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ORIGINAL ARTICLE

ctDNA detection in cerebrospinal fluid and plasma and mutational concordance with the primary tumor in a multicenter prospective study of patients with glioma☆

S. Cabezas-Camarero[1,2*], R. Pérez-Alfayate[3†], V. García-Barberán[4†], M. L. Gandía-González[5], P. García-Feijóo[5], I. López-Cade[6], V. Lorca[4], I. Casado-Fariñas[7], M. A. Cerón[6], M. Paz-Cabezas[1,4], M. J. Sotelo[8,9,10], M. García Conde[11], H. Roldán Delgado[11], Y. Sánchez Medina[12], I. Díaz-Millán[1] & P. Pérez-Segura[1,2]

[1]Department of Medical Oncology, Hospital Clínico Universitario San Carlos, IdISSC, Madrid; [2]Department of Medical Oncology Department, IOB Institute of Oncology-Madrid, Madrid; [3]Department of Neurosurgery, Hospital Clínico Universitario San Carlos, IdISSC, Madrid; [4]Molecular Oncology Laboratory, Hospital Clínico Universitario San Carlos, IdISSC, Madrid; [5]Department of Neurosurgery, Hospital Universitario La Paz, IdIPaz, Madrid; [6]Experimental Therapeutics Unit, Department of Medical Oncology, Hospital Clínico Universitario San Carlos, IdISSC, Madrid; [7]Pathology Department, Hospital Clínico Universitario San Carlos, Madrid, Spain; [8]Department of Medical Oncology, Aliada Cancer Center, Lima; [9]Department of Medical Oncology, Clínica San Felipe, Lima; [10]Department of Medical Oncology, Hospital María Auxiliadora, Lima, Perú; [11]Department of Neurosurgery, Hospital Universitario de Canarias, La Laguna, Santa Cruz de Tenerife; [12]Department of Neurosurgery, Hospital Nuestra Señora de la Candelaria, Santa Cruz de Tenerife, Spain

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Background: Cerebrospinal fluid (CSF) stands as an easily accessible reservoir for circulating tumor DNA (ctDNA) analysis in patients with central nervous system (CNS) tumors, although evidence is still limited. Our aim was to prospectively evaluate the feasibility of detecting ctDNA for mutational analysis in CSF and plasma in patients with glioma.

Methods: This was a prospective study of patients with glioma diagnosed at four third-level hospitals in Spain. A customized next-generation sequencing (NGS) eight-gene panel (*IDH1*, *IDH2*, *ATRX*, *TP53*, *PTEN*, *PIK3CA*, *EGFR*, *BRAF*) was used in paired CSF, plasma and tumor samples. Mutation concordance occurred when the same pathogenic gene variant was detected in tumor and ctDNA. The prognostic value of ctDNA and that of its median variant allele frequency (mVAF) were analyzed.

Results: Between February 2017 and March 2020, 37 patients with glioma were enrolled. The 32 patients with analyzable CSF samples comprised patients with new diagnosis ($n = 23$) and relapse ($n = 9$); World Health Organization fifth Edition types: *IDH*-mutant astrocytoma ($n = 10$), *IDH*-mutant oligodendroglioma ($n = 6$) and *IDH*-wildtype glioblastoma ($n = 16$); CSF-ctDNA-positive: 19/32 (59%); and CSF-ctDNA-negative: 13/32 (41%). CSF mutation numbers were 1 (10/19), 2 (7/19) and 3 (2/19). Frequencies of CSF-ctDNA-mutated genes were *EGFR* (8/19, 42%), *PTEN* (7/19, 37%), *TP53* (6/19, 32%), *IDH1* (5/19, 26%) and *PIK3CA* (4/19, 21%). Tumor—CSF mutation concordance was found in 16/19 (84%). Progression-free and overall survival were significantly shorter in ctDNA-positive patients with VAF equal to or greater than the mVAF compared with ctDNA-positive patients with VAF lower than the mVAF. No association was found between ctDNA in CSF and distance to closest CSF reservoir, tumor size or *IDH* status. ctDNA was detected in 2 of 14 (14%) individual plasma samples, in both cases concordant with the primary tumor.

Conclusion: CSF is a reliable reservoir for ctDNA analyses in patients with glioma. ctDNA is detectable in plasma although at a lower rate. Larger, prospective studies should be conducted to refine the potential role of liquid biopsy in this disease.

Key words: ctDNA, cerebrospinal fluid, liquid biopsy, glioma, NGS, variant allele frequency

INTRODUCTION

Liquid biopsy has been shown to play a role in the diagnosis, monitoring and outcome prediction of multiple solid and hematological malignancies. However, detection of tumor-borne components in the peripheral blood of patients with central nervous system (CNS) tumors has been elusive, with low and inconsistent detection rates.[1,2] The development of liquid biopsy in CNS tumors is challenged by the blocking effect of the blood-brain barrier (BBB) and

*Correspondence to: Dr Santiago Cabezas-Camarero, Head & Neck Cancer, Neuro-Oncology and Familial Cancer Unit, Medical Oncology Department, Hospital Clínico Universitario San Carlos, Paseo del Profesor Martín Lagos S/N, 28040 Madrid, Spain. Tel: +34 913303000

E-mail: santiago.cabezas@salud.madrid.org (S. Cabezas-Camarero).

@SantiCabezas1

†These authors contributed equally to this work.

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by anatomical and biological parameters such as tumor size, proximity to cerebrospinal fluid (CSF) reservoirs and variable biological aggressiveness.[3,4] Therefore, although gliomas are the most prevalent primary malignant CNS tumors, the use of liquid biopsy is still purely investigational. The current World Health Organization (WHO) 2021 Classification establishes two major glioma groups according to isocitrate dehydrogenase (*IDH*) mutational status with differentiated prognoses and specific molecular profiles that allow for a more accurate classification than relying only on histomorphological criteria.[5] Although tumor sampling remains the standard diagnostic procedure in patients with gliomas, CNS biopsies account for a 2%-3% risk of major intracranial hemorrhage and a 0.8% death rate that prevents the performance of serial or multiple intraprocedural biopsies.[6] On the other hand, liquid biopsy can circumvent the spatial and temporal biases and is safer to perform than conventional tissue biopsy.[7] Whereas liquid biopsy studies in blood using circulating tumor DNA (ctDNA), circulating tumor cells or extracellular vesicles have either resulted in low detection rates or remain technically cumbersome, a few studies have demonstrated the feasibility of ctDNA detection in CSF with relatively high detection rates in patients with glioma. However, evidence is still limited and the use of ctDNA has not been validated in large prospective studies, preventing its introduction into clinical practice.[8-10] Beyond allowing diagnosis in cases where tissue is not available, other potential applications of liquid biopsy in gliomas are the identification of druggable molecular targets, the detection of residual disease and outcome prediction, and to allow for a more accurate differential diagnosis between tumor progression and post-treatment changes. In addition, several unanswered technical questions remain, such as which is the most appropriate CSF reservoir and CSF collection technique, the ideal CSF volume and timing of collection, as well as the molecular assay to be used.[3,4]

The aim of the current study was to prospectively evaluate the performance of a customized next-generation sequencing (NGS) platform for ctDNA analysis in CSF collected intra-operatively in patients with newly diagnosed or recurrent glioma.

PATIENTS AND METHODS

Primary and secondary endpoints

The primary endpoints were the detection rate of ctDNA pathogenic mutations in the CSF and plasma and the concordance rate of pathogenic mutations between the primary tumor and the CSF and between the primary tumor and plasma.

The secondary endpoints were: (i) The association of ctDNA detection in CSF with progression-free survival (PFS) and overall survival (OS). PFS and OS were defined as time from initial diagnosis or from CSF collection until progression or death from any cause, respectively; (ii) The association between ctDNA detection and tumor stage (WHO fifth edition.), *IDH* status, tumor size, tumor volume and distance to the closest CSF reservoir; (iii) Safety related to the CSF collection procedure.

Study design and ethical considerations

This was a prospective study of patients with glioma diagnosed at four third-level hospitals in Spain: Hospital Clínico Universitario San Carlos and Hospital Universitario La Paz, both in Madrid, and Hospital Universitario de Canarias and Hospital Nuestra Señora de la Candelaria, both in Santa Cruz de Tenerife. The study was approved by the Institutional Review Board (IRB) of Hospital Clínico Universitario San Carlos (IRB code 16/549-E) and was conducted in accordance with the principles of the Declaration of Helsinki.

Patient enrollment

Patients had to be $\geq$18 years of age, with a strongly suspected clinicoradiological diagnosis of newly diagnosed or recurrent glioma—in all cases this was assumed after tumor board discussion. All patients signed an informed consent form before being enrolled in the study, allowing permission for collection of a sample of CSF either intra-operatively or through a lumbar puncture before the surgical manipulation of the tumor at the time of new diagnosis or at the time of tumor relapse.

This study followed the REporting recommendations for tumour MARKer prognostic studies (REMARK).[11]

Tumor sampling and CSF collection procedure

Tumor tissue was obtained during surgery for newly diagnosed or recurrent gliomas. NGS analysis of tumor tissue was performed either on formalin-fixed paraffin-embedded (FFPE) samples or on fresh tumor samples (Figure 1).

The CSF was collected before the surgical manipulation of the tumor, either intra-operatively from the convexity space or through lumbar puncture (LP). In all cases, 2-5 ml CSF was collected in non-EDTA, dry, sterile tubes. The CSF was immediately centrifuged and the CSF supernatant stored at −80°C. The collection of CSF intra-operatively from the convexity space was performed after craniotomy at the opening of the dura matter. The convexity subarachnoid space was carefully dissected, and the CSF slowly aspirated with a 5-10 ml syringe (Figure 1).

Immunohistochemistry and MGMT *methylation studies*

The *IDH*1-R132H antibody (clone H09) (catalog reference: IDAH09, Dianova, Hamburg, Germany) was used for *IDH1*-R132H mutation expression analysis by immunohistochemistry (IHC) in an autostainer Dako Omnis (Agilent Technologies, Inc., Santa Clara, CA), as previously described.[12] *IDH1*-R132H mutation expression was established as positive when tumor cells showed a strong cytoplasmic staining, while weakly diffuse tumor cell staining was considered negative.[12]

O6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation studies were performed using bisulfite-based DNA conversion with the EZ DNA Methylation-Direct

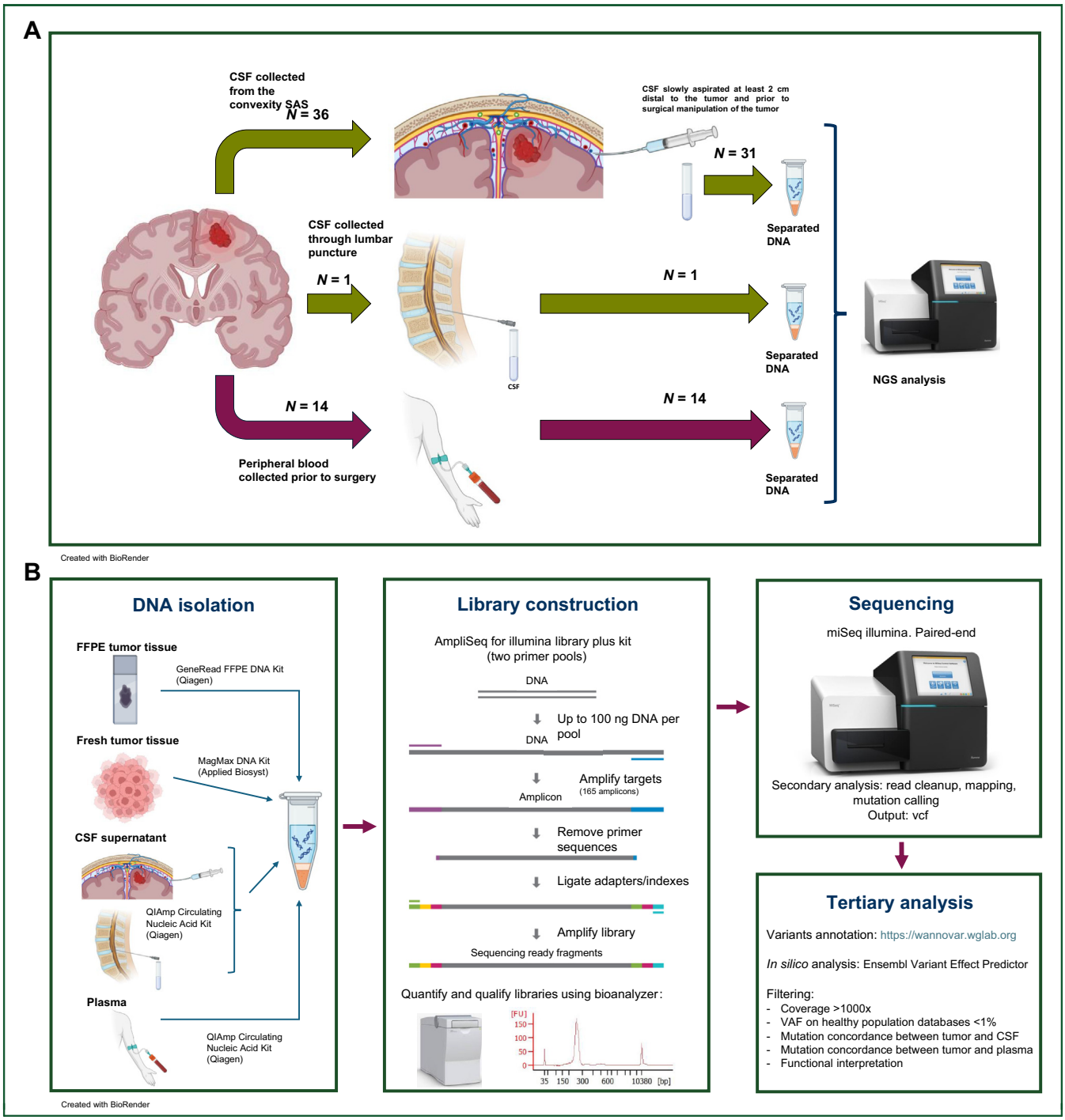

Figure 1. Sample collection (A) and methodology for DNA isolation and next-generation sequencing (NGS) analysis (B). Cerebrospinal fluid (CSF) was collected from the subarachnoid space (SAS) prior to the surgical manipulation of the tumor (baseline) in 36 patients. In one patient CSF was collected through a lumbar puncture at the time of relapse (the patient did not undergo surgery for the relapse). Among 14 of these patients, plasma was also collected at baseline (A). After DNA isolation, samples were analyzed with NGS in an miSeq Illumina device. Finally, tertiary analysis was conducted, and only clearly pathogenic gene variants using different filtering criteria were selected (B).
FFPE, formalin-fixed paraffin-embedded; VAF, variant allele frequency.

TM Kit (Zymo Research, Irvine, CA) followed by PCR DNA amplification, and run in an acrylamide-gel electrophoresis, as previously described.[13]

NGS studies in tumor tissue and CSF

Either frozen fresh tumor samples or four to eight sections of FFPE tumor tissue, with the tumor region selected by a pathologist, were obtained, and DNA extraction was performed using the MagMAX™ Total Nucleic Acid Isolation kit (Applied Biosystems, ThermoFisher Sci, Waltham, MA) and the GeneRead DNA FFPE Kit (QIAGEN, Germantown, MD), respectively. For DNA extraction from CSF the QIAmp Circulating Nucleic Acid Kit (QIAGEN GmbH, Hilden, Germany) was used. The QUBIT 3.0 fluorometer instrument

(Thermo Fisher Scientific, Waltham, MA) was used for DNA quantification (Figure 1).

High-throughput NGS by an AmpliSeq for Illumina Custom DNA Panel (Illumina, Inc., San Diego, CA) of eight genes *(IDH1, IDH2, ATRX, TP53, PTEN, PIK3CA, EGFR, BRAF)* was used in paired plasma, CSF and tumor samples—FFPE and/or fresh tumor samples, and run in an Illumina MiSeq instrument (Illumina, Inc.) (paired-end, 2 × 150). Fastq archives were generated directly by the Illumina MiSeq sequencer. Additionally, unannotated Variant Call Format (VCF) files were generated using the MiSeq built-in data processing capabilities, including alignment with the BWA (Burrows-Wheeler Aligner) software against the reference genome hg38. Subsequently, the VCF files were annotated using the ANNOVAR annotation tool.[14] Pathogenic variants were identified using public online databases such as ClinVar to select clinically significant variants. Additionally, all variants with a COSMIC identifier, which indicates a known association with cancer, were included in the analysis to ensure comprehensive coverage of potential pathogenic variants. Only pathogenic mutations—defined as pathogenic in COSMIC, CLINVAR and with in-silico predictors such as SIFT and PolyPhen-2 by Variant Effector Predictor[15]—were considered as valid and are reported here. Mutation concordance occurred when the same pathogenic gene variants (PGVs) were detected in tumor and CSF or in tumor and plasma. Variant allele frequency (VAF) was measured and expressed as the percentage of mutant alleles for each PGV as per the following formula: %VAF = (mutant copy number / mutant + wild-type copy number) × 100.[16] The median VAF (mVAF) was used as reference for evaluating ctDNA prognosis among ctDNA-positive cases. In those ctDNA-positive patients where more than one PGV was detected, the highest VAF was selected for calculating the mVAF (Figure 1).

Radiological tumor assessment

Tumor assessment was performed in all patients with magnetic resonance imaging (MRI) following the Response Assessment in Neuro-Oncology (RANO) criteria.[17] Tumor size was determined as the measured largest diameter on MRI. Distance to the closest CSF reservoir was established as the shortest distance from the tumor to any CSF reservoir (subarachnoid space, brain ventricles or basal cisterns). Tumor volume calculation and tumor three-dimensional reconstruction were performed using MRI Digital Imaging and Communications in Medicine (DICOM) images processed through the OsiriX MD version 12.0.3 (Pixmeo SARL, Geneva, Switzerland).

Sample size justification and statistical analyses

The current study was designed in 2016, when evidence regarding the feasibility of analyzing ctDNA in CSF was almost absent from the literature. We based the sample size calculation on the expected accrual of 15 *IDH*-mutant (*IDH*-mut) and 30 *IDH*-wild-type (*IDH*-wt) glioma patients in a 3-year period. Therefore, the target sample size was 45 patients including a 10% dropout rate, making a total enrollment of 49 patients.

A descriptive analysis of demographic and clinicopathological data was conducted, and quantitative variables were summarized according to their frequencies. Continuous variables were summarized using mean and standard deviation, or median and interquartile range for those variables with an asymmetric distribution.

For the comparison of qualitative variables, the chi-square test or Fisher's exact test were used if necessary. Mean comparisons between two independent groups were carried out using the Student's *t*-test if the variables followed a normal distribution, or the nonparametric Wilcoxon rank sum test for asymmetric variables. To compare continuous asymmetric variables between more than two categories, the nonparametric Kruskal—Wallis test was carried out. PFS and OS were estimated using the Kaplan—Meier method and compared with the Mantel—Cox log-rank test. All statistical calculations were conducted using R v3.6.3 under R-Studio 1.1.383 (R Development Core Team, Vienna, Austria; https://www.r-project.org).

RESULTS

Between February 2017 and March 2020, 37 glioma patients were enrolled from whom 2-5 ml CSF was collected either intra-operatively before surgical manipulation of the tumor ($n = 36$) or through a lumbar puncture ($n = 1$). Enrollment was held when 16 patients with *IDH*-mut tumors had been enrolled. The 37 patients were included in the safety study.

Table 1. Baseline characteristics of the patients at the time of CSF collection that were included in the CSF-ctDNA analysis

N	**32**[a]	
Male : Female	22 : 9	
Age, years (range)	51 (20-78)	
WHO fifth Edition (2021)	*IDH*-wt GB *IDH*-mut ASTRO *IDH*-mut OLIGO	16/32 10/32 6/32
Disease phase	New diagnosis Relapse	22/31 9/31
Type of surgery	Biopsy only STR GTR	7/31 13/31 11/31
MGMT promoter status ($n = 18$)	Methylated Unmethylated	8/18 10/18
Post-operative therapy	RT +/− TMZ Post-operative chemotherapy (TMZ or PCV)	24/31 25/31
Other tumor measures ($n = 23$)	Tumor size, cm Tumor volume, ml Distance to closest CSF reservoir (ventricle or basal cistern), mm	5.03 (2.23-11.25) 31.8 (4.13-166.56) 0.5 (0-28.6)

ASTRO, astrocytoma; CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA; GB, glioblastoma; *IDH*-mut, *IDH*-mutant; *IDH*-wt, *IDH*-wild-type; *MGMT*, O-methylguanine methyltransferase; OLIGO, oligodendroglioma; PCV, procarbazine, lomustine, vincristine; RT, radiotherapy; TMZ, temozolomide.

[a]Most demographic data were missing for 1 of the 32 patients, diagnosed with an *IDH*-mutant astrocytoma.

Of these, five patients were excluded for different reasons from the molecular analysis (Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2025.02.005). Among the remaining 32 patients, the male : female ratio was 22 : 9 (demographic and survival data were missing for one patient). Median age was 51 years (range 20-78 years). CSF was collected at new diagnosis in 23 patients and at the time of tumor recurrence in 9 patients. According to the WHO 2021 (fifth Edition) Classification of CNS Tumors, there were 16 *IDH*-wt glioblastomas, 10 *IDH*-mut astrocytomas and 6 *IDH*-mut oligodendrogliomas. For the survival analyses, data from five patients were missing. The remaining 27 patients were followed until May 2024. These and other baseline characteristics of the patients are summarized in Table 1. More detailed information for each of the 32 patients whose CSF was analyzed for ctDNA detection can be found in Supplementary Tables S1 and S2, available at https://doi.org/10.1016/j.annonc.2025.02.005.

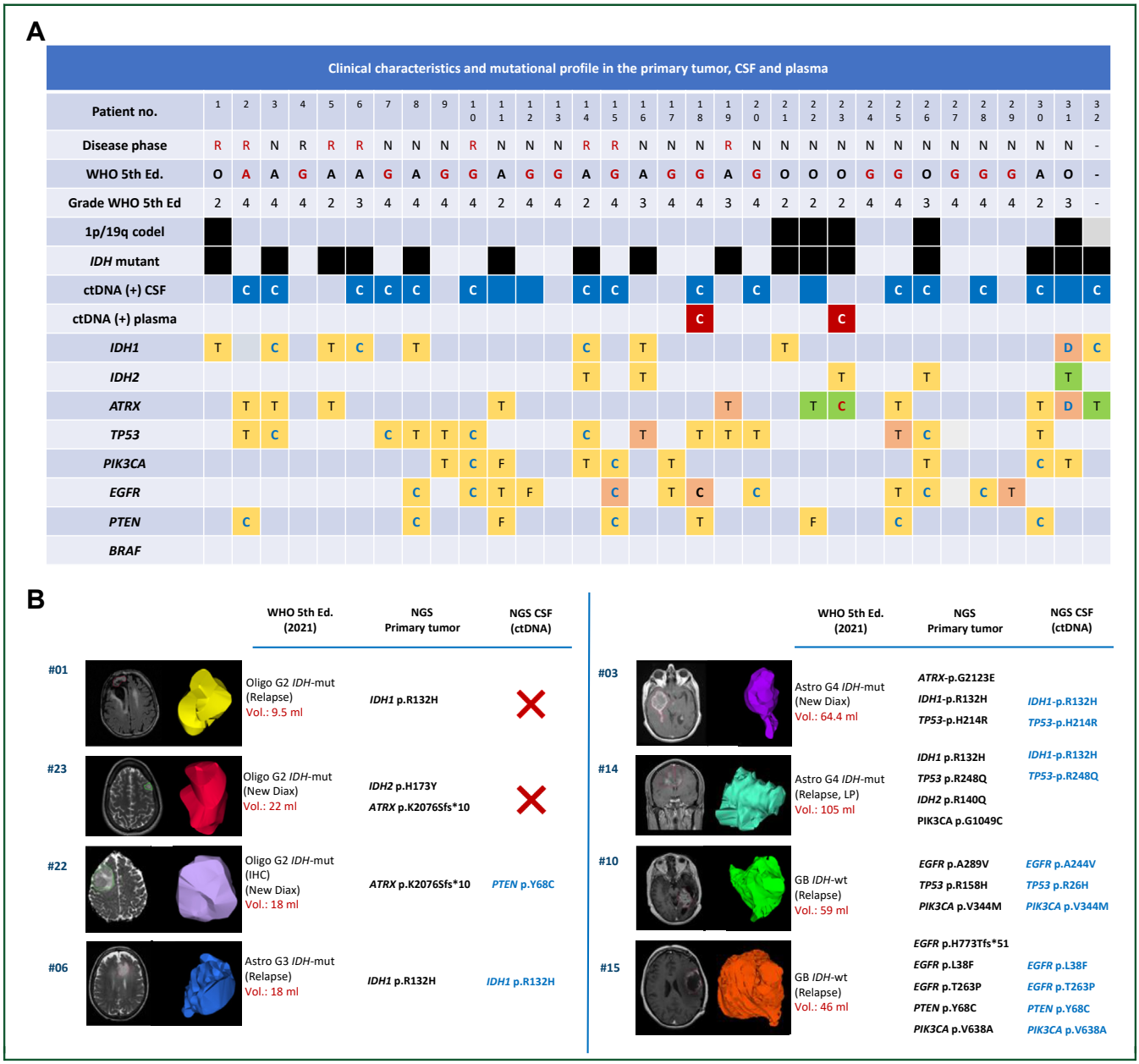

Figure 2. Pathogenic gene variant (PGV) detection in cerebrospinal fluid (CSF) and plasma and concordance status with the primary tumor (A) and examples showing the radiological tumor image and volumetric reconstruction, as well as the next-generation sequencing (NGS) result in CSF and the tumor–CSF concordance (B).
(A) Squares marked with a 'C' indicate that the same PGV was detected in the CSF (blue squares) or in plasma (red squares) and the primary tumor. Squares marked with a 'T' indicate that the PGV was only detected in the primary tumor and those marked with an 'F' indicate that the PGV was only present in the CSF. Discordant PGVs between the tumor and CSF despite occurring in the same gene are indicated with a 'D'. Yellow squares indicate that the PGV is a nonsynonymous single nucleotide variant (SNV). Green squares indicate frameshift PGVs. Purple squares indicated stop-gain PGVs. Different gene alterations in the same gene in a single patient are indicated in light red squares. (B) PGVs in the primary tumor appear in black font. PGVs in the CSF appear in blue. In patients #01 and #23 in the panel, no PGVs were detected in CSF. Note that demographic data were missing for patient 32.
A/Astro, astrocytoma; C, concordant; codel, codeletion; ctDNA, circulating tumor DNA; D, discordant PGVs between the tumor and CSF despite occurring in the same gene; F, cerebrospinal fluid; G/GB, glioblastoma; G2-4, grade 2-4; *IDH*-mut, *IDH*-mutant; *IDH*-wt, *IDH*-wild-type; N/New diax, new diagnosis; O/Oligo, oligodendroglioma; R, relapse; T, primary tumor; Vol, tumor volume; WHO 5th Ed, World Health Organization fifth Edition of the Classification of Central Nervous System Tumors.

ctDNA detection in cerebrospinal fluid and plasma

CSF was detected as ctDNA-positive for at least one PGV in 19/32 patients, reaching a 59% detection rate. Among the 19 ctDNA-positive patients, 10 patients showed one PGV in CSF, 7 patients harbored two PGV in CSF and 2 patients had three PGV in CSF. The most common genes with PGV in CSF were *EGFR* (42%), *PTEN* (37%), *TP53* (26%), *IDH1* (26%) and *PIK3CA* (21%). Among 14 patients with plasma collected at baseline, ctDNA was detected in two of them, achieving a 14% detection rate (Figure 2, Table 2, and Supplementary Tables S1 and S2, available at https://doi.org/10.1016/j.annonc.2025.02.005).

Mutation concordance between ctDNA and the primary tumor

Among the 19 patients who were positive for ctDNA in CSF, the same PGV was also present in the primary tumor in 16 cases, achieving a mutation concordance rate of 84%. For the two patients that were ctDNA-positive in plasma, one PGV concordant with the primary tumor was detected in each of them (Figure 2, Table 2 and Supplementary Tables S1 and S2, available at https://doi.org/10.1016/j.annonc.2025.02.005).

Association of ctDNA detection and median VAF in CSF and clinical characteristics

We found no association between ctDNA detection or mVAF in CSF and WHO subclass, histological type and *IDH* status, tumor size, tumor volume and distance to the closest CSF reservoir. Among 23 patients assessable for tumor size—largest diameter—and tumor volume, and in 26 patients assessable for distance to the closest CSF reservoir, no association was found with ctDNA detection or mVAF in CSF (Supplementary Tables S3 and S4, available at https://doi.org/10.1016/j.annonc.2025.02.005).

Association between ctDNA detection in CSF and survival

Among the 27 patients assessable for survival, median follow-up since initial diagnosis and since CSF collection was 31.5 months and 24 months, respectively. Median PFS and median OS from initial diagnosis in the total population were 29 months [95% confidence interval (CI) 17-81 months] and 49 months [95% CI 23 months-(not reached)], respectively. PFS and OS were significantly shorter in *IDH*-wt glioblastomas compared with *IDH*-mut tumors, both since initial diagnosis and since CSF collection (Table 2, and

Table 2. Results of ctDNA analysis in CSF and relationship of ctDNA with survival

		***N* = 32, *n* (%)**			
CSF-ctDNA-positive		19/32 (59)			
CSF-ctDNA-negative (false-negative rate)		13/32 (41)			
Tumor—CSF mutation concordance		16/19 (84)			
Number of PGV in CSF	1	10/19 (53)			
	2	7/19 (37)			
	3	2/19 (10)			
Frequency of CSF-ctDNA-mutated genes	*EGFR*	8/19 (42)			
	PTEN	7/19 (37)			
	TP53	5/19 (26)			
	IDH1	5/19 (26)			
	PIK3CA	4/19 (21)			
	ATRX	1/19 (5)			
		***n* = 27**[a]			
		PFS	*P* value	OS	*P* value
Survival since initial diagnosis, months (95% CI)		29 (17-81)	NA	49 (23-NR)	NA
	IDH-wt GB versus *IDH*-mut Astro versus *IDH*-mut Oligo, (months)	8 versus 40 versus 120	0.014	23 versus 41 versus NR	0.034
Survival since CSF collection, months		26 (8.0-NR)	NA	49 (11-NR)	NA
	IDH-wt GB versus *IDH*-mut Astro versus *IDH*-mut Oligo (months)	7.5 versus 12 versus 52	0.0066	8 versus 13 versus NR	0.043
ctDNA-positive versus ctDNA-negative (months)					
Total population, months	Total population	10 versus 29	0.29	49 versus 55	0.45
	IDH-wt	3.5 versus 18.5	NC	5 versus 8	NC
	IDH-mut	47 versus 44	NC	NR versus 55	NC
Newly diagnosed population, months	Total population	26 versus 29	NC	49 versus 55	NC
	IDH-wt	3.5 versus 18.5	NC	26 versus 8	NC
	IDH-mut	47 versus 44	NC	NR versus 55	NC
ctDNA-positive & mVAF (≥ versus < mVAF) (*N* = 17)[a]		**PFS**	***P* value**	**OS**	***P* value**
Total population, months		6 versus 42	0.037	6 versus 49	0.018
Newly diagnosed population, months	Total population	7.5 versus 42	0.21	8.5 versus 49	0.098
	IDH-wt	1.5 versus 15	NC	1.5 versus 49	NC
	IDH-mut	9 versus 47	NC	11 versus NR	NC

Astro, astrocytoma; CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA; GB, glioblastoma; *IDH*, isocitrate dehydrogenase; mut, mutant; mVAF, median variant allele frequency; NA, not applicable; NC, not calculated due to small sample size; NR, not reached; Oligo, oligodendroglioma; OS, overall survival; PFS, progression-free survival; PGV, pathogenic gene variants; wt, wild type.

[a]Survival data were missing for 5/32 patients, and therefore only available for 27 patients. Among the 19 ctDNA-positive patients, survival data were only available for 17 patients.

Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2025.02.005). There were no differences in PFS ($P = 0.29$) or OS ($P = 0.45$) according to ctDNA detection status in the total population or in newly diagnosed patients, although there was a numerically worse survival for ctDNA-positive patients in the total and newly diagnosed subpopulations (due to the small sample size statistics were not calculated according to *IDH* status; Table 2 and Figure 3).

Among ctDNA-positive patients, mVAF was 1.64% (range 0.19%-35.29%). PFS and OS were significantly shorter with ctDNA greater or equal to mVAF in the total population ($P = 0.037$ and $P = 0.018$, respectively) and in newly diagnosed patients (due to the small sample size statistics were not calculated according to *IDH* status; Table 2 and Figure 4).

There were no differences in PFS (44 versus 42 versus 10 months, $P = 0.55$) and OS (NR versus NR versus 16 months; $P = 0.758$) between patients in whom 0 ($n = 13$), 1 ($n = 10$) or ≥ 2 ($n = 9$) PGVs were detected in CSF.

Safety related to the ctDNA collection procedure

There were no adverse events related to the CSF collection procedure in any of the 37 patients enrolled in the study.

DISCUSSION

Among 31 patients in this study, the ctDNA detection rate in CSF was 59% and the PGV concordance rate between tumor and CSF reached 84%. These results are in line with those from the few other studies analyzing ctDNA in CSF that have been reported to date. They differ in the collection procedure (intra-operatively versus lumbar puncture versus CSF reservoir implantation) as well as in the type of sequencing technology used (NGS versus digital PCR). ctDNA detection rates vary between 38% and 92% depending on the study design, genomic sequencing technology and number of genes analyzed[8-10,18-26] (Table 3). These data provide detection rates far higher than the detection rates of ctDNA in plasma in gliomas, which range between 0% and 15% in most studies.[1,2] Indeed, we found

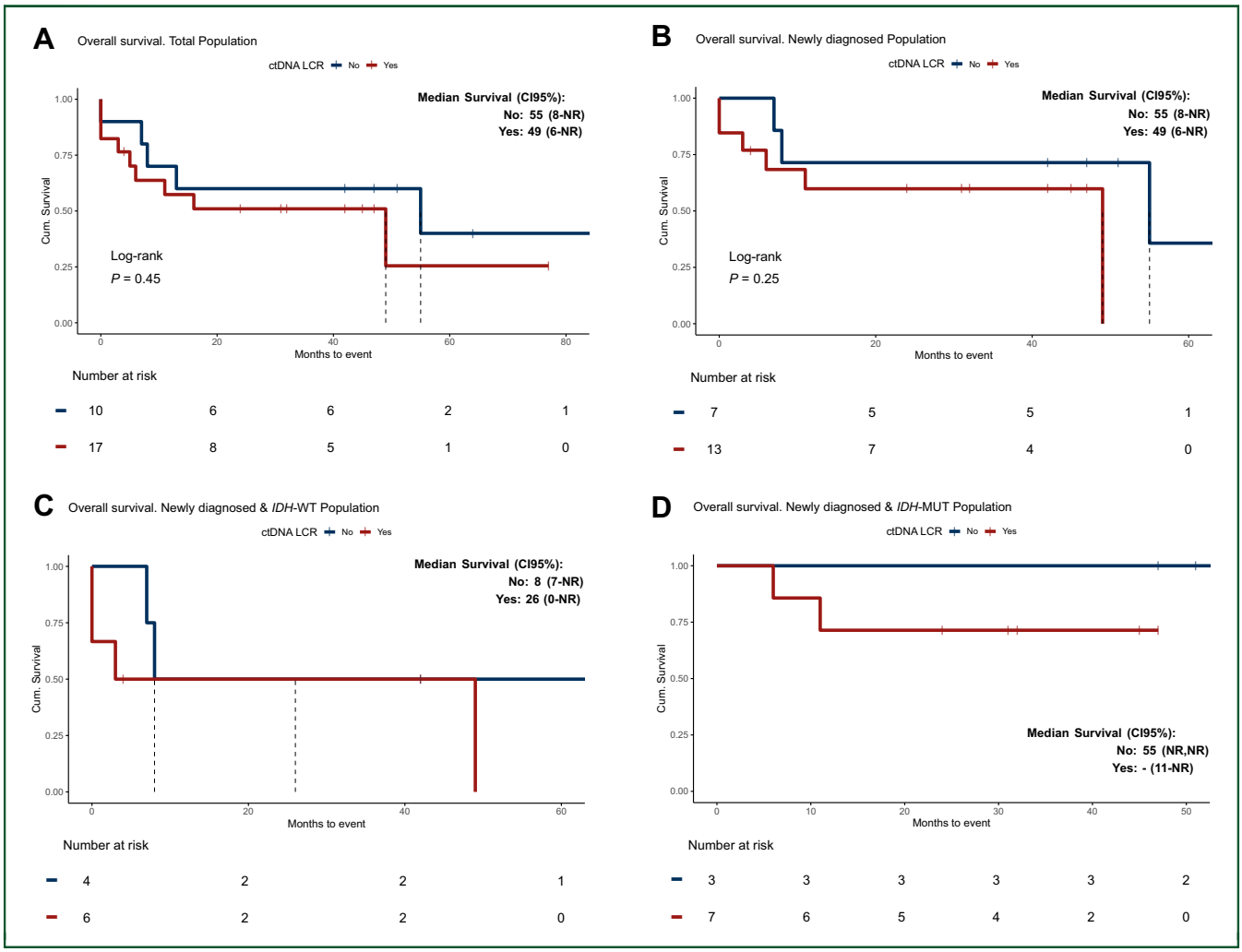

Figure 3. Kaplan–Meier curves for overall survival (OS) (A-D) and progression-free survival (PFS) (E-H) since cerebrospinal fluid (CSF) collection as per the circulating tumor DNA (ctDNA) detection status. (Due to the small sample size, statistics were not calculated for the newly diagnosed *IDH*-wt and *IDH*-mut populations).
IDH, isocitrate dehydrogenase; *IDH*-MUT, *IDH*-mutant; *IDH*-WT, *IDH*-wild-type; ND, newly diagnosed; NR, not reached.

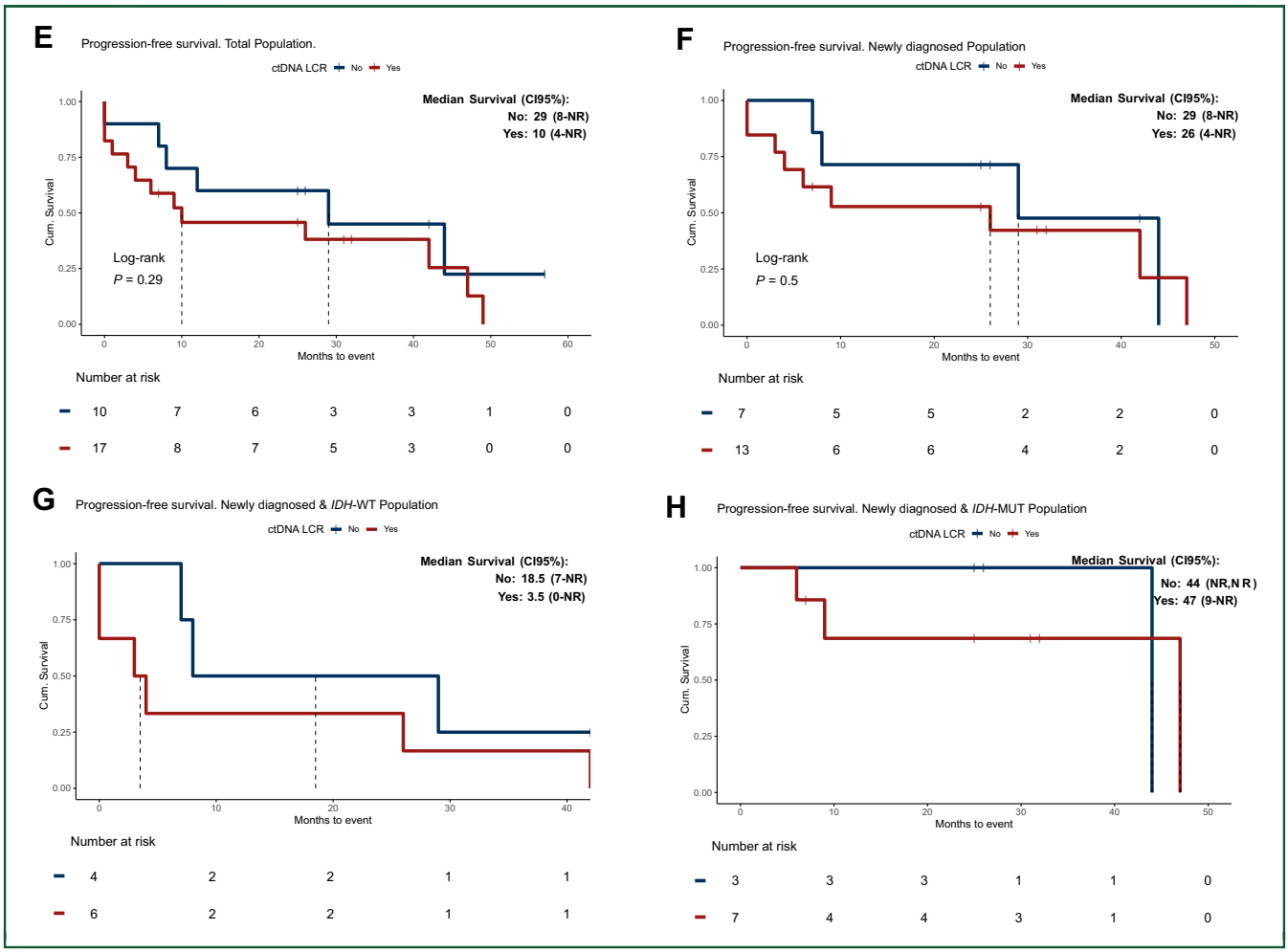

Figure 3. Continued.

only a 14% detection rate of ctDNA in plasma in our study. Juratly et al.,[8] in a study in which CSF was collected intraoperatively, analyzed mutations in the TERT promoter (TERTp) in 38 patients with TERTp-mut tumors, reporting a 92.1% detection rate and a 100% concordance rate. Martínez-Ricarte et al.,[9] in a study of 20 patients where CSF was collected through lumbar puncture, reported a 59% ctDNA detection. Miller et al.,[10] using a 410-gene panel, analyzed CSF samples from 85 patients, collected during surgery from an intraventricular catheter, or through lumbar puncture, and reported a ctDNA detection rate of 49.4%. Fujioka et al.,[22] among 34 patients with glioma, detected ctDNA pathogenic mutations in CSF in 28, and achieved a precise molecular diagnosis from CSF in 71%. Pentsova et al.,[20] reported a 50% ctDNA detection rate among 12 patients with glioma. Panditharatna et al.,[27] among 23 patients with pediatric diffuse midline glioma, detected the H3K27M mutation in CSF in 75%, 67% and 90% of cases collected at diagnosis, during therapy and at postmortem, respectively.

In our study we found no association between ctDNA detection in CSF and tumor grade or subtype according to the WHO fifth edition of the Classification of CNS Tumors. However, as we show in Figure 1, it seems that the more biologically aggressive the tumor, the greater likelihood of detecting mutations in CSF. In this regard, Martínez-Ricarte et al.[9] found a higher detection rate in high-grade tumors, and Fujioka et al.[22] also reported a statistically significant association between higher grade tumors and an accurate molecular diagnosis in CSF.

Although we did not find any association between ctDNA detection in CSF and tumor volume, tumor size or distance to the closest CSF reservoir, Orzan et al.[25] found recently that the amount of ctDNA is lower when collected through lumbar puncture compared with collection from reservoirs proximal to the tumor.

Although most liquid biopsy studies in CSF have centered on the mutational analysis of ctDNA, other promising ctDNA-based modalities have been tested in CSF. Mouliere et al.[21] demonstrated a higher detection of ctDNA using DNA size selection, also known as DNA fragmentation, in multiple cancer types including glioma. Zuccato et al.[23] recently published the results of a study evaluating the CSF methylome from 57 patients with brain metastases, glioblastoma and CNS lymphoma, reliably distinguishing between these three entities. In addition, CSF methylome closely mirrored the methylome in tumor tissue in external controls with the same entities.

In our study, OS was numerically longer in ctDNA-negative patients both in the total population and in the newly diagnosed subpopulation. Although the difference

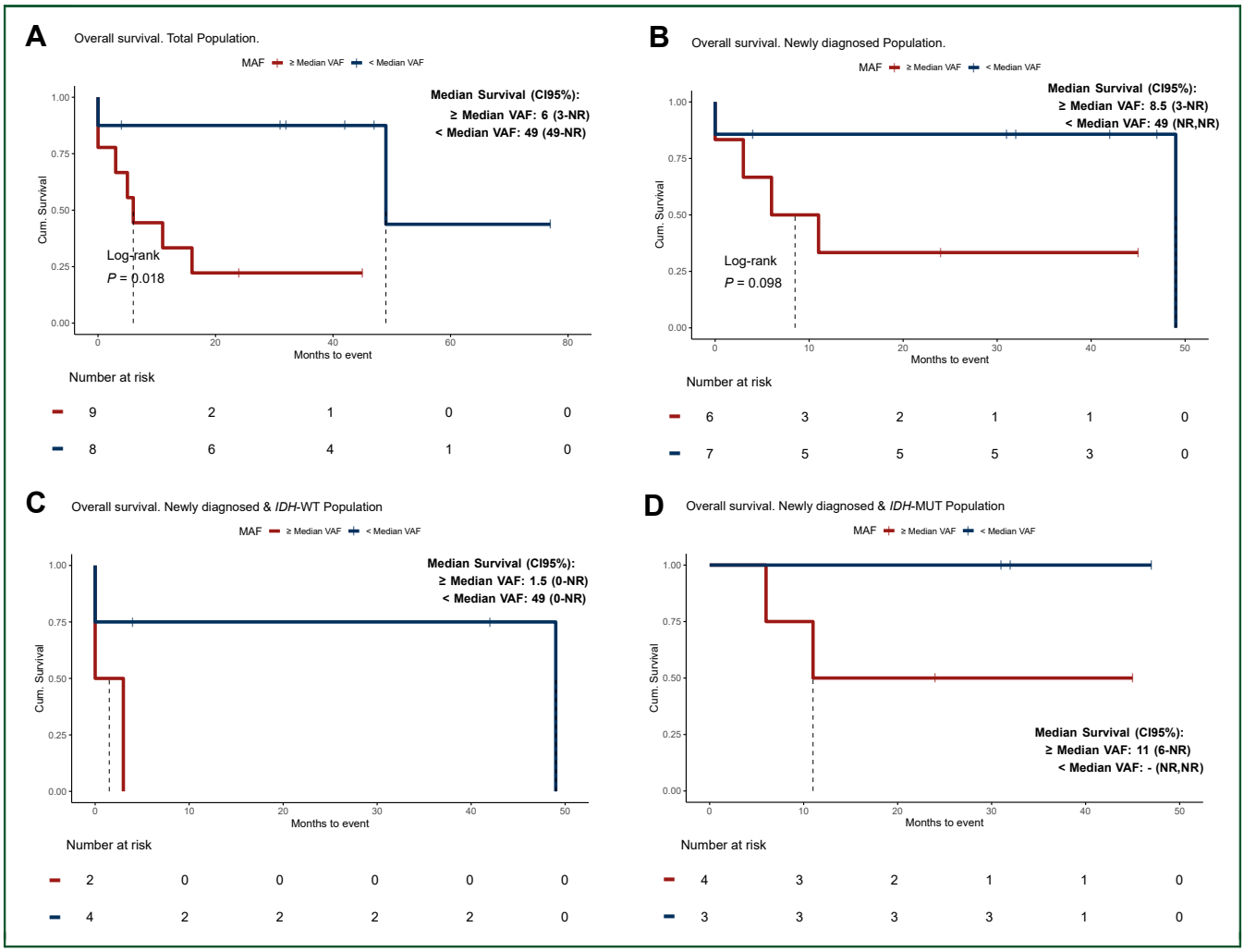

Figure 4. Kaplan–Meier curves for overall survival (OS) (A-D) and progression-free survival (PFS) (E-H) since cerebrospinal fluid (CSF) collection depending on the variant allele frequency (VAF) cut-off point in the circulating tumor DNA (ctDNA)-positive total population. The VAF cut-off point was the median VAF value for this analysis. (Due to the small sample size, statistics were not calculated for the newly diagnosed *IDH*-wt and *IDH*-mut populations.)
IDH, isocitrate dehydrogenase; *IDH*-MUT, *IDH*-mutant; *IDH*-WT, *IDH*-wild-type; ND, newly diagnosed; NR, not reached.

did not reach statistical significance, possibly due to the small sample size, these findings are in line with those from prior studies. Miller et al.[10] demonstrated a significantly longer OS in ctDNA-negative patients compared with ctDNA-positive cases both in the total population and in patients with *IDH*-wt glioblastoma.

On the other hand, using the mVAF of ctDNA-positive cases, we found that PFS and OS were significantly shorter when ctDNA was greater or equal to mVAF in the total population, and a similar trend was found in the newly diagnosed total, *IDH*-wt and *IDH*-mut subpopulations. Interestingly, Juratly et al.[8] reported that among ctDNA-positive patients, those with higher VAFs had a shorter OS, and Panditharatna et al.[27] found a higher VAF in CSF in pediatric gliomas adjacent to CSF reservoirs. Therefore, ctDNA VAF could be used as a reliable prognostic marker in CSF in patients with glioma.

We obtained all but one (collected through lumbar puncture) of the CSF samples intra-operatively before the surgical manipulation of the tumor. None of the patients suffered from adverse events attributable to the CSF collection procedure in our study. While most prior studies collected CSF either through lumbar puncture or from an implanted ventricular reservoir, Fujioka et al.[22] collected CSF during craniotomy in a series of 34 patients and from lumbar puncture in 11 of these. Although they did not conduct a safety analysis regarding the procedure, intra-operative CSF collection from the convexity space seems to be the safest procedure for obtaining CSF when surgery is indicated for diagnostic or therapeutic purposes.

Unfortunately, in our study we did not perform serial CSF collection to evaluate the ctDNA dynamics in our population. Miller et al.[10] serially collected CSF samples demonstrating the genomic evolution of the tumors in CSF. The more time elapsed between initial surgery and CSF collection, the less similar was the CSF mutational profile compared with that of the primary tumor. Panditharatna et al.,[27] in 10 patients treated with either radiotherapy or targeted therapy, found an overt reduction in CSF VAF during therapy and an increase in ctDNA VAF at progression.

Although not the focus of this study, we demonstrated that ctDNA is detectable in plasma in patients with glioma,

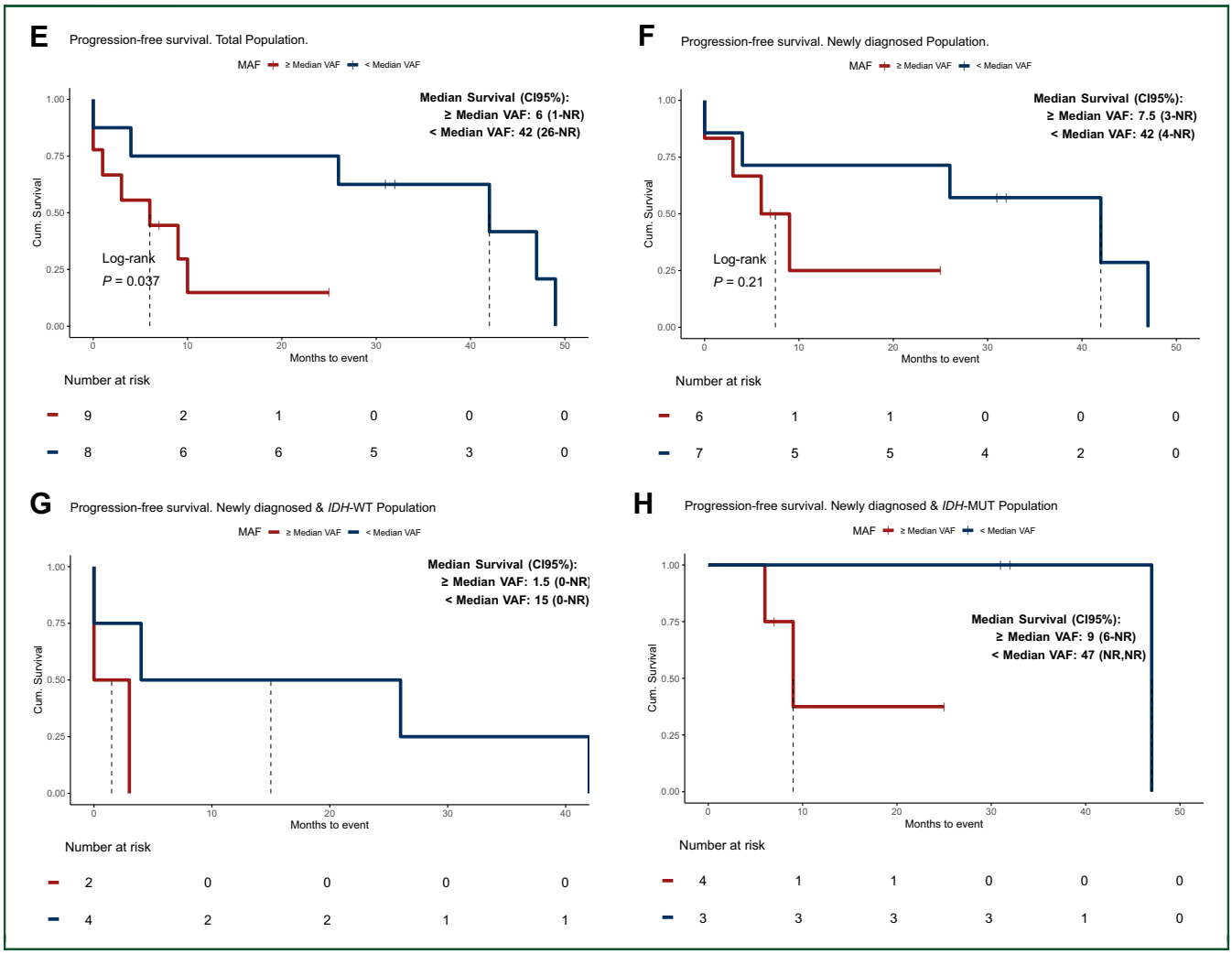

Figure 4. Continued.

although with a low detection rate. This agrees with prior reports showing detection rates of ctDNA in plasma <20% in patients with glioblastoma.[1] However, more recent studies have reported considerably higher detection rates in plasma using larger and highly sensitive multigene panels and/or digital PCR technologies such as Beaming.[28-31]

Our study has several limitations. Besides the low number of participants, it was a heterogenous sample that included both newly diagnosed and recurrent tumors, as well as both *IDH*-wt and *IDH*-mut tumors, which are well known to have a different clinical behavior that may have influenced different ctDNA shedding rates, thereby making it difficult to estimate the actual ctDNA detection rate in each of these specific scenarios. There were also several commonly mutated genes in gliomas (i.e. *TERT* promoter, *CDKN2A/B*, *CIC*, *FUBP1*, *NOTCH1*, *NF1*, *PIK3R1*, etc.) that were not included in our multigene NGS panel, and therefore the detection and concordance rates may have been artificially lowered.[32,33] Only established pathogenic or likely pathogenic missense mutations were analyzed, and therefore some other potential PGVs may have been overlooked, lowering the ctDNA detection rate. Finally, as already mentioned, we did not perform serial CSF collections, and therefore we could not study the ctDNA dynamics during treatment and tumor follow-up in our population.[10]

Larger, prospective studies aiming to validate the results from the currently available evidence should be conducted to allow for the generalizability of CSF liquid biopsy in clinical practice. In addition, other potential applications of liquid biopsy in CSF remain to be studied, such as the evaluation of minimal residual disease after local or systemic therapies, its role in the differential diagnosis between post-treatment changes and tumor progression and its accuracy in the diagnosis of patients not eligible for tumor biopsy.[4] Finally, the use of low-intensity pulsed ultrasound (LIPU) for the temporary opening of the BBB is being investigated to favor greater access for CNS agents active against brain tumors and other CNS diseases.[34] This same technology could be explored in parallel with therapeutic assays, to evaluate if the temporary opening of the BBB could increase ctDNA shedding both into the CSF or into the peripheral blood.

Table 3. Summary of studies of ctDNA in CSF in patients with adult glioma

Author	*N*	CSF collection method	Sequencing method	CSF detection rate, *n/N* (%)	Tumor–CSF concordance, *n/N* (%)	Other findings
De Mattos-Arruda (2015)[18]	4	LP	ddPCR	3/4 (75)	3/4 (75)	—
Wang (2015)[17]	22	Surgery	NGS WES	15/22 (68)	15/22 (68)	ctDNA+ if close to CSF reservoir
Pentsova (2016)[19]	12	LP	NGS (341 genes)	6/12 (50)	3/6 (50)	—
Martínez-Ricarte (2018)[9]	20	LP and intracranial CSF reservoirs	ddPCR (*TERTp*, *IDH*, *ATRX*, TP53, *H3F3A K27M*)	10/17 (59)	10/17 (59)	Higher ctDNA VAF in high-grade gliomas
Mouliere (2018)[20]	13	LP presurgery	sWGS (sCNA and DNAfp)	5/13 (38.5)	—	DNA fragmentation useful as a screening tool. DNA fragmentation different in CSF and plasma
Juratli (2018)[8]	38	Surgery	ddPCR (*TERTp*)	35/38 (92.1)	35/35 (100)	Worse prognosis with higher ctDNA VAF
Miller (2019)[10]	85	LP, IVC, surgery	NGS (410 genes)	42/85 (49.4)	—	Worse prognosis in ctDNA+ Serial CSF shows genomic evolution
Fujioka (2021)[21]	34	Surgery (34) LP (11)	dPCR (*IDH1-R132H*, *TERTp-C228T*, *TERTp-C250T*, *H3F3A-K27M*)	Surgery: 20/28 (71) LP: 56/11 (54.5)	—	Higher probability between high-grade tumors and CSF-ctDNA detection
Zuccato (2023)[22]	57 (BM, GB, PCNSL, controls)	IVC (Ommaya)	cfDNA methylome (cfMeDIP-seq)	–	—	CSF methylome accurately distinguished between entities CSF methylome finding externally validated in tissue samples
Fujita (2023)[23]	6 *IDH*-mut gliomas	LP, IVC (Ommaya), surgery	ddPCR (*IDH1-R132H*)	3/6 (50)	3/6 (50)	—
Orzan (2023)[24]	32 GB, 19 other gliomas	Surgery	NGS	GB: 75% Other glioma: 52.6% GB + other: 66.7%	—	Worse survival in patients with informative cfDNA in CSF
Iser (2024)[25]	45 ndGB: CSF proximity of tumor 39 ndGB: CSF through LP	Surgery, LP	NGS, ddPCR	Proximity: 90% LP: 60.5%	Proximity: 69.4% LP: 60.5%	ctDNA more abundant in proximity of tumor compared with LP Longer PFS in patients with noninformative ctDNA compared with informative ctDNA
Cabezas-Camarero (2025)	32	Surgery (31), LP (1)	NGS (*IDH1, IDH2, ATRX, TP53, PTEN, PIK3CA, EGFR, BRAF*)	19/32 (59)	16/19 (84)	Worse prognosis among ctDNA+ patients with ctDNA ≥ median VAF NS worse prognosis in ctDNA+ Low detection rate of ctDNA in plasma (2/14, 14%)

BM, brain metastases; cfDNA, cell-free DNA; CNVs, copy number variants; CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA; ddPCR, droplet-digital PCR; dPCR, digital PCR; GB, glioblastoma; IVC, intraventricular catheter; LP, lumbar puncture; ndGB, newly-diagnosed glioblastoma; NGS, next generation sequencing; NS, non-significant; PCNSL, primary CNS lymphoma; SNVs, single-nucleotide variants; VAF, variant allele frequency; WES, whole-exome sequencing.

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CONCLUSION

CSF is a reliable reservoir for ctDNA analyses in patients with glioma and its intraoperative collection is safe. Among 32 patients analyzed, the detection rate and concordance with the primary tumor were 59% and 84%, respectively. A poorer prognosis was found in ctDNA-positive patients with a ctDNA VAF equal to or above the median. Larger, prospective studies should be conducted to refine the potential role of liquid biopsy in this disease.

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