI; 18.2, 100). Biomarker analyses confirmed benefit in children with germline MMRD, microsatellite instability, higher activated and lower regulatory circulating T-cells. Stochastic mutation accumulation driven by underlying germline MMRD impacted the tumor microenvironment, contributing to delayed responses. No benefit was observed in the single patient with a MMR-proficient tumour and TMB 7.4 mut/Mb.

Conclusions: Nivolumab resulted in durable responses and prolonged survival for the first time in a pediatric trial of refractory hypermutated cancers including malignant gliomas. Novel biomarkers identified here need to be translated rapidly to clinical care to identify children who can benefit from checkpoint-inhibitors, including for upfront management of their cancers.

INTRODUCTION

Immune-checkpoint inhibition (ICI) targeting programmed-death 1 (PD1) and its ligand (PD-L1) has shown improved survival in adults with advanced cancers[1-4]. The best responses have been observed in cancers exhibiting high tumor mutation burden (TMB), mismatch-repair deficiency (MMRD), microsatellite instability (MSI), or PD-L1 expression[3-5]. Notably, hypermutant gliomas have failed to respond due to the immune-privilege of the central nervous system (CNS), their immunosuppressive microenvironment[6], and the sub-clonal nature of MMRD in these cancers[7-9].

Previous clinical trials in children using anti-PD1 (nivolumab[10], pembrolizumab[11]), anti-PD-L1 (atezolizumab[12], avelumab[13]) and anti-CTLA4 (ipilimumab[14]) have shown limited efficacy restricted to lymphomas and rare solid tumors. In addition to low TMB and immunogenicity contributing to an 'immune-cold' microenvironment[11], this has been attributed to low expression of major histocompatibility complex[15], an immature immune system[16] and gut microbiome[17], and the presence of an immunosuppressive microenvironment enriched for macrophages[18]. The failure of ICI in unselected pediatric cancers has led to recommendations of avoiding ICI monotherapy trials in children without robust biological rationale[19,20].

The improved outcomes using ICI in refractory MMRD cancers in adults[21] and detection of hypermutation in 5% of pediatric cancers[22], majority of which were driven by MMRD, prompted us to hypothesize that such a molecularly selected cohort of cancers in children may respond to ICI monotherapy. Hence, we developed NCT02992964, an investigator-initiated, multicenter, open-label, single-arm pilot study in which pediatric patients with relapsed/refractory cancers

with increased TMB and/or MMRD were treated with nivolumab. The trial included an initial 'Part 1: Molecular Profiling', allowing patients to consent to analyses to confirm eligibility. This required the presence of a mutation in MMR genes (*MLH1, MSH2, MSH6, PMS2, EPCAM, MSH3*) or a loss of MMR protein expression on immunohistochemistry (IHC), hypermutation, or a corresponding mutational signature (COSMIC)[23] on sequencing, microsatellite instability, a history of germline conditions linked to hypermutation (constitutional mismatch-repair deficiency syndrome/CMMRD, Lynch syndrome, xeroderma pigmentosum), or prior treatment with temozolomide. Patients were eligible for 'Part 2: Treatment and Companion Biomarker Studies' provided they separately consented for therapy, had a measurable or evaluable relapsed/refractory cancer with no alternative treatment, and confirmation of MMRD and/or hypermutation.

Herein we report the unique anti-tumor responses and survival benefit using nivolumab observed in NCT02992964 for children with advanced, non-hematological malignancies with elevated mutation burden and/or MMRD. We also present the results of exploratory analyses that suggest that definite genomic and immune biomarkers can help identify children whose cancers can benefit from ICI monotherapy.

METHODS

Trial design and treatment

NCT02992964 was an investigator-initiated, multicenter, open-label, single-arm pilot study in which pediatric patients aged ≥12 months and <25 year of age with relapsed/refractory cancers with elevated TMB and/or MMRD were treated with nivolumab 3mg/kg every 2-weeks until

confirmed disease progression, intolerable toxicity, or for a maximum of 24-months. The study was approved by institutional ethics review boards and competent authorities in each center, and conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Written informed consent was obtained from all patients or parents/guardians according to local regulations. Nivolumab and funding to support the conduct of the study was provided by Bristol Myers Squibb.

Patients

The protocol included an initial 'Part 1: Molecular Profiling' option allowing patients to consent to sequencing to confirm eligibility. This required, either in the recurrent or primary tumor, the presence of a mutation in MMR genes (*MLH1, MSH2, MSH6, PMS2, EPCAM, MSH3*) or loss of protein expression on immunohistochemistry (IHC), hypermutation or a corresponding mutational signature (COSMIC)[23] on sequencing performed locally, microsatellite instability, a history of germline conditions linked to hypermutation (constitutional mismatch-repair deficiency syndrome/CMMRD, Lynch syndrome, xeroderma pigmentosum), or prior treatment with temozolomide.

Patients were eligible for 'Part 2: Treatment and Companion Biomarker Studies' provided they separately consented for therapy, had a relapsed/refractory cancer for which there was no known curative therapy, or therapy proven to prolong survival with an acceptable quality of life, baseline imaging within 14-days of starting treatment with measurable or evaluable disease, and confirmation of MMRD and/or TMB ≥5 mutations/megabase. The latter was determined by measurement of TMB either via FoundationOne (Foundation Medicine, Cambridge, MA), or an

equivalent next-generation sequencing cancer-gene panel. Patients with a TMB ≥5 mutations/megabase (mut/Mb) were eligible, with separate cohorts for TMB 5-10 mut/Mb and >10 mut/Mb. Patients with history of autoimmune diseases, HIV, chronic hepatitis B/C, prior allogeneic or solid organ transplant or ICI treatment, uncontrolled infection or requiring corticosteroid therapy at greater than physiological doses were excluded.

Study Assessments and End-points

Disease status was evaluated every 2-months while on therapy and response assessed using iRECIST criteria for solid tumors[24], and adapted according to revised INRC for neuroblastoma[25], and iRANO for CNS malignancies[26], and was labeled as complete (CR) or partial remission (PR) or progressive disease (PD). The primary objective was to evaluate objective response rate (ORR = CR + PR). For correctly assigning immune responses following possible pseudoprogression, clinically stable patients could continue therapy beyond initial unconfirmed progression (iUPD), with subsequent scans being used to establish confirmed progression, or delayed "immune" CR or PR, which could then be included in the best overall response (iBOR). iBOR was defined as the best response recorded from the start of the treatment until disease progression/ recurrence), even after discontinuation of protocol therapy[24,25,27,28] (Details in tables 5-9 of trial protocol: Supplement Appendix). It was mandatory for all patients who demonstrated CR, PR, or SD lasting beyond six cycles to have central review of the radiology at the Hospital for Sick Children. If feasible, histological evaluation was allowed to resolve ambiguity regarding immune responses.

Secondary objectives included determination of progression free survival (PFS), overall survival (OS), and evaluating the safety and toxicity of treatment. PFS was defined from time of first study medication dose to first progression that either involved significant clinical deterioration and/or was unambiguously confirmed radiologically as PD. OS was defined as time from the first study medication to death. Adverse events were graded according to the NCI's Common Terminology Criteria for Adverse Events (CTCAE) v.4.03.

Biomarker analyses

Exploratory biomarker analyses included whole exome sequencing and analyses of total and microsatellite in(sertion)-del(etions) (MSI) burden, plus IHC for CD8 and PD-L1 expression, and peripheral blood immune profiling using flow cytometry, and T-cell receptor (TCR) rearrangement using methods previously published by our group[29]. All bioinformatics analyses were performed on the SickKids High Performance Cluster (HPF) and the UHN High Performance Cluster for Health (H4H). The details are provided below.

For whole exome sequencing, genomic DNA, along with matched germline blood samples for available cases was extracted using the PaxGene Blood DNA Extraction Kit (Cat No./ID: 761133) for blood samples, Qiagen DNeasy Blood & Tissue Kits (Cat No./ID: 69504) for frozen tissue, MasterPure Complete DNA and RNA Purification Kit (Epicentre #MC85200) for paraffin embedded tissue). WES was performed at The Centre for Applied Genomics (TCAG), SickKids, using SureSelect Agilent All Exon v5 kit, followed by sequencing (150X) on Illumina HiSeq 2500. The software bcl2fastq2 v2.17 was used to generate raw fastq files. Alignment to the hg38 reference genome, followed by pre-processing and QC was adapted from the GATK standard

pipeline, using BWA-MEM 0.7.12 (alignment), BAMQC, Picard 2.6.0 (QC). Somatic variant calling was done post-alignment, using processed bam files from tumour and matched normal samples, to call both single nucleotide variants (SNVs) and insertion deletion (indel) variants. A consensus vcf file of shared variants across 2 or more of 4 variant callers (Mutect v1.1.5, GATK v3.6/Mutect2, Strelka v1.0.14, and Varscan2 Somatic v2.4.2) was generated for SNVs and indels separately, using VCFtools 0.1.15, and these vcfs were annotated using VEP v83. We used the built-in algorithms in the GATK mutect2 pipeline sequence to correct for context-dependent artifacts related to tissue source and processing by using the read-orientation bias filter and excluding low allele fraction SNPs whose evidence for the alternate allele consists almost entirely F1R2 reads or F2R1 reads. The tumor mutation burden (SNVs per megabase) from WES data was calculated by counting total number of somatic SNVs divided by total number of callable bases in megabases (~50Mb). DeconstructSigs[30] was used to determine COSMIC signatures[23,31] in the mutation spectrum within a tri-nucleotide context for each sample.

Microsatellite indels were called on the bam files of tumour and matched normal samples, using an in-house pipeline using MSMuTect. The detailed methods for this algorithm have been previously reported.[32,33] Briefly, repeats of five or more nucleotides were considered to be MS loci, and using the PHOBOS algorithm and the lobSTR approach, tumor and normal BAM files were aligned with their 5' and 3' flanking sequences. Each MS-locus allele was estimated using the empirical noise model, which is the probability of observing a read with a microsatellite (MS) length k and motif m, where the true length of the allele is j with the motif m. This was used to call the MS alleles with the highest likelihood of being the true allele at each MS-locus. The MS alleles of each tumor and matched normal pair were called individually, which were

compared to identify the mutations on the tumor MS-loci. The Akaike Information Criterion (AIC) score was assigned to both the tumor and normal models, and a threshold score that was determined by using simulated data was applied to make the final MS-indel call.

For immunohistochemical analysis of the immune microenvironment, four-micron thick sections of formalin-fixed paraffin-embedded (FFPE) surgical specimens were stained using an automated stainer (Dako OMNIS) with the following primary antibodies: PD-L1 (clone28-8, Abcam, 1/500) and CD8 (Clone c8/144B, Dako OMINS). Measurements were recorded as the number of positive cells per tissue surface unit in square millimetres. Quantitative evaluation of the immunohistochemical stains was performed by examine each section using at least five to seven different high-power fields with the most abundant tumor infiltrating lymphocyte areas.

For T-cell receptor rearrangement repertoire profiling, genomic DNA was extracted (methods as above) from tumors. These were transferred to the Pugh laboratory at the University Health Network (UHN) in Toronto, where library preparation and capTCRseq[34] hybrid capture were performed. Following library preparation, the samples were sequenced first on a MiSeq for QC purposes and then 300ng of each sample, pooled in a ratio of 1:1:1, was processed for a 3-step capture using target hybrid capture panel.8 Post-capture QC was performed on a MiSeq, followed by sequencing of up to a depth of ~2 millions reads on the NextSeq. Post-sequencing the raw data were analyzed using MiXCR version 2.1.12, 'iNext', 'immunarch' R packages and Pugh Lab customized functions on R version 3.5 to look at T-cell receptor rearrangements in the form of unique clonotypes (VDJ rearranged sequences) for T-cell receptors alpha, beta, gamma and delta. As the total read depth varied across the cohort, affecting the total successfully aligned

reads, all raw fastq reads were downsampled to ~1 million reads. QC parameters of percent aligned reads, reads used in clonotypes, final clonotype count and the total number of clonotypes per 1000 reads were considered.

For flow cytometry analysis, viable frozen peripheral blood mononuclear cells were incubated with Fc block (BD Biosciences) prior to staining for surface markers (anti-CD3 - clone UCHT1, anti-CD4 – clone RPA-T4, anti-CD8 – clone RPA-T8, anti-4-1BB– clone 4B4-1, anti-TIGIT – clone MBSA43, anti-Ki67 – clone 20Raj1) and viability dye (eBioscience). Cells were fixed and permeabilized for intercellular staining with the Foxp3 transcription factor staining buffer set (BD). Flow cytometry voltages were set using Rainbow beads (Spherotech) with the same setting between experiments. Samples were acquired on a BD LSR Fortessa flow cytometer and data were analyzed using the FlowJo software.

Statistical Analyses

The trial was originally planned as a pilot study for which the initial aim was to accrue 20 pediatric patients with CMMRD. Following encouraging results in adult trials for hypermutant cancers, an amendment was made and the study inclusion criteria was expanded to include patients with tumours harboring high TMB, as well as those with CMMRD, and consequently the total enrollment was increased to 50 patients. We planned to recruit patients into two cohorts of TMB 5-10 (cohort A) and ≥10 mutations/Mb (cohort B) using a Simon two-stage optimal design within each cohort. Cohort A was designed with power of 85% and one-sided alpha of 0.05 to test a true response rate of 35% again a null hypothesis ORR of 10%. If there were ≥2 responders within the first 10 patients, the cohort would expand to 21 patients and the outcome would be positive if there were ≥5 responses. Cohort B was designed with power of 80% and

one-sided alpha of 0.05 to test a true response rate of 30% again a null hypothesis ORR of 10%. If there were ≥2 responders within the first 10 patients, the cohort would expand to 29 patients and the outcome would be positive if there were ≥6 responses. Unfortunately, the study was terminated prematurely due to slow recruitment and loss of funding. As per pre-defined protocol criteria, patients exhibiting objective disease progression prior to the end of cycle 1 were considered evaluable for response. For all other patients, only those patients who have measurable disease present at baseline, had received at least one cycle of therapy (two doses of nivolumab), and had their disease re-evaluated were considered evaluable for response. All enrolled patients who had received at least one dose of nivolumab were evaluable for the safety data. Patients who are removed from protocol therapy continued to have the required follow up observations and documentation of additional treatments received, with the only exception for this continued documentation being the patient's withdrawal of consent, in which case only data collected prior to the withdrawal of consent could be evaluated.

PFS and OS for treated patients were estimated using the Kaplan-Meier method with patients censored, as necessary, at date of last follow-up. For correlative biomarker analyses, best overall response (iBOR) was used as a manifestation of true immune, including delayed, responses. For the serial immune correlates, landmark analysis was performed at 3 and 6 months. Furthermore as these correlates changed over time, we performed time-dependent covariate analysis using a modified Cox model as previously described (https://cran.r-project.org/web/packages/survival/vignettes/timedep.pdf). All statistical analyses were performed with R v.4.2.1. All p-values were 2-sided, with a cut-off of 0.05 for significance. Adjustments to the p-values for multiple comparisons to control for false discovery rates was

performed using methods as previously reported by Benjamini et al[35,36]. Plots were edited for aesthetics using Adobe Illustrator v.23.0.1.

Data Availability

All data relevant to this work will be available at the European Genome Phenome Archive and will be accessible through communication with the corresponding authors. All codes are publicly available.

RESULTS

Patient demographics

Twenty patients were enrolled in 'Part 1' and screened for eligibility. Nine did not meet criteria for enrollment for 'Part 2.' Between May 2017 and November 2020, eleven patients received nivolumab on study at a dose of 3mg/kg every 2-weeks until confirmed disease progression, intolerable toxicity, or for a maximum of 24-months. Data cut-off for outcomes was March 2022. All patients had failed first-line therapies. Radiation was delivered >6 months prior to recurrence and trial enrolment for all patients receiving prior radiotherapy. Though the protocol allowed palliative radiation at least 14 days before trial commencement, none of the patients received (re)-irradiation prior to the initiation of nivolumab. Patients were eligible only after recovery of acute toxic effects of previous anti-cancer therapy (Supplement Appendix).

One individual withdrew consent from all aspects of the trial immediately following the first dose without any evidence of progression or toxicity. As per the trial mandate requiring completion of at least one cycle (two doses) for end-point assessments, this patient was excluded

from further follow-up and outcome analyses (Fig.1). Median age of the final cohort (n=10) was 14-years (range 9-18; Table 1 and Table S1). Cancer diagnoses included malignant gliomas (n=7; glioblastoma n=5), neuroblastoma (n=1), colorectal carcinoma (n=1; CRC) and adrenocortical carcinoma (n=1; ACC) (Fig.2A).

Baseline genomic characteristics and cancer predisposition

Three children had been enrolled based on loss of MMR protein expression (all had TMB> 10 mutations/Mb), six based on TMB >10 mutations/Mb (five of whom also had IHC loss), while a single patient was enrolled for TMB of 7.4 mutations/Mb with an MMR-proficient tumor (Table 1, Fig.2B, C). Mutational signature (COSMIC)[23] analyses demonstrated single base substitution signatures of MMR deficiency in 8/10 cancers, and concomitant DNA polymerase-proofreading deficiency signatures along with a pathogenic *POLE* somatic variant in three of these eight MMRD cancers. Germline cancer predisposition was detected in 9/10 patients [CMMRD: n=2; Lynch syndrome: n=5; one patient each with *PALB2* variant and Li-Fraumeni syndrome (LFS); Table 1]. The single patient without germline predisposition (P8) had been previously treated with temozolomide, showed focal loss of staining for MSH6 in subpopulations of tumor cells suggesting sub-clonal MMR deficiency, and demonstrated signature 11[23], suggesting that the MMR deficiency was acquired and driven by temozolomide treatment (Fig.2B). None of the patients with germline MMR deficiency and high-grade gliomas had received temozolomide[37,38].

Toxicity

Toxicity data was evaluated for all patients who received at least one dose of nivolumab and are summarized in Table 2 (n=11). Treatment was well tolerated. Seven patients experienced grade-

≤2 treatment-related adverse events. The only significant auto-immune adverse event leading to treatment discontinuation was in a patient with glioblastoma (P6), who developed grade-3 pancreatitis (with grade-4 lipase elevation), after having previously experienced other immune-related side effects including grade-2 autoimmune gastritis and grade-1 non-infective cystitis. A second patient (P1) with glioma stopped following symptomatic hydrocephalus due to a rapid progression of a posterior fossa mass that subsequently needed to undergo de-bulking surgery.

Antitumor activity and patient survival

Disease status was evaluated every two months and patients could continue therapy beyond initial unconfirmed progression (iUPD), with subsequent scans being used to establish confirmed progression, or delayed "immune" responses, which could then be included in the best overall response (iBOR) even after discontinuation of protocol therapy[24,25,27,28]. Among 10 patients, nine had measurable disease, with evaluable-only disease in the patient with neuroblastoma. The initial radiological responses were complete response (CR) in one (P4), partial response (PR) in one (P6), stable disease (SD) in four, and progressive disease (PD) in three patients (Table 1, Fig.2D). Remarkably, delayed responses (iBOR) were observed in multiple patients even where the trial was terminated due to initial progression. This resulted in best overall responses to be reclassified as CR in three (P2, P4, P7), and PR in two patients (P6, P3) (Fig.2D, 2E). Hence while initial objective response rate was 20%, the best overall response was 50%. Recurrent tumors in patients who achieved iBOR exhibited significant to complete radiological regression (Figs. 3,4) and all who continued ICI treatment are alive at the time of reporting at a median follow-up of 37-months (range: 32.4-60) from trial enrolment. The best percentage change from baseline in target lesions is shown in Fig.2D.

The median follow-up for the cohort was 20.8-months (95% CI; 11.2, 30.4). The median OS was 23.7-months (95% CI; 7.2, not reached), culminating in an estimated 2-year OS of 50% (95% CI; 27, 93). The median PFS was 3.6-months (95% CI; 0.9, not reached), with an estimated 2-year PFS of 20% (95% CI; 6, 69) (Fig.2F). For patients with malignant gliomas, the median OS was 16.2-months (95% CI; 7.2, not reached) with an estimated 2-year OS of 43% (95% CI; 18.2, 100), while the median PFS was 2.7-months (95% CI; 0.9, not reached) with an estimated 2-year PFS of 14% (95% CI; 2.3, 87.7) (Fig.2G). The delayed immune responses following initial progression contributed to the differences observed between PFS and OS, as only minority received additional non-immune therapies after progression (Table 1).

Tumor genomic and immune biomarkers and neuroimaging response trajectories

Based on previous studies showing impact of tumoral genomic and immune biomarkers in determining outcome to ICI treatment[5,29,39-42], we investigated several biomarkers in responders and non-responders at baseline (Table 1, Fig. 2C) and their spatio-temporal variability (Figs.3, 4, S1) in select cases where repeat biopsies were performed. The unique trajectories to best overall responses in multiple patients in our trial in the context of these biomarkers revealed the following interesting observations.

An adolescent with Lynch syndrome and multifocal recurrent glioblastoma (P4), where the baseline tumor had elevated TMB, MSI, CD8 T-cell infiltration and PD-L1 expression[29] (Fig.3A, Table 1) developed initial tumor 'flare[29]' (pseudo-progression or iUPD; Fig.2E, 3A) at multiple sites after the second dose of nivolumab. Clinical deterioration mandated admission to the

intensive care unit. Symptoms subsided with supportive management without any immunosuppressive (steroid) treatment. He continued on protocol treatment, achieved CR, and is alive 3-years following initiation of nivolumab and one year after completing the trial.

The second survivor (P6) with CMMRD and recurrent multifocal glioblastoma demonstrated initial radiological progression without worsening symptoms (iUPD; Fig.2E, 3B) and continued protocol therapy, eventually achieving PR prior to stopping the trial due to severe pancreatitis (Fig.2E). This tumor also has extreme mutation and microsatellite burden, along with elevated CD8 and PD-L1 expression. After a local relapse six months after stopping protocol treatment due to severe toxicity, he was re-challenged with nivolumab (compassionate access) and remains in CR without toxicity recurrence at data cut-off.

A third survivor (P7) with CMMRD achieved delayed CR after sustained massive disease progression on serial imaging for 3-months on protocol treatment, prompting discontinuation of trial therapy (Fig.2E, 3C). Notably, the tumor at baseline had highly elevated TMB and MSI, but lacked CD8 and PDL1 expression. Continued clinical improvement while at home on hospice care prompted an imaging more than 6-months after stopping therapy. Remarkably, even in the absence of any intervening therapy, this demonstrated PR. She was restarted on nivolumab (compassionate access) and is alive in CR after completing two years of therapy. All these three patients with aggressive CNS tumors also harbored a second, somatic *POLE* mutation in their tumors contributing to the ultra-hypermutation (TMB >100 mutations/Mb)[43] in their tumors.

The fourth survivor had Lynch syndrome and a metastatic CRC (P2) with moderately elevated TMB but a very high MSI burden (Table 1). She demonstrated radiological SD, but pathological CR was demonstrated when the residual lesion was resected and showed only inflammation with no detectable tumor cells (Fig.S1). Further there was elevation of CD8 and PDL1 expression in this second biopsy as compared to the primary specimen. She remains in remission for 60-months from enrolment (Fig.2E).

Interestingly, two additional deceased patients with Lynch syndrome had mixed responses attributable to divergent TMB and/or tumor locations that deserve further elaboration. Sustained symptomatic progression in patient P3 following initial disease stability prompted a biopsy (Fig.4A). An increase in TMB and MSI burden were noted. Additionally, 83% of mutations in the second biopsy were novel and distinct from the primary specimen. There was also higher CD8 T-cell infiltration and PD-L1 expression as compared to baseline. The tumor continued to symptomatically progress following re-challenge of immunotherapy post-surgery, leading to midline shift and visual deterioration, and no further ICI treatment was administered thereafter. Remarkably, there was delayed partial response 5-months later without further intervention. Ultimately, there was another progression after 12-months which culminated in fatality ~16-months from trial enrolment.

Another patient with metastatic recurrence (P1) demonstrated progression of two of her lesions, including the primary target temporal glioma that had a moderately elevated TMB and high MSI burden (Table 1). On trial, a rapidly enlarging posterior fossa mass caused significant symptomatic hydrocephalus, requiring surgical debulking. This specimen did not have any CD8

or PD-L1 expression in contrast to the primary lesion. Further this posterior fossa lesion and the primary tumor had only 1% of mutations shared between them, suggesting that the two lesions were genomically disparate, plausibly with distinct neoantigen expression that resulted in different immune microenvironment characteristics. Though treatment was stopped culminating in rapid fatality, strikingly, a third lesion detected pre-therapy that was never biopsied showed reduction in size with nivolumab treatment (Fig.4B).

Overall, within this cohort of cancers with elevated TMB, we observed that children whose tumors harbored higher (≥median) total indel and microsatellite indel burden had improved survival (median OS not reached for both; Fig.5A). Association of CD8 and PDL1 expression with response and survival were variable (Fig.2C).

Immune biomarkers in peripheral blood

We performed exploratory immune cell subset analyses from serial blood samples collected at pre-determined time-points as indicated in the trial protocol that provided additional insights. First, responders, including patients who had delayed responses after sustained initial progression (P7, P3), had higher 41BB+ CD8 T-cells in blood at baseline, as well as achieved a higher peak of these activated populations of T-cells on immunotherapy (Fig.5B). Non-responders had higher T-regulatory cells (CD4+) in blood at baseline, and their nadir counts in serial measurements stayed higher than responders (Fig.5C). Furthermore, patients with persistently elevated T-regulatory cells after first 3 months on trial had a higher risk of death (Supplementary Table S2). Lastly, a pilot analyses of serial peripheral blood TCR-beta (TRB) rearrangement were performed for 3 responders and 3 non-responders over the first 3 months of

treatment[44]. The responders had higher total clone count, as well as higher richness (measured by Shannon's diversity) and evenness (measured by Gini coefficient) of clonal distribution than non-responders[44] (Fig.5D).

DISCUSSION

In this report, we present the first prospective clinical trial using nivolumab exclusively for children with refractory solid and brain tumors with high TMB. Best overall responses (50%) exceeded initial objective radiological response (20%), translating to ongoing prolonged ongoing survival in 4/5 responders with rapidly-fatal cancers when continued on ICI treatment. Despite a premature termination for slow accrual and loss of funding, our trial, like select others[45], could still provide us with several important clinical and biological insights for this unique cohort of childhood cancers with elevated mutation load.

We noted that the high TMB in our cohort of refractory pediatric cancers had diverse etiologies which plausibly impacted ICI response, as had also been recently proposed by an international multi-stakeholder group on ICI use in pediatric cancers[20]. All five responders in this trial exhibited MMRD, which uniquely for our cohort of pediatric patients was predominantly of germline origin and related to well-characterized cancer predisposition syndromes. Cancers in the two children with constitutional (biallelic) MMRD demonstrated ultra-hypermutation (TMB >100 mut/Mb) with concomitant DNA polymerase-proofreading deficiency[43] and responded following ICI. Remarkably, we also witnessed responses among 3/5 patients with Lynch syndrome, where the cancers had both hypermutation and evidence of somatic MMRD as was demonstrated using IHC and mutational signature analysis [COSMIC single base substitution

(SBS) signatures 6, 15, 20[31]]. It is likely that germline MMRD contributes to improved survival on immunotherapy because of the continuous obligatory mutation accumulation[43] leading to higher neoantigen expression. An additional important contributor could be the concomitant presence of high MS-indel burden[29], as frameshift mutations can generate 'higher-quality' neoantigens, especially if the indels are in microsatellites in coding loci[29,46]. It is important to note that for pediatric cancers, MSI panels developed for adults have low sensitivity and appropriate genomic assays should be incorporated for incorporation of MSI-high pediatric cancers in future ICI studies[33,47].

In this context, it is important to highlight that the patient with GBM where MMRD was acquired following temozolomide treatment (P8) did not respond to nivolumab despite having a high TMB. As observed in adult glioblastomas that historically do not respond to ICI,[7] this tumor harbored SBS 11[7,31] and had only limited focal areas of loss of MMR staining (Fig.2B), suggesting sub-clonality for acquired MMRD, plausibly contributing to the low MSI burden and lack of ICI response[7,29]. Similarly, an elevated TMB arising from other etiologies was not associated with response to ICI. This included a patient with neuroblastoma (P5) with defect in the homologous recombination repair (HRD) pathway harboring SBS 3 (HRD signature),[31] and a patient with Li-Fraumeni syndrome and ACC (P10). Together, these data suggest that in children, hypermutation driven by germline MMRD is likely essential for ICI response.

The dynamic responses that we observed in several patients with germline MMRD induced hypermutation are worth further discussion. While early tumor 'flare' or pseudo-progression has been previously reported[29] (P4; Fig.3A), we witnessed clear sustained and symptomatic tumor

progression on nivolumab prior to delayed responses and prolonged survival in three children (P3, P6, P7; Figs.3B,C, 4A). Such prolonged survival with continuation of ICI post-progression has been noted in adult cancers like melanoma.[48] The patient with ultra-hypermutant MMRD glioblastoma (P7) had low CD8 expression at diagnosis, and sustained progression for >3-months resulting in trial termination. Repeat scans 6-months without additional therapy demonstrated response, leading us to hypothesize that MMRD-driven genomic instability can contribute to stochastic mutation accumulation over time, conferring delayed immunogenicity[43]. This was objectively demonstrated in another patient (P3), where a biopsy during progression indeed showed that the glioma now harbored a higher TMB, leading to higher immunogenicity, as demonstrated by both higher CD8 and PD-L1 expression[29] in the tumor, plausibly contributing to the delayed response. Similar evidence of radiological progression for several weeks was also noted in patient P6 before delayed and sustained response, contributing to prolonged ongoing survival. Of note, among CNS tumors, responders with lower tumor burden (P4, P6) had relatively earlier response than those with higher disease burden (P3, P7), where despite favorable genomic biomarkers, response was delayed beyond 6 months (Fig.2E). There is increasing evidence that a higher tumor burden negatively impacts the microenvironment and can modulate response to checkpoint inhibitors[49].

It is also important to note that we observed different lesions in the same patient, which are conventionally considered as metastatic tumors at relapse, could be biologically heterogeneous and consequently have different responses (P1; Fig.4B). This could be attributed to random mutation accumulation secondary to MMRD in lesions at different sites. These findings highlight the challenges of conventional radiological assessments in children treated with immunotherapy

519 and combined with the delay in responses observed by us, may account for limited responses
520 seen in some previous studies in children receiving ICI treatment.
521
522 Lastly, although limited by the small numbers, continuous collection of biomarkers in the blood
523 and in tumor tissue at progression in our trial provided us with additional biological insights. We
524 did not find a reliable association between PD-L1 expression and patient outcome (Fig.2C)
525 plausibly linked to cancer-driven immune-editing[50], as MMRD tumors in our trial exhibited
526 change in PD-L1 expression over time (Fig.4A) [51,52]. Similarly, while we observed variable and
527 consistent elevation of CD8 (+) T-cells in the microenvironment plausibly driven by neoantigen
528 expression caused by the high TMB, this too could change over time (Fig.4A,B), confounding
529 associations of baseline measurements on diagnostic biopsies with response at recurrence
530 following ICI treatment.
531
532 Interestingly, serial peripheral blood analysis during treatment on trial did show important
533 correlation between tumor response and immune activation. Activated 41BB+CD8+ T-cells,
534 higher levels of which have been previously associated with anti-tumor immune responses[53,54,29],
535 were uniformly and significantly elevated in all five responders in our study. Interestingly,
536 patient P1, who demonstrated divergent responses at three sites including radiological response
537 in one lesion, also had elevated activated 41BB+CD8+ T-cell counts (Fig.5B). This suggested
538 that asymmetric and atypical responses could be potentially identified by peripheral biomarkers.
539 We also found lower T-regulatory cells in the peripheral blood of responders, as has been
540 recently linked to more effective anti-tumor responses [55]. Additionally, we observed that
541 responders had both higher richness and evenness of their TRB clonal repertoire. While we did

observe increase in skewness (Gini coefficient) of the TRB repertoire over time, we did not find enrichment of specific TCR sequences (measured by reduction of Shannon's entropy) as has been previously reported in responders following ICI treatment[56-60]. This is plausibly related to the high, ongoing mutation accumulation and neoantigen expression in MMRD cancers that leads to a dominance of multiple TRB clones. These intriguing findings maybe specific to germline MMRD where cancers arise in the backdrop of constitutional genomic instability need to be evaluated in more detail in future studies.

In summary, our trial shows benefit of nivolumab treatment in pediatric patients with MMRD cancers with elevated TMB, including lethal malignant gliomas, with an acceptable safety profile. During ICI treatment, clinicians need to be aware of early pseudo-progression, differential responses at distinct sites, and delayed responses due to changing tumor immunogenicity. Future approaches could involve biomarker-driven front-line use in aggressive tumors like malignant ultra-hypermutant gliomas with favorable biomarkers[61,62], and exploration of combinatorial approaches in patients failing anti-PD1 monotherapy[63]. Routine use of MMR IHC, the incorporation of TMB and mutational signatures into cancer sequencing analyses, and screening patients from low and middle-income countries[64,65] with higher prevalence of germline MMRD can improve outcome for these patients worldwide.

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AUTHOR CONTRIBUTIONS:

DM, EB and UT planned the study. DM and LSN developed the study documentation. AD and DM analysed the data and wrote the manuscript. BW reported the radiology. AL and CH reported the pathology. SSR, SS, ABE, JC, LG, YM, GG and TP performed the bioinformatics. SS and PSO did the flow cytometry studies. LS, EB, VB, LS and ME were involved in patient enrolment and sample coordination. Those involved in patient management included DM, EB, UT, AD, NC, RD, CFC, THE, RD, JEM. All authors have reviewed and agreed to the contents of the manuscript.

COMPETING INTERESTS: None

TABLES

Table 1. Summary of the demographics, clinical features, biomarkers, treatment details, and outcomes of patients treated on NCT02992964 (n=10).

Id	Age (y)	Sex	Genetic predisposition	Race	Cancer Diagnosis	MMR Immuno-histochemistry	TMB (mut/Mb)	Total indels	MS-indels	*POLE/ POLD1* variants	COSMIC SBS v.2	Baseline CD8 expression	Baseline PD-L1 expression	Pre-trial palliative radiation	Objective response on trial (Criteria)	Best overall response	Nivolumab post-trial termination	Other non-immune systemic therapies	OS (months)	Status
P1	13	F	Lynch syndrome	Caucasian	Anaplastic astrocytoma	PMS2, MLH1 lost in tumor only	14.3	595	1588	Absent	1, 15	7%	Faint	No	Progressive disease (iRANO)	Progressive disease	No	None	1.8	Dead
P2	17	F	Lynch syndrome	Caucasian	Colorectal carcinoma	MSH2, MSH6 lost in tumor only	25.4	892	704	Absent	21, 30	2.70%	Faint	No	Stable disease (RECIST)	Pathological complete remission	No	None	53.12	Alive
P3	12	F	Lynch syndrome	Caucasian	Glioblastoma	MSH2, MSH6 lost in tumor only	15.9	434	892	Absent	1, 6, 18	0%	Absent	No	Stable disease (iRANO)	Delayed partial response	Yes	After 12 months: Nilotinib, Everolimus	15.85	Dead
P4	15	M	Lynch syndrome	Caucasian	Glioblastoma	MSH2, MSH6 lost in tumor only	253.64	801	509	*POLE; p.S459F*	1, 5, 12, 20	2%	>1%	No	Complete response (iRANO)	Complete response	No	None	35.14	Alive
P5	16	F	Germline *PALB2*	Caucasian	Neuro-blastoma	All MMR stains retained	11.35	x	x	Absent	3, 8, 12, 18	x	x	No	Stable disease (Revised INRC)	Stable disease	No	MIBG, PARP-inhibitors	31.29	Dead
P6	13	M	CMMRD	Asian	Glioblastoma	PMS2 lost in both tumor and germline	714.46	1091	1291	*POLE; p.S461P*	1, 14, 15	7.30%	>1%	No	Partial response (iRANO)	Partial response	Yes	None	26.56	Alive
P7	9	F	CMMRD	Native American	Glioblastoma	PMS2 lost in both tumor and germline	581.84	903	989	*POLE; p.S459F*	1, 10, 14, 15	0.60%	Absent	No	Stable disease (iRANO)	Delayed complete response	Yes	None	25.45	Alive
P8	14	M	None	Caucasian	Anaplastic astrocytoma	Focal, heterogeneous MSH6 loss	106.42	68	19	Absent	11	>10%	>1%	No	Progressive disease (iRANO)	Progressive disease	No	None	7.2	Dead
P9	18	M	Lynch syndrome	African American	Glioblastoma	MSH2, MSH6 lost in tumor only	33.46	133	185	Absent	1, 15	0.70%	Absent	No	Stable disease (iRANO)	Stable disease	Yes	None	16.24	Dead
P10	11	M	Li Fraumeni syndrome	Hispanic	Adrenocortical carcinoma	All MMR stains retained	7.4	14	29	Absent	4, 23, 29	x	x	No	Progressive disease (iRECIST)	Progressive disease	None	None	0.72	Dead

Abbreviations: y=Years, F: Female, M=Male, MMR: Mismatch repair, CMMRD: Constitutional MMR Deficiency, SBS: Single base substitution signatures, mut/Mb: Mutations/megabase.MS: Microsatellite, MIBG: iodine meta-iodobenzylguanidine, PARP: Poly (ADP-ribose) polymerase, X: data not available.

Table 2. Treatment-related adverse events that were considered possibly, probably or definitely related to study therapy for children treated on NCT02992964 (n=11)

Adverse Event	Number of patients		
	All grades	Grade 3	Grade 4
Headache	3	1	0
Fatigue	3	0	0
Hydrocephalus	1	0	1
Papilledema	1	0	0
Other neurological [1]	2	0	0
Lipase increased	1	1	1
Pancreatitis	1	1	0
Other gastro-intestinal [2]	3	0	0
Other skin [3]	2	0	0
Other [4]	3	0	0
Lymphopenia	1	1	0
Other laboratory [5]	1	0	0

[1] gait disturbance, dizziness, tremor, seizure
[2] vomiting, nausea, diarrhea, blood in stool, gastritis
[3] alopecia, dry skin
[4] dyspnea, 'flu-like symptoms, non-infective cystitis, arthralgia
[5] ALT increased, hypokalemia, hyponatremia, anemia

FIGURES AND LEGENDS

Fig.1. CONSORT diagram showing flow of patients

Fig.2. Characteristics and outcomes of patients: (A) Cancer types included in the final analysis cohort. **(B) Immunohistochemistry patterns of MMR-deficiency** highlighted using representative patients: complete loss of PMS2 expression in all cells in a patient with CMMRD (P6); loss of PMS2 in tumor cells with retention in normal cells in a patient with Lynch syndrome (P1) and focal loss of MSH6 in only a subset of tumor cells in a patient with therapy-associated MMRD (P8) (Magnification: 100X, inset 400X). **(C) Oncoplot** summarizing clinical and genomic features. Patients are arranged by their tumor's total single nucleotide variants per megabase (on a semi-logarithmic scale). **(D) Waterfall plot summarizing radiological responses** of target lesions (++: rapid enlargement and exact measurements at progression not available; x: Divergent responses including response at non-target site; #: Pathological complete response; *: Delayed response). This demonstrates that while initial ORR was 20% (P4, P6), the iBOR was 50% (including P2, P3 and P7). **(E) Swimmer's plot summarizing patient course**. Each solid box denoted time while on-trial protocol. Some patients had termination of their trial protocol treatment due to progression or toxicity but thereafter received nivolumab through compassionate or commercial access, the duration of which are shown as dashed boxes. **(F) Survival for entire cohort:** OS (blue) and PFS (yellow) **(G) Survival for CNS tumors:** OS (blue) and PFS (yellow). The PFS curves are based on prior-defined radiologic criteria in the trial protocol (iRANO/ iRECIST) and did not include the delayed responses (i.e.. iBOR).

Fig.3. Response after pseudo and true progression following ICI treatment in three survivors with glioblastoma. (A) Early 'flare' on ICI treatment (P4). Two lesions in the posterior fossa at time of progression measured 6x6 mm (right) and 5x3 mm (left). On week 3, there was significant clinical deterioration needing intensive care and MRI demonstrated increase to 9x9 mm and 8x9 mm respectively. Supportive care without use of steroids led to clinical improvement and next MRI showed reduction to 6x5 mm and 6x4 mm, respectively. The patient continued on trial protocol and the 6 months' scan confirmed CR. **(B) Early interim progression and response (P6)**. Multi-focal disease, with two of the largest lesions measuring

10.5x9 mm (target lesion) and 9x8 mm at progression. After start of protocol, MRI at 3 months showed mild reduction in size to 10x8 mm and 9x7 mm, respectively. The next MRI showed progression, with increase to 18.6x11mm and 14x8.2mm, but the patient was stable. Continued treatment on protocol showed reduction to 10.7x6.8mm and 9.6x7.5 mm respectively at 6 months. Patient attained PR for target site and the other sites of disease had disappeared completely when trial had to be stopped due to severe pancreatitis. **(C) Delayed response after initial massive and sustained progression (P7).** The target lesion at the primary surgical bed at start of protocol treatment was 6x5 mm, which continued to show significant progression continuously through months 2 (31x29 mm) and 3 (47x37 mm), along with metastatic progression at other sites, following which patient was shifted to palliative home care without any anti-cancer therapy or steroids. Clinical improvement after 9 months prompted MRI that showed reduction to 5x3 mm, the patient was restarted on nivolumab, achieved CR and completed 24 months of treatment.

Fig.4. Biological explanation for unique and divergent response trajectories for two deceased children with high-grade gliomas. (A) Tumor evolution on ICI and impact on microenvironment (P3). At protocol initiation, lesion was 55x45mm, progressed to 66x45 mm after a month and 85x51mm in the 3rd month. Debulking was done and biopsy showed increase in TMB and novel mutation accumulation, as shown using a Venn-diagram depicting the overlap of variants at baseline (blue) and at the time of progression (dark pink). Simultaneously, there was increase in CD8 and PD-L1 expression at the time of the second biopsy. The tumor progressed symptomatically to 90.5x62 mm after restarting ICI post-surgery, and treatment was stopped. Serial scans without further treatment showed stabilization and subsequently reduction to 38x30 mm, before further progression and death. All IHC images are 100X (inset: 400X). **(B) Divergent responses following ICI in biologically distinct lesions in the same patient (P1)**. Massive radiological progression in lesion 1 (from 10.9x8.8 mm to 47x29 mm) which was the target lesion along with rapid symptomatic progression in lesion 2 (from 28x23 mm to 53x48 mm) soon after trial initiation mandated debulking of lesion 2. Comparison of lesions 1 (biopsy at baseline) and 2 (after rapid progression on nivolumab) showed different TMB and MSI burden, with insignificant overlap of mutations as shown using a Venn-diagram for the primary (blue) and posterior fossa (dark pink) lesions. This suggested that these were biologically distinct

gliomas with very different immune microenvironment. Lesion 2 in the posterior fossa showed massive regrowth very rapidly after debulking. Lesion 3 (initially 16x15 mm) was never biopsied but showed radiological response on nivolumab treatment. All IHC images are 100X (inset: 400X).

Fig.5. Biomarker analyses. Tumor biomarker analyses. (A) Microsatellite indel and total indel burden. Responders had higher levels of both MS and total indels and higher levels stratified overall survival probability. (Cut-off for high and low for Kaplan-Meier survival curves are medians for respective cohorts. Shaded portions signify 95% CI). **Peripheral Blood. (B) Activated T-cells** (41BB+ CD8 + T-cells as % of total T cells): At treatment initiation, peak, and trajectory during the first year. Responders had higher activated T-cells at baseline and achieved higher peaks during ICI treatment. **(C) T-regulatory cells** (%): At treatment initiation, nadir, and trajectory during the first year. Responders had lower regulatory T-cells at baseline and lower nadir during ICI treatment. Both stratified overall survival probability. **(D) T-cell receptor-beta clonotype analysis**: clone count and diversity indices compared between responders and non-responders, with peak and nadir measurements while on treatment.

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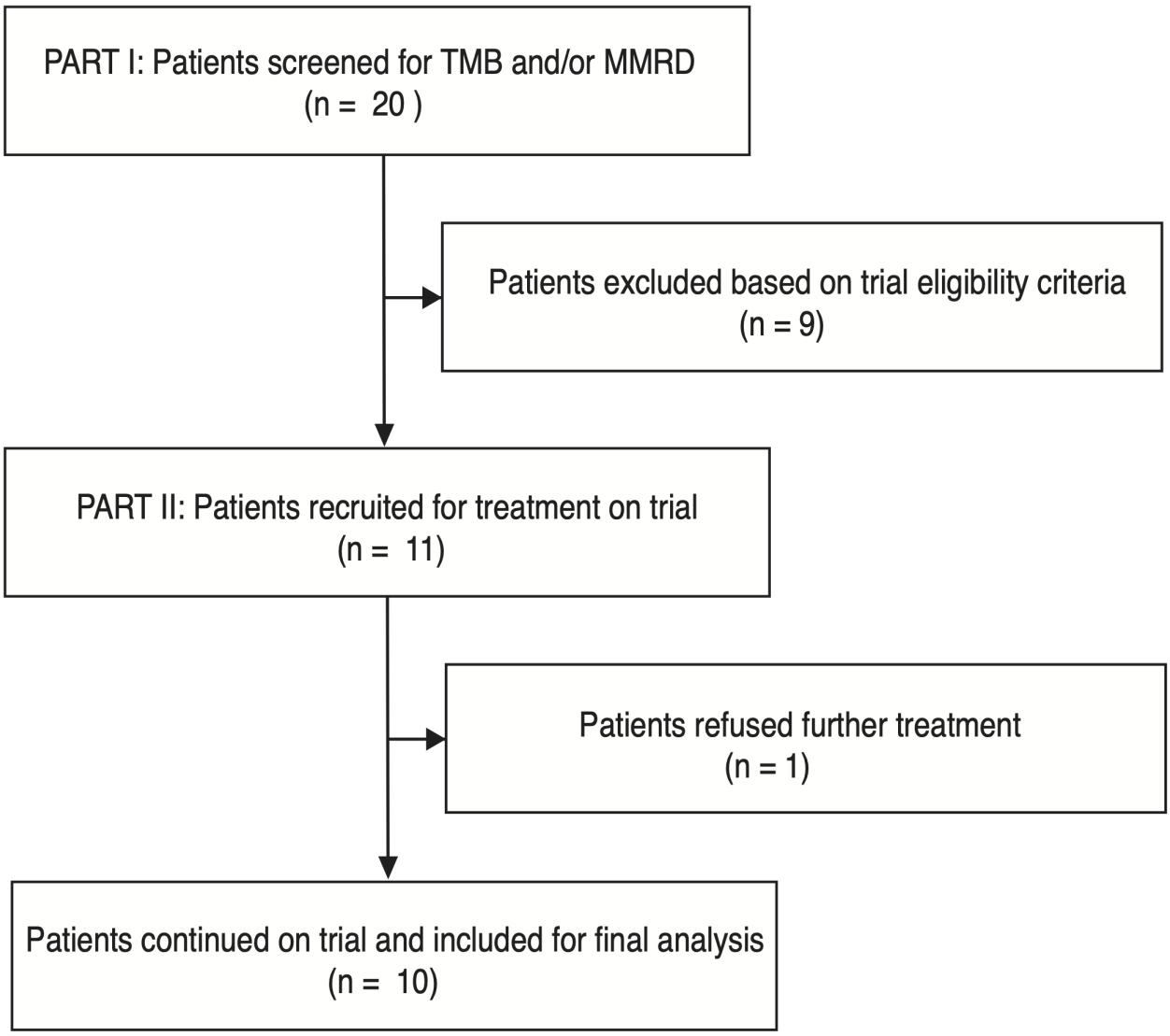
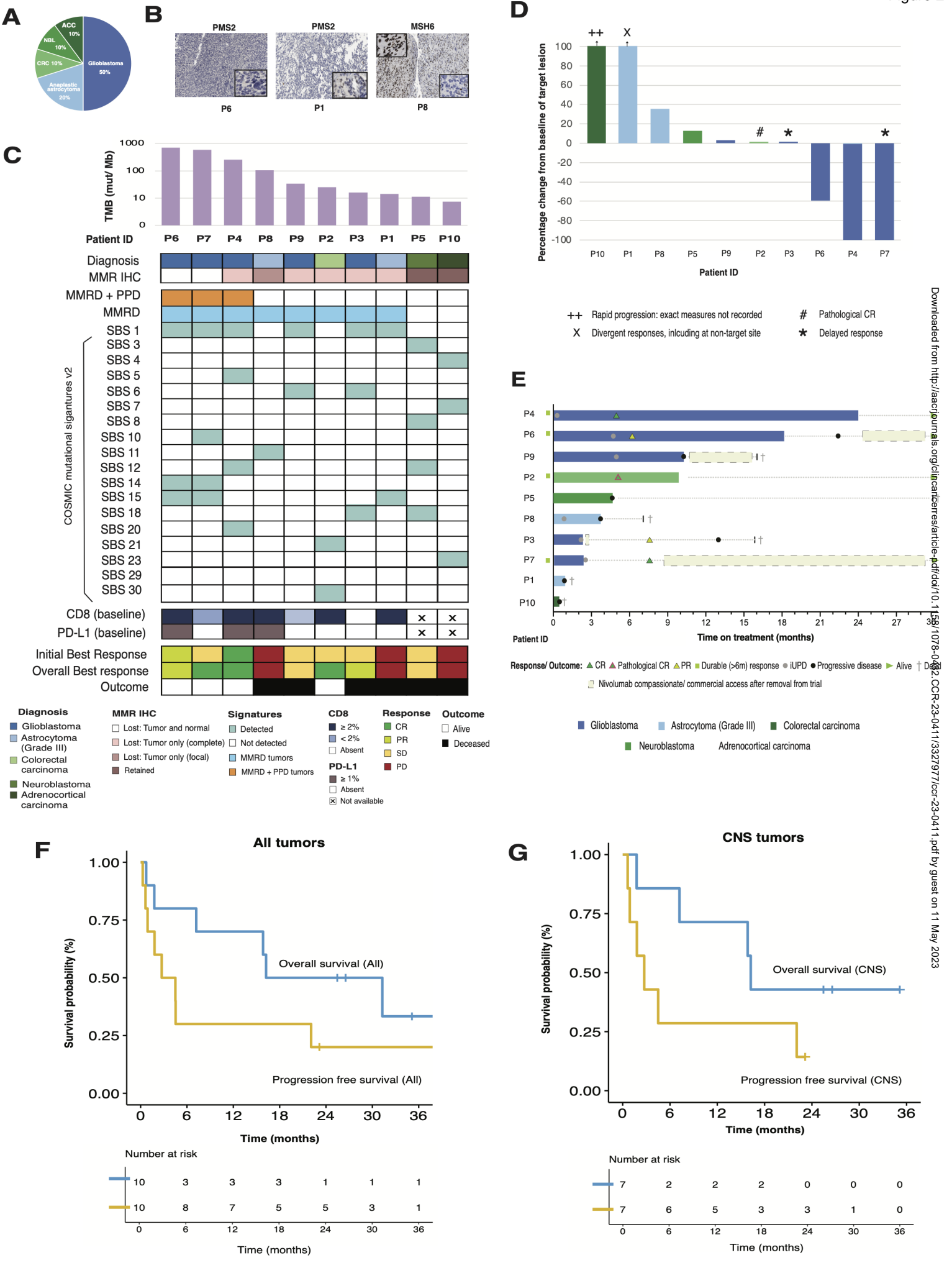
Figure 1





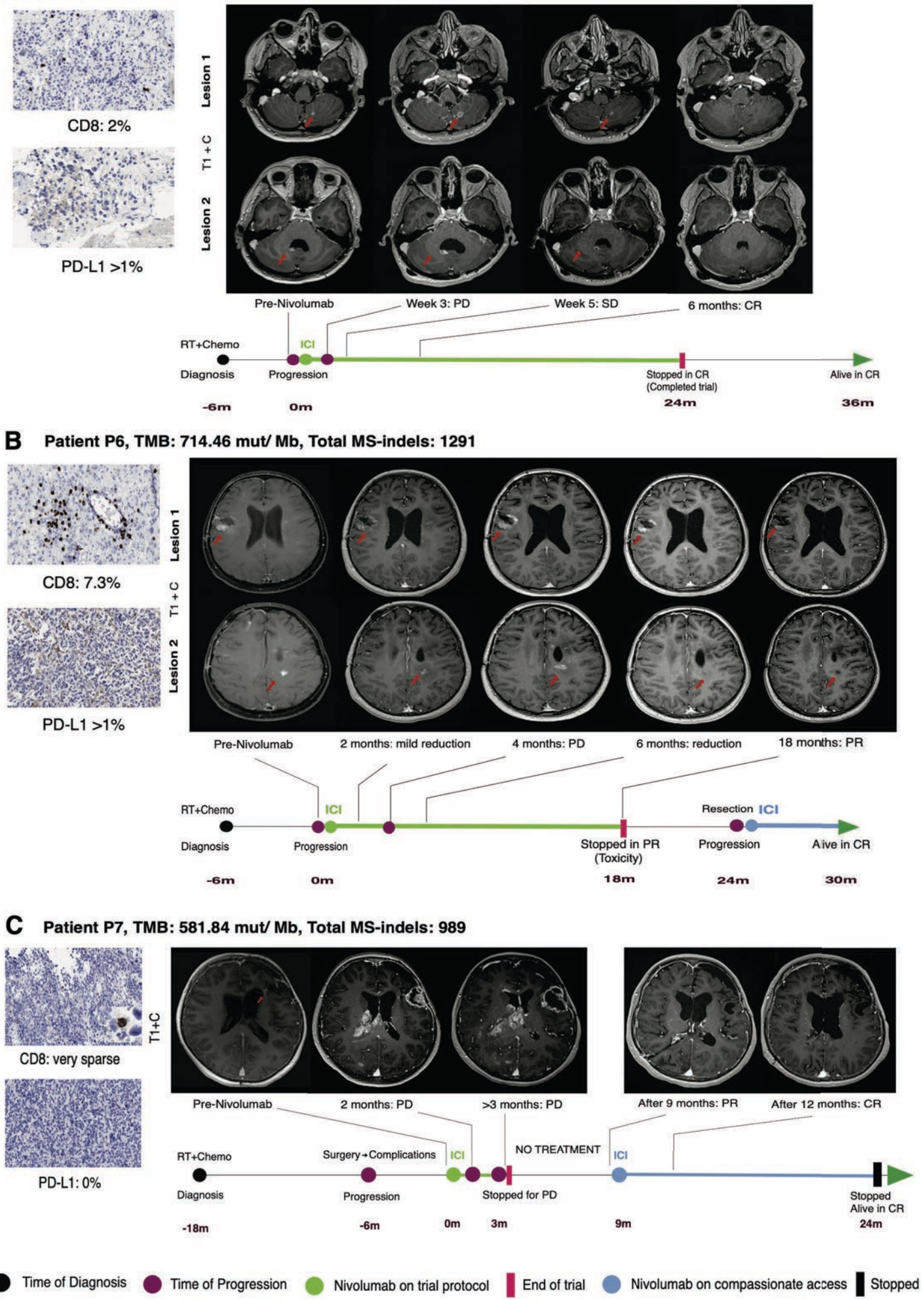


Figure 4