Neurosurgical Review (2025) 48:592
https://doi.org/10.1007/s10143-025-03747-z

RESEARCH

Determination of longitudinal Circulating levels of miR-21-5p, miR-23b-3p and miR-34a-5p in plasma of patients with glioblastoma using droplet digital PCR

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Received: 26 November 2024 / Revised: 4 June 2025 / Accepted: 19 July 2025

Abstract

Glioblastoma (GBM) is characterized by molecular heterogeneity and rapid progression. Liquid biopsy is increasingly recognised as a promising non-invasive biomarker for GBM diagnosis, prognosis and treatment monitoring. The aim of the study was to explore circulating microRNAs (miRNA) as potential GBM-biomarkers. We developed droplet digital PCR (ddPCR) assays to quantify plasmatic concentrations of miR-21-5p, miR-23b-3p and miR-34a-5p both in healthy volunteers and GBM patients at diagnosis and over follow-up (1, 3, 6 and 12 months after surgery). Furthermore, we investigated the correlations between peripheral miRNA levels and clinical, neuroradiological, pathological features, extent of tumour resection, overall (OS) and recurrence-free (RFS) survivals. Our findings showed that these miRNAs were detectable in all samples, even if with different profiles, showing a similar longitudinal course characterized by a gradual increase 1 and 3 months after surgery, followed by a progressive decrease 6 and 12 months after surgery. miR-34a-5p levels were significantly higher in GBM patients compared to healthy volunteers (AUC = 0.664, $p = 0.039$; cut-off: 1.25 copies/µL). RFS (7.6 vs. 15.6 months, $p = 0.049$) and OS (8.2 vs. 24.5 months, $p = 0.032$) were significantly shorter in patients with miR-34a-5p levels below the mean at diagnosis and 3 months after surgery, respectively. Similarly, OS (13.3 vs. 24.4months, $p = 0.024$) was significantly shorter in patients with miR-21-5p levels below the mean 6 months after surgery. This study highlights the potential clinical utility of ddPCR-based quantification of plasmatic miRNAs in GBM. Longitudinal analysis revealed consistent dynamic expression patterns for all three investigated miRNAs, with miR-34a-5p and miR-21-5p emerging as potential prognostic biomarkers. Although the diagnostic performance of miR-34a-5p was intermediate and the small cohort size limited definitive conclusions, these preliminary findings support further exploration of these miRNAs as part of a multi-marker panel to enhance diagnostic and prognostic accuracy in GBM. Larger, prospective studies are required to validate these results and to elucidate the biological underpinnings of peripheral miRNA dynamics in the context of GBM pathophysiology and treatment.

Keywords Glioblastoma · Liquid biopsy · Non-coding micro-RNA · MiR-21-5p · MiR-23b-3p · MiR-34a-5p · ddPCR

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Published online: 07 August 2025

Introduction

In the neuro-oncology field, liquid biopsy has recently surged in popularity as a promising tool for diagnosis, prognosis and treatment monitoring [3, 20, 26, 35, 45]. Glioblastoma (GBM), the most common primary malignant brain tumour in adults, is characterised by rapid progression and poor prognosis [25, 43]. GBM is diagnosed based on the fifth edition of the World Health Organization (WHO) Classification of Tumours of the Central Nervous System (CNS) [25, 26] and exhibits significant molecular and genetic heterogeneity [46]. The identification of biomarkers for GBM remains challenging due to several limitations, including the low abundance of circulating analytes, which may compromise the sensitivity and specificity of detection methods [2, 18, 20, 26, 36, 47]. MicroRNAs (miRNAs), a class of small non-coding RNAs frequently dysregulated in cancer, have emerged as reliable tumour-biomarkers, owing to their enhanced peripheral stability compared with other circulating nucleic acids [6, 11, 16, 17, 34].

The purpose of this study was to achieve absolute quantification of plasmatic miRNA concentrations and assess their potential as circulating tumour- biomarkers using the droplet digital PCR (ddPCR). According to the literature, we selected three candidate miRNAs implicated in GBM pathogenesis: miR-21-5p, miR-23b-3p and miR-34a-5p [1, 2, 7, 13, 18, 19, 22, 36, 39, 41]. The primary aims were to evaluate the plasmatic concentrations of these miRNAs in both healthy volunteers and patients, and to characterise their longitudinal expression profile during follow-up. Furthermore, we examined correlations between miR-21-5p, miR-23b-3p and miR-34a-5p levels and clinical, neuroradiological, pathological features, as well as extent of tumour resection and adjuvant treatments. Finally, we investigated their prognostic value in terms of overall (OS) and recurrence free survival (RFS).

Materials and methods

Study population

Twenty-three patients operated at the Neurosurgical Department of the ASST Cremona between April and December 2021 were prospectively enrolled in the study. Inclusion criteria were: age over 18 years, estimated prognosis of more than 6 months, Karnofsky performance status (KPS) of 70 or higher and pathological diagnosis consistent with either GBM IDH-wildtype WHO grade 4 or astrocytoma IDH-mutant WHO grade 4. Furthermore, 32 healthy volunteers, older than 18 years and with no past medical history of cancer, were included in the study.

The study adhered to the STROBE Guidelines [42]. All the procedures were conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from patients and healthy volunteers. The local ethics committee approved the study (NP 32219, dated on 2 October 2019).

Clinical, neuroradiological, histological features and treatments

Clinical and neuroradiological data were recorded in an anonymized database. Clinical features included: age at diagnosis, gender, KPS, modified Rankin scale (mRS) and medical therapies. Patients were classified as "independent" (KPS $\geq$ 90) or "dependent" (KPS $\leq$ 80) in daily life activities and "presenting" (mRS $>$ 2) or "non-presenting" (mRS $\leq$ 2) significant disabilities [40].

The neuroradiological tumour characteristics and extent of tumour resection were assessed by two independent investigators on preoperative and early postoperative magnetic resonance imaging (MRI). Near total (NTR) and subtotal resection (STR) were defined if the tumour removal was greater or lesser than 95%, respectively [21]. Preoperative and postoperative residual tumour volumes were computed as detailed in Supplementary materials (Figs. 1S and 2S) [21, 31, 32].

All patients underwent microsurgical fluorescence-guided tumour resection or frameless neuronavigated robotic-assisted biopsy, in keeping with the patient's and tumour's characteristics.

One month after surgery, patients with newly diagnosed tumours received conformal radiotherapy (RT) and concomitant chemotherapy (CT) with temozolomide (TMZ) [33, 37, 38]. Patients presenting with tumour recurrence during adjuvant TMZ and those with recurrent GBM were started on second-line CT with regorafenib [24]. The histological diagnosis followed the fifth edition of the WHO Classification of Tumours of the CNS [25, 43], with *IDH1* and *IDH2* mutations [14] and O6-methylguanine methyltransferase (*MGMT*) promoter methylation status [4] determined as described in Supplementary Materials.

Plasma collection

Peripheral blood samples were collected in EDTA-coated collection tubes (BD Vacutainer K2E 10.8 mg) at diagnosis (before surgery) and during follow-up at regular intervals (1, 3, 6 and 12 months after surgery), specifically in 23 patients at diagnosis, 16 patients 1 month after surgery (before starting the concomitant RT and CT in case of newly-diagnosed GBM and the adjuvant second-line CT in case of recurrent

GBM), 8 patients 3 months after surgery (before starting adjuvant CT with TMZ in case of newly-diagnosed GBM and after the first 3 cycles of regorafenib in case of recurrent GBM), 9 patients 6 months after surgery and 5 patients 12 months after surgery (during adjuvant CT) (Fig. 1). Plasma samples were obtained within 30 min from the withdrawal by a first centrifugation of peripheral blood at 3,000 rpm for 10 min at 4 °C followed by a second centrifugation at 4,000 rpm for 20 min at 4 °C. The aliquots were then stored at – 80 °C until RNA isolation.

RNA isolation from plasma

Total RNA was isolated from 200 µL of plasma using the miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Plasma samples were lysed in 1 mL of QIAzol Lysis Reagent (Qiagen; Hilden, GE) and 2.5 µL of 5 nM synthetic cel-miR-39-3p (Integrated DNA Technologies; Coralville, IA, USA) from *C. elegans* were added to each sample as a spike-in control. RNA was eluted from the spin columns in 35 µL of nuclease-free water. The total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.; Waltham, MA, USA) and RNA quality was assessed using the 260/280 ratio [29].

Reverse Transcription (RT) and droplet digital PCR (ddPCR)

For each miRNA, 2.5 µL of purified total RNA were reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.; Waltham, MA, USA) and specific stem-loop primer in a 7.5 µL reaction. The RT reaction was performed at 16 °C for 30 min, followed by incubation at 42 °C for 30 min and 85 °C for 5 min. The ddPCR experiments were performed according to the QX200 TaqMan ddPCR protocol, as previously described [27, 28]. For each miRNA, 1.33 µL of RT product, 11 µL of 2X ddPCR Supermix for probes (Bio-Rad Laboratories, Inc.; Hercules, CA, USA) and 11 µL of specific 20X TaqMan PCR probe assay (miR-21-5p: assay ID 000397; miR-23b-3p: assay ID 000400; miR-34a-5p: assay ID 000426) (Thermo Fisher Scientific, Inc.) were mixed to obtain a final volume of 22 µL. 20 µL of the ddPCR assay mixture and 70 µL of droplet generation oil for probes (Bio-Rad Laboratories, Inc.) were loaded into a disposable droplet generator cartridge (Bio-Rad Laboratories, Inc.). Once the droplet generation process has been completed by the QX200 droplet generator (Bio-Rad Laboratories, Inc.), 45 µL of the mix containing the droplets were transferred to a 96-well PCR plate (Bio-Rad Laboratories, Inc.) using a multichannel pipette. The plate was heat-sealed with foil and placed in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.) set as follows: 95 °C for 10 min, 40 cycles at 94 °C for 30 s, 58 °C for 1 min, 98 °C for 10 min, and 4 °C for 40 min. A no

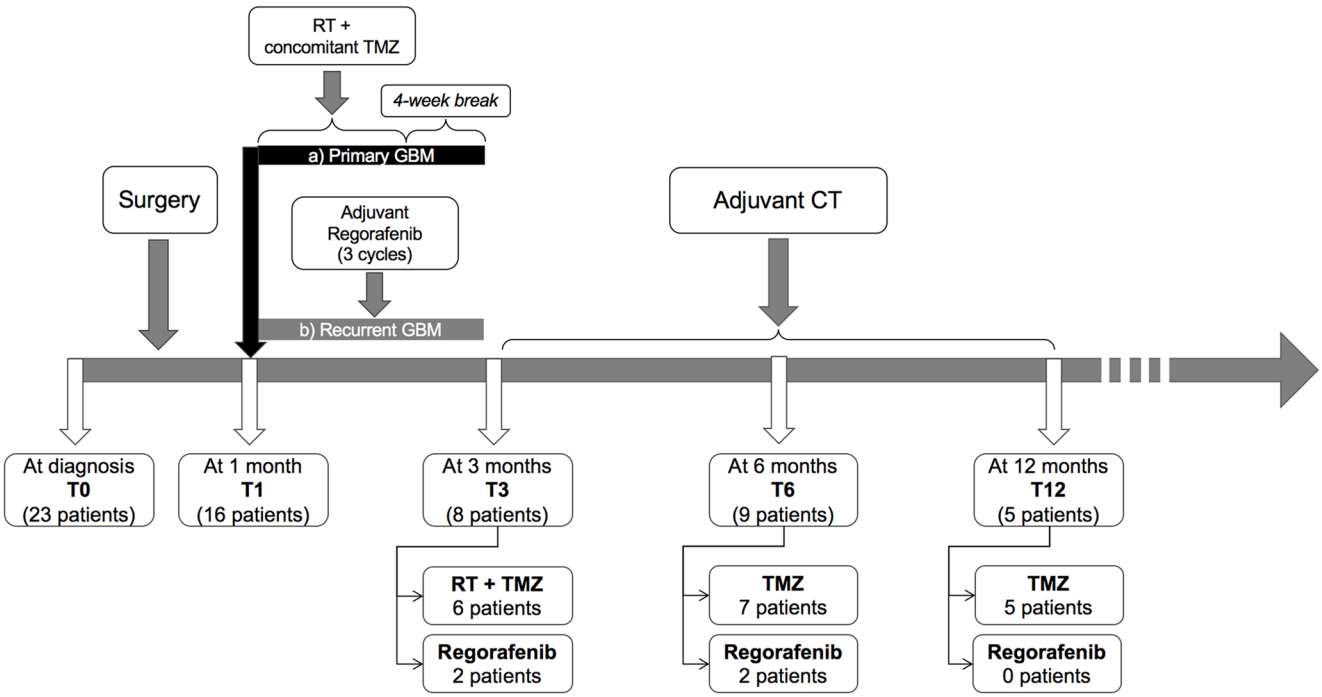

Fig. 1 Timing line of blood samples collection reporting the standard adjuvant therapy scheme, based on radiotherapy (RT) and chemotherapy with temozolomide (TMZ)

template control and a negative control for each RT reaction were included in every assay. The cel-miR-39 assay was performed to monitor the efficiency of the RT reaction. Data were considered only for samples that generated more than 12,000 droplets. Clear thresholds were drawn between positive and negative droplet clusters to calculate the concentration, expressed as copies/µL, using QuantaSoft Software, version 1.7 (Bio-Rad Laboratories, Inc.).

Statistical analysis

Categorical variables were presented as numbers (percentages) and compared with either the Pearson's chi-square test with Yates' correction or Fisher's exact test, as appropriate. Continuous variables were expressed as means (± standard deviation, SD) or medians (interquartile range, IQR) and compared with the unpaired Student's *t* test or Mann-Whitney U test as appropriate, after confirmation of normality of distribution by histograms' visual inspection and the Shapiro-Wilk test.

Correlations between plasmatic miRNA levels and clinical or tumour variables were assessed using the Pearson's or rho Spearman's correlation coefficients (*r*) in case of linear and non-linear associations, respectively. Adjusted R^2 estimates were also reported as results of linear regression.

Receiver operating characteristics (ROC) curves were generated to evaluate sensitivity, specificity and predictive power of each miRNA for GBM diagnosis. The area under curve (AUC) values ranged from 0 to 1 and the cut-off values of miRNA plasmatic concentrations were identified according to the Youden index (YI) [8, 10].

Multivariate linear regression analysis was used to identify which variables independently predicted plasmatic miRNA levels at different timing points. Results of multivariate modelling were expressed as odds ratios (OR), 95% confidence interval (95% CI) after adjustment for covariates and *p* for significance.

OS and RFS analyses were performed according to the Kaplan-Meier method from the date of surgery to the date of death, tumour recurrence/progression or end of observation. Censoring was applied to the last follow-up date for patients who remained alive. The log-rank test was used to compare survivals [8].

Cox regression analysis was adopted to determine which variables independently predicted OS and RFS. Results of multivariate modelling were expressed as hazard ratios (HR), 95% CI after adjustment for covariates and *p* for significance.

A two-tailed probability (*p*) value ≤ 0.05 was considered statistically significant.

Statistical analysis was performed using the package SPSS for Windows, Version 23.0 (SPSS Inc.; Chicago, IL, USA).

Results

Patient clinical characteristics and tumour features

The mean age at diagnosis was 57.9 years (± 9.6) with a male prevalence (60.9%). At diagnosis, 18 patients (78.3%) were "independent" in daily life activities (KPS ≥ 90) and 21 (91.3%) did not present significant neurological disabilities (mRS ≤ 2).

Lesions were left-sided in 11 cases (47.8%), right-sided in 9 cases (39.1%) and sited along the midline in 3 cases (13.1%). The mean tumour volume was 33.8 cc (± 19.1).

Craniotomy was performed in 21 patients (91.3%) and NTR was achieved in 15 of them (71.4%). The mean percentage of tumour resection was 99.3% (± 1.0) and 80.7% (± 7.9) in case of NTR and STR resection, respectively.

Nineteen (82.6%) were newly diagnosed tumours. The pathological diagnosis was consistent with GBM IDH-wild-type WHO grade 4 in 22 cases (95.7%) and astrocytoma IDH-mutant WHO grade 4 in the remaining one (4.3%). The MGMT promoter resulted un-methylated in 12 cases (52.2%), methylated in 4 (17.4%) and hyper-methylated in the remaining 7 (30.4%), with no statistical differences in distribution ($p = 0.119$).

Of the 19 patients with newly diagnosed tumours, 17 (89.5%) received RT with concomitant TMZ. The RT protocol was standard in 15 (88.2%) and hypo-fractionated in the remaining 2 (11.8%). Overall, 12 patients (52.2%) were deemed suitable for adjuvant CT: 8 were started on TMZ and 4 with recurrent GBM were treated with regorafenib (Fig. 2).

Plasmatic levels of miR-21-5p, miR-23b-3p and miR-34a-5p

We developed ddPCR assays for circulating miR-21-5p, miR-23b-3p and miR-34a-5p in order to assess their absolute quantification in plasma of GBM patients. These miRNAs were all detectable in healthy volunteers (Fig. 3, panels A, C and E) with mean plasmatic concentrations of 114.28 copies/µL (± 125.41), 4.43 copies/µL (± 5.40) and 0.68 copies/µL (± 0.62), respectively. Patients presented on average 115.49 copies/µL (± 132.57) of miR-21-5p, 7.18 copies/µL (± 10.15) of miR-23b-3p and 1.28 copies/µL (± 1.10) of miR-34a-5p at diagnosis (Fig. 3, panels A, C and E). miR-34a-5p showed significantly higher mean circulating levels in patients at diagnosis than healthy volunteers (1.28 vs. 0.68

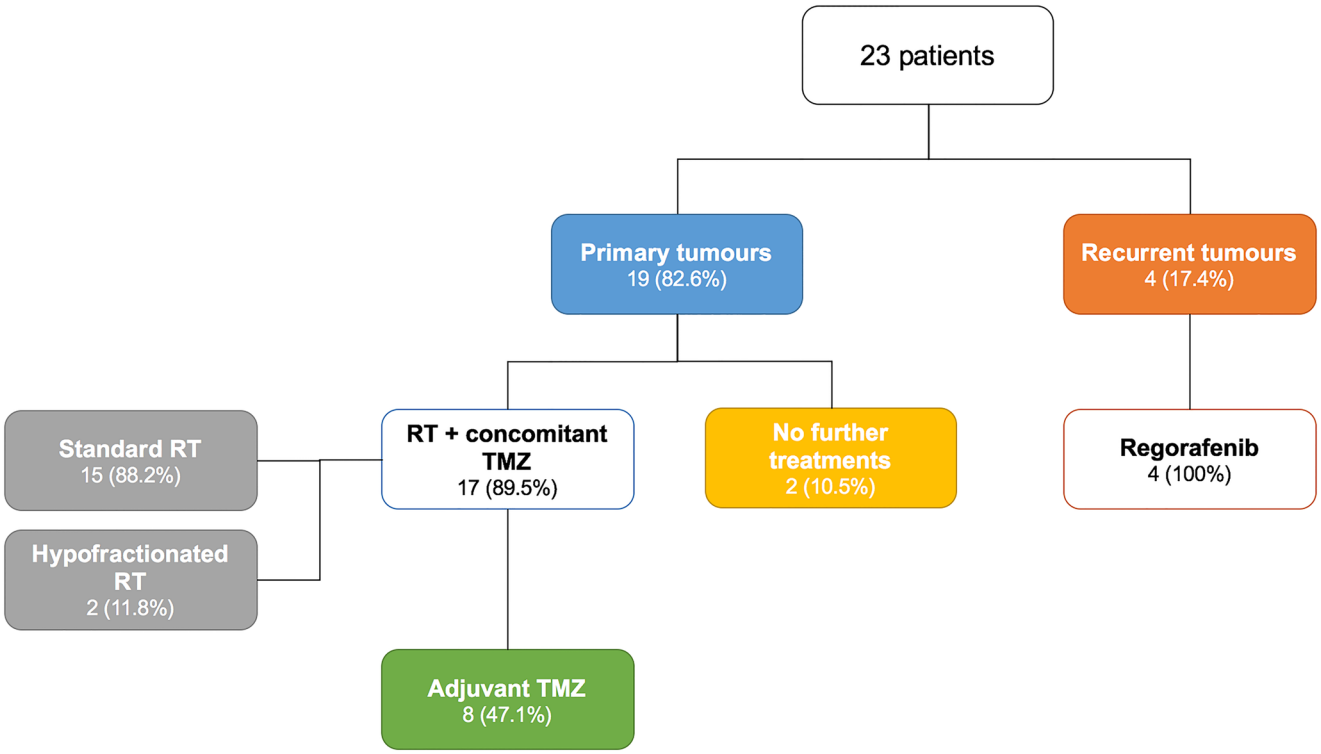

Fig. 2 Flow chart showing patients' distribution according to adjuvant therapies (RT: radiotherapy; TMZ: temozolomide)

copies/µL; $p=0.012$) (Fig. 3, panel E). As shown in Fig. 3 (panels B, D and F), the ROC analysis documented an AUC of 0.484 for miR-21-5p (95% C.I. 0.326–0.643, $p=0.844$), 0.537 for miR-23b-3p (95% C.I. 0.375-0.700, $p=0.639$) and 0.664 for miR-34a-5p (95% C.I. 0.515–0.814, $p=0.039$) with a cut-off value for the latter of 1.25 copies/µL (sensitivity: 47.8%; specificity: 87.5%; YI: 0.353).

The ddPCR assays enabled the quantification of circulating levels of each miRNA throughout the follow-up. As displayed in Fig. 4 (panels A, B and C), the mean plasmatic concentrations of each miRNA exhibited a consistent longitudinal trend: a gradual increase 1 and 3 months after surgery, followed by a progressive decrease 6 and 12 months after surgery.

The mean plasmatic concentrations of miR-21-5p (356.16±303.89 vs. 114.28±125.41 copies/µL; $p=0.001$) and miR-23b-3p (22.26±19.04 vs. 4.43±5.40 copies/µL; $p<0.001$) reached significantly higher levels in patients 3 months after surgery compared to healthy volunteers. However, the mean circulating levels of miR-34a-5p remained consistently higher in patients at all time points compared to healthy individuals (Fig. 4, panel C).

All the investigated miRNAs displayed significantly higher plasmatic concentrations 3 months after surgery compared to the time of diagnosis (miR-21-5p: 356.15±303.89 vs. 115.49±132.57 copies/µL, $p=0.004$; miR-23b-3p: 22.26±19.04 vs. 7.18±10.15 copies/µL, $p=0.008$; miR-34a-5p: 4.79±5.17 vs. 1.28±1.10 copies/µL, $p=0.004$) (Fig. 5, panels A, B and C). Furthermore, miR-21-5p and miR-23b-3p showed significantly lower circulating levels 12 months after surgery (miR-21-5p: 27.42±17.27 copies/µL; miR-23b-3p: 0.91±0.42 copies/µL) compared to 3 months (miR-21-5p: 356.15±303.89 copies/µL, $p=0.037$; miR-23b-3p: 22.26±19.04 copies/µL, $p=0.031$) and 6 months after surgery (miR-21-5p: 183.74±141.87 copies/µL, $p=0.033$; miR-23b-3p: 7.77±6.16 copies/µL, $p=0.031$).

No significant differences were observed between plasmatic miRNA concentrations and clinical, neuroradiological or histological features apart from preoperative corticosteroid therapy and ATRX status. Patients who had not received preoperative dexamethasone (2, 8.7%) exhibited significantly higher mean circulating levels of miR-23b-3p (6.14±10.01 vs. 18.10±1.56 copies/µL, $p<0.001$) and miR-34a-5p (1.16±1.07 vs. 2.56±0.35 copies/µL, $p=0.021$) at diagnosis (Supplementary Results – Table 1). Furthermore, the plasmatic concentrations of all miRNAs were significantly higher at the time of diagnosis in tumours showing retained ATRX expression (miR-21-5p: 136.05±137.44 vs. 8.15±5.87 copies/µL, $p=0.001$; miR-23b-3p: 8.51±10.73 vs. 0.36±0.40 copies/µL, $p=0.004$; miR-34a-5p: 1.47±1.11 vs. 0.37±0.03 copies/µL, $p<0.001$) (Supplementary Results – Table 2). However, the correlation and multivariate analyses did not yield any statistically

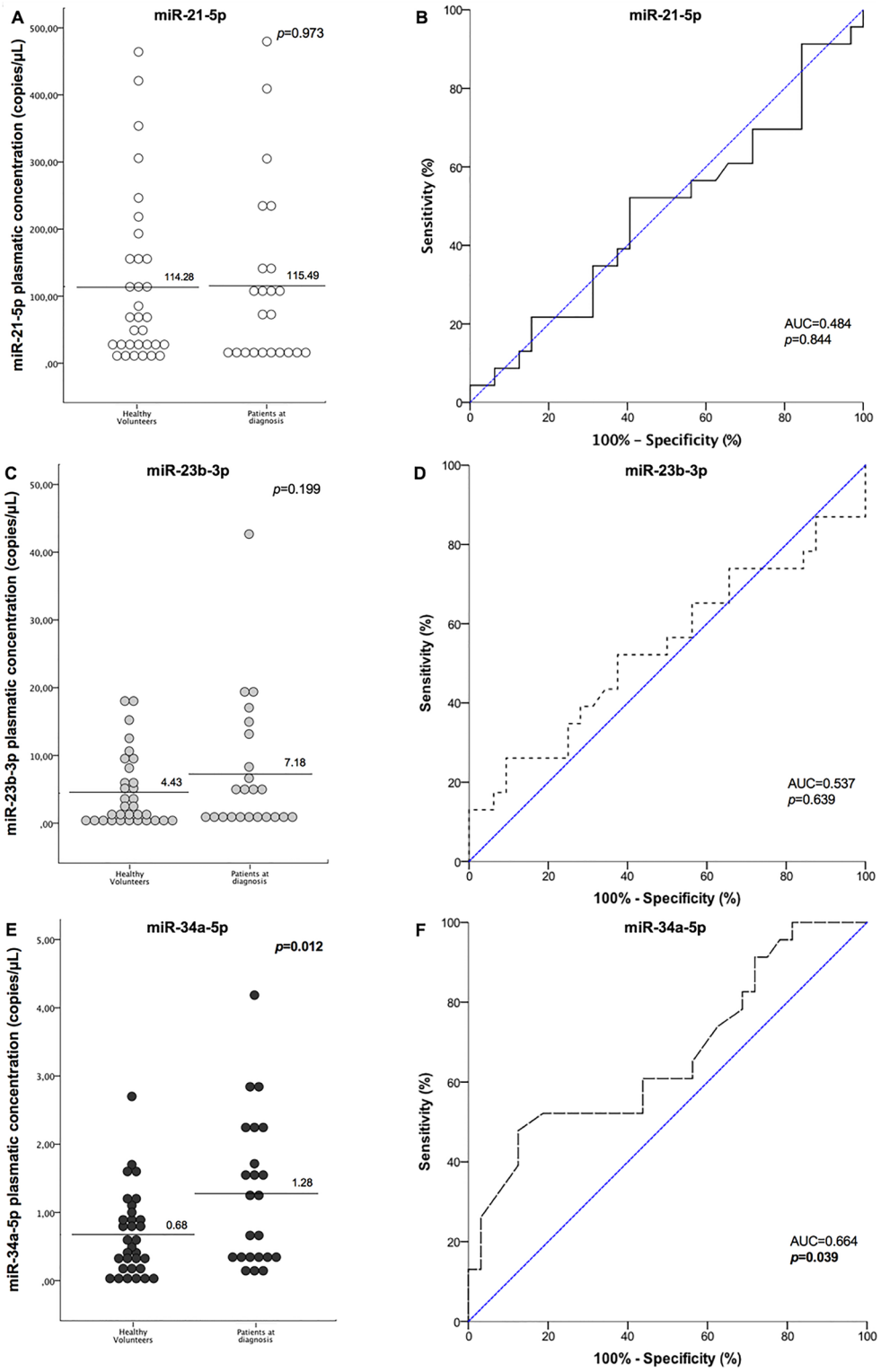

Fig. 3 ddPCR determination of plasmatic levels of miR-21-5p, miR-23b-3p and miR-34a-5p in healthy volunteers and GBM patients at diagnosis. Panels**A**, **C** and **E** display dot plots showing the plasmatic concentration of each miR (mean values represented by lines). Panels **B**, **D** and **F** display ROC curves with respective area under the curve (AUC) and *p* values

significant results (Supplementary Results – Tables 3, 4, 5 and 6).

Analysing miRNA levels over the follow-up, a significant difference was observed only in the plasmatic concentrations of miR-34a-5p one month after surgery that resulted significantly higher in patients who underwent NTR compared to STR (2.70±1.93 vs. 1.13±0.61 copies/µL, $p=0.040$). Nevertheless, correlation and multivariate analyses did not document any statistically significant

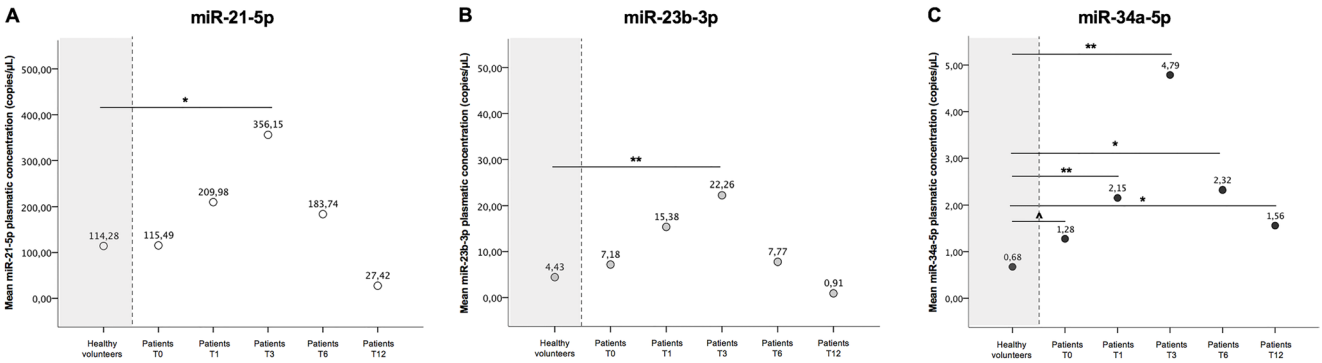

Fig. 4 Comparison of mean plasmatic concentrations of miR-21-5p (panel **A**), miR-23b-3p (panel **B**) and miR-34a-5p (panel **C**) quantified by ddPCR between healthy volunteers and patients affected by GBM at different timing points (**: $p<0.001$; *: $p=0.001$; ^: $p<0.05$)

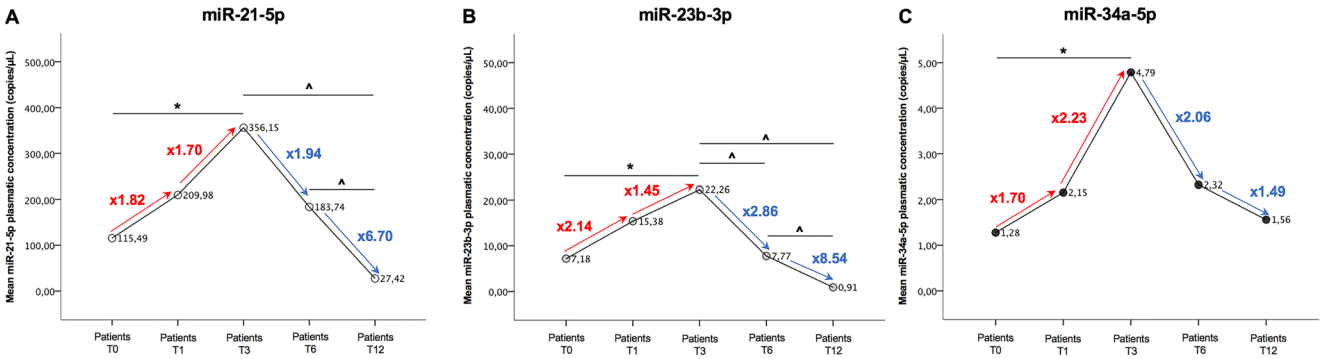

Fig. 5 Longitudinal course of mean plasmatic concentrations of miR-21-5p (panel **A**), miR-23b-3p (panel**B**) and miR-34a-5p (panel **C**) quantified by ddPCR (*: $p<0.01$; ^: $p<0.05$)

findings (Supplementary Results – Tables 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17).

Correlation analyses of miRNA plasmatic concentrations at different time points were detailed in Tables 1, 2, 3, 4 and 5.

The multivariate linear regression analyses (Table 6) showed that circulating levels of miR-23b-3p (OR 11.50, 95% C.I. 9.53–13.47, $p<0.001$) and miR-34a-5p (OR 23.80, 95% C.I. 5.57–42.02, $p=0.013$) at diagnosis were predictors of miR-21-5p circulating levels at diagnosis, such as miR-21-5p plasmatic concentrations at diagnosis independently predicted miR-23b-3p (OR 0.08, 95% C.I. 0.06–0.09, $p<0.001$) and miR-34a-5p (OR 0.01, 95% C.I. 0.00–0.02, $p=0.013$) plasmatic concentrations at diagnosis. Furthermore, miR-23b-3p plasmatic concentrations 1 month after surgery predicted miR-21-5p levels 1 month after surgery (OR 10.83, 95% C.I. 8.34–13.32, $p<0.001$) and vice versa (OR 0.08, 95% C.I. 0.06–0.10, $p<0.001$). miR-21-5p levels 6 months after surgery predicted miR-23b-3p levels 6 months after surgery (OR 0.02, 95% C.I. 0.01–0.03, $p=0.022$).

Overall survival and recurrence free survival

The mean length of follow-up was 11.9 months (±9.4; median 8.0, IQR: 3.0–17.0). The mean OS was 12.4 months (95% C.I. 8.4–16.4) with 39.1% and 21.7% of predicted OS at 1 and 2 years, respectively (Fig. 6, panel A). After splitting the population above and below the mean values of circulating miRNAs at different time points, the mean OS resulted significantly shorter for patients with miR-34a-5p levels below the mean 3 months after surgery (8.2 months, 95% C.I. 5.2–11.1 vs. 24.5 months, 95% C.I. 19.6–29.4; $p=0.032$; Fig. 6, panel B) and for those with miR-21-5p levels below the mean 6 months after surgery (13.3 months, 95% C.I. 7.4–19.2 vs. 24.4 months, 95% C.I. 20.4–28.4; $p=0.024$; Fig. 6, panel C).

The mean RFS was 11.4 months (95% C.I. 6.8–16.0) with 31.9% and 26.6% of predicted RFS at 1 and 2 years, respectively (Fig. 7, panel A). After splitting the population above and below the mean values of circulating miRNAs at different timing points, the mean RFS was significantly shorter in patients with miR-34a-5p levels below the mean at diagnosis (7.6 months, 95% C.I. 3.1–12.1 vs. 15.6 months, 95% C.I. 8.0–23.1, $p=0.049$; Fig. **7**, panel B).

Table 1 Correlation analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) at diagnosis (T0) and miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) at diagnosis (T0), 1 (T1), 3 (T3), 6 (T6) and 12 (T12) months after surgery

	miR-21-5p T0			miR-23b-3p T0			miR-34a-5p T0		
	R	R^2	*p*	R	R^2	*p*	R	R^2	*p*
T0									
miR-21-5p	-	-	-	**0.931**	**0.867**	**<0.001**	**0.420**	**0.176**	**0.046**
miR-23b-3p	**0.931**	**0.867**	**<0.001**	-	-	-	0.253	0.064	0.244
miR-34a-5p	**0.420**	**0.176**	**0.046**	0.253	0.064	0.244	-	-	-
T1									
miR-21-5p	**0.651**	**0.424**	**0.006**	**0.796**	**0.634**	**<0.001**	0.013	0.000	0.961
miR-23b-3p	**0.672**	**0.452**	**0.004**	**0.819**	**0.671**	**<0.001**	0.018	0.000	0.947
miR-34a-5p	0.160	0.026	0.554	0.182	0.033	0.501	**0.522**	**0.272**	**0.038**
T3									
miR-21-5p	−0.325	0.106	0.432	−0.141	0.020	0.738	**−0.750**	**0.563**	**0.032**
miR-23b-3p	0.227	0.052	0.589	0.386	0.149	0.345	−0.424	0.180	0.295
miR-34a-5p	−0.572	0.327	0.139	−0.480	0.230	0.228	−0.242	0.059	0.564
T6									
miR-21-5p	−0.226	0.051	0.559	−0.289	0.084	0.451	−0.130	0.017	0.738
miR-23b-3p	0.029	0.001	0.940	0.041	0.002	0.917	−0.282	0.080	0.462
miR-34a-5p	−0.227	0.052	0.556	−0.145	0.021	0.710	−0.242	0.059	0.530
T12									
miR-21-5p	0.839	0.704	0.075	0.137	0.019	0.826	0.220	0.048	0.722
miR-23b-3p	−0.007	0.000	0.991	−0.001	0.000	0.999	−0.354	0.125	0.559
miR-34a-5p	−0.479	0.229	0.415	−0.607	0.368	0.277	0.757	0.573	0.138

Table 2 Correlation analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 1 month (T1) after surgery and miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 1 (T1), 3 (T3), 6 (T6) and 12 (T12) months after surgery

	miR-21-5p T1			miR-23b-3p T1			miR-34a-5p T1		
	R	R^2	*p*	R	R^2	*p*	R	R^2	*p*
T1									
miR-21-5p	-	-	-	**0.987**	**0.974**	**<0.001**	0.310	0.096	0.242
miR-23b-3p	**0.987**	**0.974**	**<0.001**	-	-	-	0.283	0.080	0.289
miR-34a-5p	0.310	0.096	0.242	0.283	0.080	0.289	-	-	-
T3									
miR-21-5p	0.115	0.013	0.829	0.154	0.024	0.770	−0.343	0.118	0.505
miR-23b-3p	0.675	0.456	0.141	0.707	0.500	0.116	−0.214	0.046	0.683
miR-34a-5p	−0.387	0.150	0.449	−0.343	0.118	0.505	−0.717	0.514	0.109
T6									
miR-21-5p	−0.032	0.001	0.946	0.063	0.004	0.894	−0.039	0.002	0.934
miR-23b-3p	0.420	0.176	0.349	0.540	0.292	0.211	0.298	0.089	0.517
miR-34a-5p	−0.146	0.021	0.754	−0.085	0.007	0.856	−0.048	0.002	0.919
T12									
miR-21-5p	0.431	0.186	0.569	−0.063	0.004	0.937	−0.108	0.012	0.892
miR-23b-3p	−0.659	0.434	0.341	−0.940	0.884	0.060	−0.113	0.013	0.887
miR-34a-5p	−0.456	0.208	0.544	−0.443	0.196	0.557	0.895	0.801	0.105

Discussion

The identification of circulating biomarkers suitable for liquid biopsy has attracted growing interest in oncology, offering promising applications in early tumour detection, disease monitoring, treatment stratification, and targeted therapy [3, 20, 26, 35, 45]. In GBM, various circulating molecules, including non-coding RNAs, have been investigated. However, results reported in the literature remain inconsistent and, at times, contradictory, likely due to heterogeneity in patient populations, biological sample types (e.g. cerebrospinal fluid, serum, plasma) and analytical approaches, all of which challenge reproducibility and clinical applicability.

Table 3 Correlation analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 3 months (T3) after surgery and miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 3 (T3), 6 (T6) and 12 (T12) months after surgery

	miR-21-5p T3			miR-23b-3p T3			miR-34a-5p T3		
	R	R^2	*p*	R	R^2	*p*	R	R^2	*p*
T3									
miR-21-5p	-	-	-	**0.760**	**0.578**	**0.028**	0.689	0.475	0.059
miR-23b-3p	**0.760**	**0.578**	**0.028**	-	-	-	0.385	0.148	0.346
miR-34a-5p	0.689	0.475	0.059	0.385	0.148	0.346	-	-	-
T6									
miR-21-5p	0.585	0.342	0.300	0.717	0.134	0.849	**0.940**	**0.884**	**0.018**
miR-23b-3p	0.737	0.543	0.156	0.860	0.009	0.062	**0.945**	**0.893**	**0.015**
miR-34a-5p	**0.983**	**0.966**	**0.003**	0.849	0.924	0.069	0.448	0.201	0.171
T12									
miR-21-5p	-	-	-	-	-	-	-	-	-
miR-23b-3p	-	-	-	-	-	-	-	-	-
miR-34a-5p	-	-	-	-	-	-	-	-	-

Table 4 Correlation analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 6 months (T6) after surgery and miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 6 (T6) and 12 (T12) months after surgery

	miR-21-5p T6			miR-23b-3p T6			miR-34a-5p T6		
	R	R^2	*p*	R	R^2	*p*	R	R^2	*p*
T6									
miR-21-5p	-	-	-	**0.847**	**0.717**	**0.004**	0.400	0.160	0.287
miR-23b-3p	**0.847**	**0.717**	**0.004**	-	-	-	0.349	0.122	0.358
miR-34a-5p	0.400	0.160	0.287	0.349	0.122	0.358	-	-	-
T12									
miR-21-5p	**0.968**	**0.937**	**0.032**	0.830	0.689	0.170	0.259	0.067	0.741
miR-23b-3p	0.559	0.312	0.441	0.634	0.402	0.366	−0.688	0.473	0.312
miR-34a-5p	0.200	0.040	0.800	−0.001	0.000	0.999	−0.729	0.531	0.271

Table 5 Correlation analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations (copies/µL) 12 months after surgery (T12)

	miR-21-5p T12			miR-23b-3p T12			miR-34a-5p T12		
	R	R^2	*p*	R	R^2	*p*	R	R^2	*p*
T12									
miR-21-5p	-	-	-	0.447	0.200	0.451	−0.164	0.027	0.792
miR-23b-3p	0.447	0.200	0.451	-	-	-	−0.027	0.001	0.965
miR-34a-5p	−0.164	0.027	0.792	−0.027	0.001	0.965	-	-	-

In this observational study, we developed a ddPCR-based workflow for the absolute quantification of three plasmatic miRNAs – miR-21-5p, miR-23b-3p, and miR-34a-5p – in GBM patients. ddPCR offers several advantages over conventional qPCR, including increased sensitivity and reproducibility, especially for low-abundance targets, and the use of fixed input volumes, thereby reducing inter-assays variability. Although these miRNAs are known to be dysregulated in different tumours and pathological conditions, their selection was informed by previous evidence implicating miR-21-5p and miR-23b-3p as upregulated and miR-34a-5p as a tumour suppressor in GBM [1, 2, 7, 13, 18, 19, 22, 23, 36, 41, 44].

We first assessed the plasmatic levels of these miRNAs in GBM patients compared to healthy volunteers using ddPCR. Notably, miR-34a-5p levels were significantly elevated at diagnosis in GBM patients, with an AUC of 0.664. While statistically significant, this intermediate diagnostic performance suggests that miR-34a-5p alone may have limited clinical utility as a standalone biomarker. These findings underscore the need for cautious interpretation and further validation in larger cohorts. Given the complexity of GBM biology and the multifactorial regulation of circulating miRNAs, a multi-marker approach may yield improved diagnostic accuracy.

Table 6 Multivariate linear regression analysis between miR-21-5p, miR-23b-3p and miR-34a-5p plasmatic concentrations

	ANOVA		OR	95% CI	*p* value
	R	*p* value			
miR-21-5p at T0	0.950	<0.001			
miR-23b-3p at T0			11.50	9.53–13.47	<0.001
miR-34a-5p at T0			23.80	5.57–42.02	0.013
miR-23b-3p at T0	0.943	<0.001			
miR-21-5p at T0			0.08	0.06–0.09	<0.001
miR-34a-5p at T0	0.563	0.022			
miR-21-5p at T0			0.01	0.00–0.02	0.013
miR-21-5p at T1	0.988	<0.001			
miR-23b-3p at T1			10.83	8.34–13.32	<0.001
miR-23b-3p at T1	0.989	<0.001			
miR-21-5p at T1			0.08	0.06–0.10	<0.001
miR-23b-3p at T6	1.000	0.022			
miR-21-5p at T6			0.02	0.01–0.03	0.022

Despite considerable interpatient variability, longitudinal profiling revealed a common temporal trend for all three miRNAs, with circulating levels gradually increasing 1 and 3 months after surgery and then declining 6 and 12 months post-surgery. Sampling time points were aligned with major clinical milestones – before and after surgery, as well as before and after concomitant RT and CT in newly-diagnosed GBM or adjuvant second-line CT in recurrent GBM – and routine follow-up assessments, in order to capture biologically and clinically relevant plasmatic fluctuations. While these findings are preliminary and hypothesis-generating, they warrant further investigation to elucidate the mechanisms driving temporal miRNA expression dynamics and to disentangle treatment-induced effects from intrinsic tumour biology evolution.

Interestingly, higher circulating levels of miR-23b and miR-34a were found in patients not receiving dexamethasone at diagnosis. While inflammation is known to affect circulating miRNA profiles and may be involved in GBM, the regulatory impact of corticosteroids on plasmatic miRNAs expression remains challenging to define. Additionally, we observed an association between ATRX loss and reduced plasmatic levels of miR-21-5p, miR-23b-3p, and miR-34a-5p in GBM patients, suggesting a possible interaction between chromatin remodelling and miRNA regulation [5]. However, the very small number of patients not receiving corticosteroids at diagnosis (2, 8.7%) and those exhibiting ATRX loss (3, 13.0%), coupled with the lack of statistical significance in multivariate analyses, limits the robustness and generalizability of these observations.

Contrary to our results, previous studies have reported reduced serum levels of miR-34a-5p in GBM patients [41]. Such discrepancies may arise from pre-analytical differences between serum and plasma, such as susceptibility to haemolysis, platelet degranulation and variations in extracellular vesicle content, all of which can influence circulating miRNA profiles [9, 15, 29].

The association between low miR-34a-5p levels and poor prognosis (Fig. 8) aligns with its known tumour-suppressive function and preclinical evidence demonstrating its capacity to inhibit GBM cell proliferation, migration, and tumour progression [13, 19, 22, 41, 44]. Conversely, miR-21-5p, a well-established onco-miR, may also be influenced by inflammatory processes, potentially contributing to its prognostic relevance during follow-up [30]. In our cohort, miR-23b-3p did not exhibit clear diagnostic or prognostic significance. Its role in GBM appears context-dependent, functioning as either an onco-miR or tumour suppressor miR in various cancer types [12]. However, its consistent correlation with miR-21-5p across time points suggests

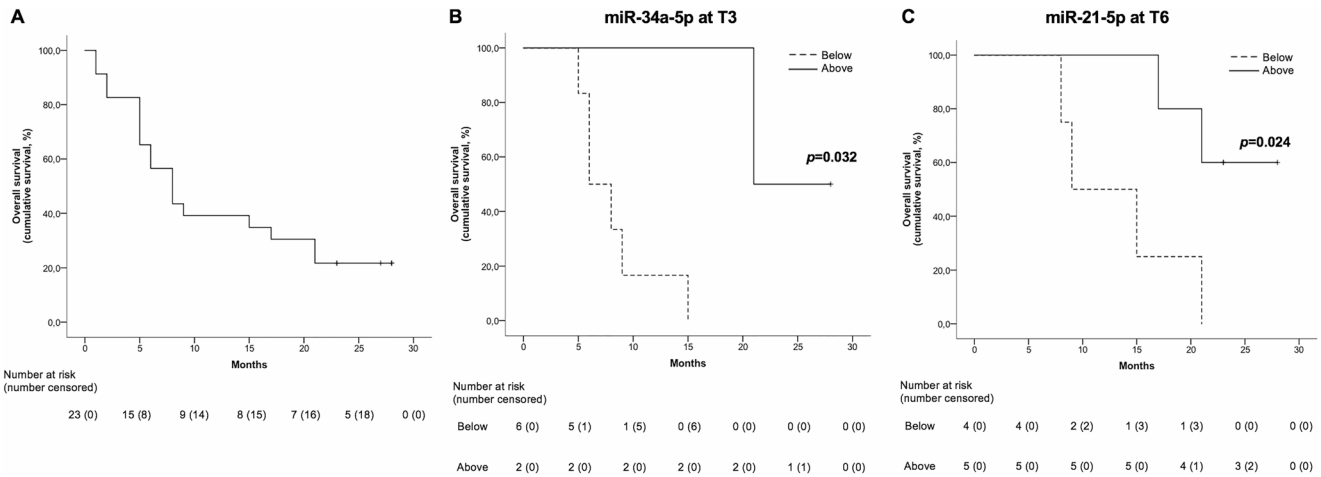

Fig. 6 Panel **A**: overall survival (OS) Kaplan-Meier curve; panel **B**: OS Kaplan-Meier curves comparing patients with circulating levels of miR-34a-5p below (dashed line) and above (solid line) the mean 3 months after surgery; panel **C**: OS Kaplan-Meier curves comparing patients with circulating levels of miR-21-5p below (dashed line) and above (solid line) the mean 6 months after surgery

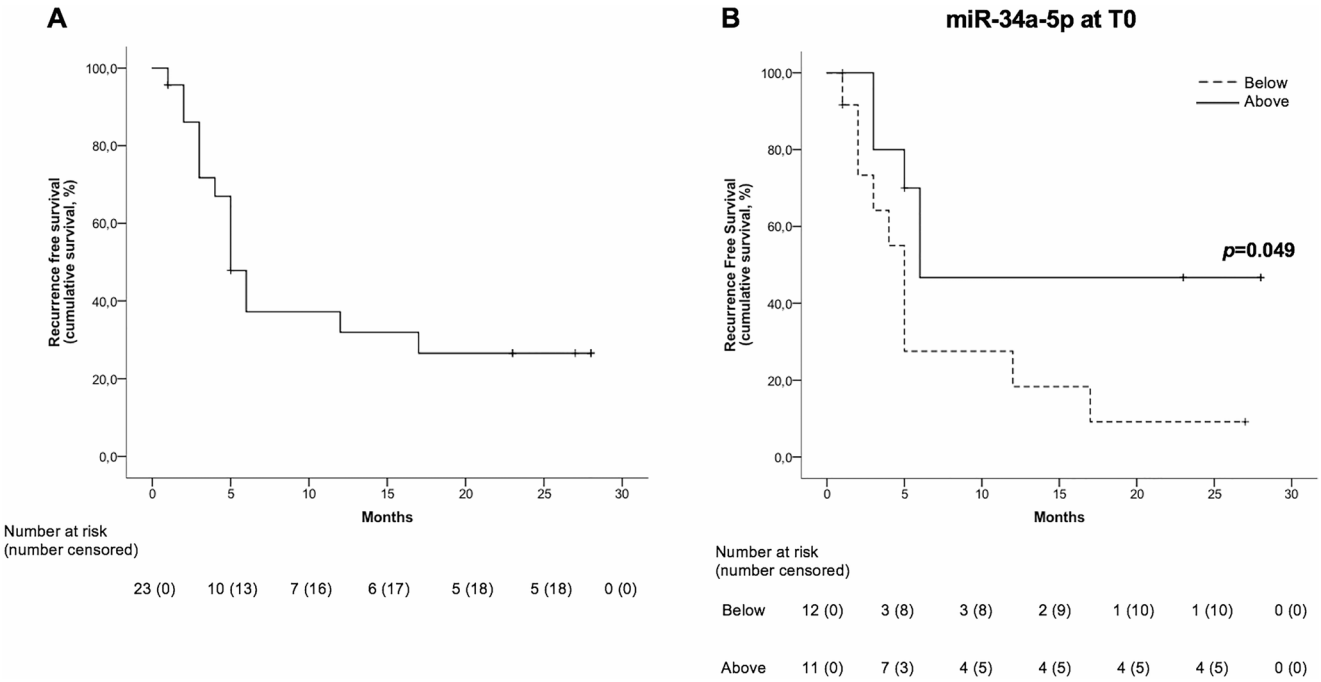

Fig. 7 Panel **A**: recurrence free survival (RFS) Kaplan-Meier curve; panel **B**: RFS Kaplan-Meier curves comparing patients with circulating levels of miR-34a-5p below (dashed line) and above (solid line) the mean at diagnosis

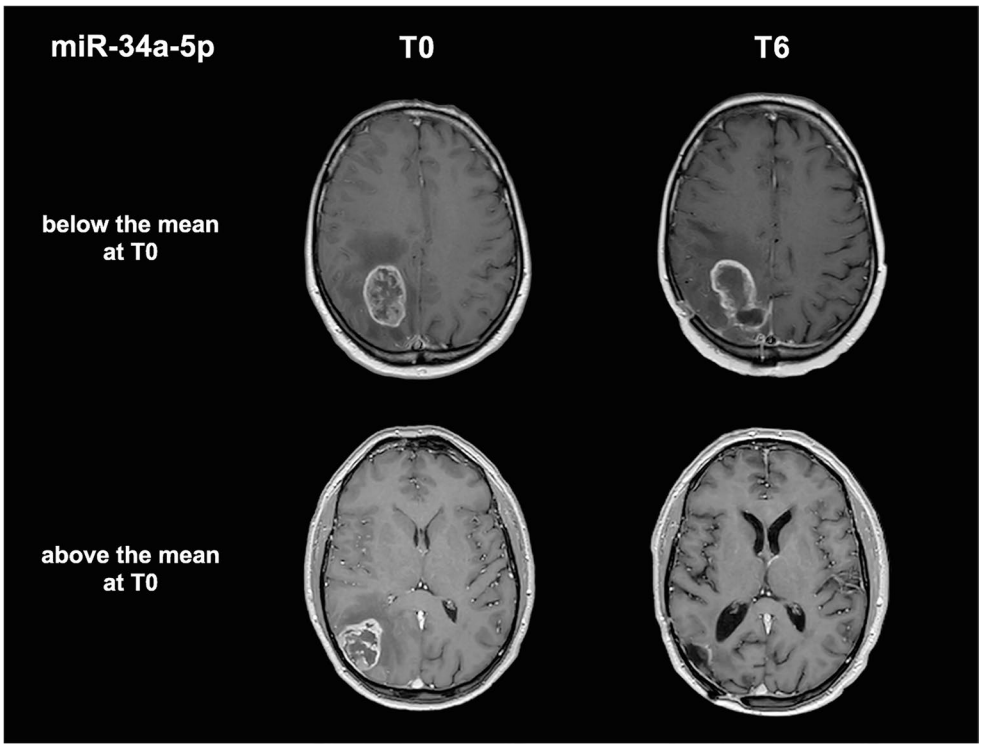

Fig. 8 Axial MRI T1-weighted contrast enhanced images of two cases presenting with miR-34a-5p plasmatic concentrations below and above the mean (T0) and showing tumour recurrence and stability (T6), respectively

possible co-regulation or involvement in shared molecular pathways, warranting further investigation [1, 7, 23].

Survival analysis demonstrated that lower miR-34a-5p levels at diagnosis and 3 months after surgery were associated with reduced RFS and OS, respectively. Similarly, reduced miR-21-5p levels 6 months after surgery correlated with decreased OS. Although a mean-based dichotomization of miRNA levels for survival analysis was adopted, we acknowledge its limitations. Advanced statistical models, such as time-dependent ROC analyses, could provide

dynamic and refined prognostic evaluations. However, the limited number of events and reduced population size at later time points constrained the feasibility of such analyses, underscoring the need for larger prospective studies.

Study limitations

The main limitation of our study is the relatively small cohort size, which may restrict the generalizability of the findings, despite the clinical homogeneity of the patient population. The reduced number of available samples at later time points hampers the interpretation of longitudinal trends. Moreover, the observational nature of the study and the fixed sampling schedule may have failed to capture the full dynamics of miRNA expression related to tumour evolution and treatment effects. Future prospective, multi-centre studies with standardized treatment regimens and sampling schedules are essential to validate and extend these findings.

Although ddPCR offers high sensitivity and specificity for absolute quantification of circulating targets without the need for normalization to endogenous controls, the biological basis underlying extracellular miRNA fluctuations remains poorly understood. Further mechanistic investigations are crucial to clarify the relationships between miRNA dynamics, tumour biology and therapeutic response.

Conclusion

This study highlights the potential clinical utility of ddPCR-based quantification of plasmatic miRNAs in GBM. Longitudinal analysis revealed consistent dynamic expression patterns for all three investigated miRNAs, with miR-34a-5p and miR-21-5p emerging as potential prognostic biomarkers. Although the diagnostic performance of miR-34a-5p was intermediate and the small cohort size limited definitive conclusions, these preliminary findings support further exploration of these miRNAs as part of a multi-marker panel to enhance diagnostic and prognostic accuracy in GBM. Larger, prospective studies are required to validate these results and to elucidate the biological underpinnings of peripheral miRNA dynamics in the context of GBM pathophysiology and treatment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10143-025-03747-z.

Acknowledgements The authors want to acknowledge the University of Brescia, Mednote s.r.l., a spin-off of the University of Trieste, and the "Associazione Provinciale Lega Italiana Lotta ai Tumori (LILT) di Cremona" which supported this research.

Author contributions Conceptualization, GDP, CAD and AS; methodology, AS, IG, CAD and IAP; formal analysis, AS and CAD; investigation, CAD, IG, IAP and AS; resources, CAD, LR, AF, MC, MRC, FS and DG.; experimental data curation, CAD, AS and GDP; clinical data curation, CAD, LR, AF, MC, MRC and DG; writing—original draft preparation, CAD, IG, AS and GDP; writing—review and editing, CAD, IG, AS, GDP, LR, AF, MC, MRC, DG and FS; supervision, CAD, AS and GDP; funding, GDP, AS, CAD, AF and DG. All authors have read and agreed to the published version of the manuscript.

Funding This research was supported by the University of Brescia (local grants), MedNote srl Milano, a spin-off of the University of Trieste, and the "Associazione Provinciale Lega Italiana Lotta ai Tumori (LILT) di Cremona".

Data availability The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Declarations

Ethics approval This study was conducted according to the guidelines of the Declaration of Helsinki and the Good Clinical Practice (GCP) guidelines. The study protocol was approved by the Ethical Committee of the ASST Cremona (NP 32219, dated on 2 October 2019).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publish The authors affirm that human research participants provided informed consent for publication of the images.

Clinical trial number Not applicable.

Competing interests The authors declare no competing interests.

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