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Pre-treatment circulating microRNA signatures predict outcomes of anti-CD19 CAR T-cell therapy

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Running Title: Predictive miRNA signature for CART-cell therapy outcome

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Contributions
MB, MF and FB are responsible for the study concept; RR, NL, BF, GD are responsible for the generation of smallRNA sequencing data; MN, FDF, EZ, IP, FI, GS are responsible for the analysis and interpretation of smallRNA sequencing data; FDF, FI, IS, GS, MU, MR, FB, FA, BS, LZ, ED, CP, MG, EM and BC collected the clinical samples, compiled the clinical information and omics sequencing data; MN, SDM, FR, FV and MT are responsible for the FACS analysis and cytokine dosage; SNB, DM, ET, RCD, gave technical support; MN, FDF wrote the original draft; MN, FDF, RR, MB, MF and FB wrote, reviewed and edited the manuscript; PLZ, MB, MF, FB supervised the study.

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Disclosure of Conflicts of Interest
PLZ: scientific advisory boards: Secura Bio BIO, Celltrion, Gilead, Janssen-Cilag, BS, Servier, Sandoz, MSD, TG Therap., Takeda, Roche, EUSA Pharma, Kiowa Kirin, Novartis, ADC Therap., Incyte, Beigene; consultancy: EUSA Pharma, MSD, Novartis; speaker's bureau: Celltrion, Gilead, Janssen-Cilag, BMS, Servier, MSD, TG Therap., Takeda, Roche, EUSA Pharma, Kiowa Kirin, Novartis, Incyte, Beigene. FB: scientific advisory boards and speaker fees: NEOVII, NOVARTIS, KITE, GILEAD, PFIZER, CELGENE, MSD.
The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement
The sequencing data presented in this study have been submitted to the Sequence Read Archive (SRA) online repository. The samples can be retrieved at https://www.ncbi.nlm.nih.gov/sra using the accession numbers SAMN39307284-SAMN39307344.

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Chimeric antigen receptor (CAR) T-cell therapy has been one of the major therapeutic advances of the last decade, substantially improving the prognosis of several relapsed/refractory hematologic malignancies. Anti-CD19 CAR T-cell products, including axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel), and lisocabtagene maraleucel (liso-cel), are approved for relapsed/refractory large B-cell lymphoma (LBCL), primary mediastinal B-cell lymphoma (PMBCL), and transformed follicular lymphoma (tFL) [1, 2]. Nevertheless, 50–60% of patients eventually relapse and only a minority achieve durable remissions [3]. In addition, CAR T-cell therapy may be complicated by severe toxicities, most notably cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [4, 5]. Reliable biomarkers able to predict both efficacy and toxicity before infusion are therefore urgently needed.

Several pre-infusion variables, including tumor burden, response to prior therapy, and baseline inflammatory status, have been associated with outcome [6, 7]. More recently, plasma CAR+ extracellular vesicles measured within 24 hours after infusion were shown to predict ICANS onset [8]. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally and are involved in cancer biology, inflammation, and immune regulation [9]. Because circulating miRNAs are highly stable in plasma and can be readily quantified, they represent attractive noninvasive biomarkers [10, 11] and may reflect both tumor- and immune-related biological processes relevant to CAR T-cell therapy outcomes.

Here, we profiled the circulating miRNome at the pre-lymphodepletion (PLD) time point in patients with relapsed/refractory B-cell non-Hodgkin lymphoma (NHL) treated with anti-CD19 CAR T cells, to investigate whether plasma miRNAs could predict toxicity and clinical outcome.

We conducted a prospective observational study in 53 patients with relapsed/refractory B-cell NHL treated at IRCCS AOU Bologna with approved anti-CD19 CAR T-cell products. The study was approved by the Ethics Committee, registered at www.clinicaltrials.gov (NCT04892433, NCT05807789), and run-in agreement with the Helsinki declaration.

Plasma miRNA profiling was performed by small-RNA sequencing, starting from 5 µl of RNA for library preparation, using QIAseq miRNA Library Kit (Qiagen). Library pools were diluted to 1.5 pM and sequenced using NextSeq 500 platform (Illumina). Differentially expressed miRNAs were identified using a log fold change >1 and Wald test $p<0.05$. Unsupervised hierarchical cluster analysis of normalized intensity values was performed using GeneSpring GX software (Agilent Technologies).

Correlation between variables was assessed using Spearman's rank coefficient, with $p<0.05$ considered statistically significant. Group comparisons were performed using Fisher's exact test for categorical variables and the Kruskal–Wallis (KW) test for continuous variables. For toxicity analyses, patients were stratified according to CRS (grade <2 vs ≥2) and ICANS (presence vs absence; grade <2 vs ≥2). To reduce dimensionality and account for class imbalance, weighted LASSO regression was applied for variable selection [12]. Variables most frequently selected were included in multivariable logistic regression models together with significant clinical covariates. Model performance was evaluated by accuracy, sensitivity, specificity, and precision, using leave-one-out cross-validation (LOOCV). For response

prediction, logistic regression models were developed for 3- and 12-month overall response rate (ORR). To address the high dimensionality of miRNA data, LASSO-based variable selection and repeated training-validation splits were used to identify the most stable predictors, which were then tested in a final model. Time-to-event outcomes (PFS, TTP, OS) were analyzed using Kaplan–Meier estimates and log-rank tests. Cut-off values for stratifying patients according to miR-542-5p levels were determined using *surv_cutpoint* R function.

The study cohort comprised 53 patients: 46 (87%) with LBCL and 7 (13%) with PMBCL (see **Supplementary Table 1** for patient characteristics). Twenty-eight patients (53%) received axi-cel and 25 (47%) tisa-cel, after a median of two previous lines of therapy. CRS of any grade occurred in 45 patients (85%), including 16 (30%) with grade ≥2 CRS. Patients with grade ≥2 CRS were more frequently female (p=0.043) and had higher PLD C-reactive protein (CRP) levels (p=0.027). ICANS developed in 18 patients (34%), including 14 with grade ≥2 events; two patients died from grade 5 ICANS. ICANS was significantly associated with female sex (p=0.022), PMBCL histotype (p=0.004), ECOG performance status ≥1 (p=0.013), and elevated CRP levels at PLD (p=0.006). Median onset was 1 day for CRS and 5 days for ICANS. Among 49 evaluable patients, the overall response rate (ORR) was 61% at 3 months and 39% at 12 months. No prophylactic corticosteroids were administered to axi-cel–treated patients.

Small-RNA sequencing identified 573 circulating miRNAs detectable in at least one sample. Unsupervised clustering of the PLD miRNome revealed three patient clusters (**Figure 1**). One cluster was enriched for patients who failed bridging therapy and for those who subsequently developed grade ≥2 CRS or ICANS, suggesting that baseline circulating miRNA patterns reflect disease biology associated with unfavorable outcomes.
To identify biomarkers of CRS, we compared the PLD miRNome of patients who developed grade ≥2 CRS (n=16) with that of patients with grade <2 CRS (n=37). Twenty-five miRNAs were differentially expressed (**Figure 2A** and **Supplementary Table 2**). After weighted LASSO selection and multivariable logistic regression including sex and PLD CRP, low miR-136-5p and high miR-4511 were independently associated with grade ≥2 CRS. The resulting model achieved 81% accuracy.
Comparison of patients who developed ICANS (n=18) with those who did not (n=35), identified 33 differentially expressed miRNAs (**Figure 2B and Supplementary Table 2**). After weighted LASSO selection and multivariable analysis including sex, histology, ECOG, and PLD CRP, high levels of miR-1290, PMBCL histology, and PLD CRP were associated with ICANS. This model predicted ICANS with 87% accuracy. Moreover, when stratified by ICANS grading, patients with severe manifestations (grade ≥2, n=14) had significantly higher levels of circulating miR-1290 at PLD compared to patients (n=39) with grade <2 ICANS symptoms (median levels: 2.3 vs. 0, KW test, p<0.0007).

We next investigated whether PLD miRNAs could predict response at 3 and 12 months. These time points were selected to capture both initial and sustained treatment responses. Weighted LASSO was applied to miRNA and clinical variables. A three-variable model

including CAR T-cell product, let-7d-3p, and miR-542-5p predicted 3-month response with 75% accuracy (**Table 1**). A second model including miR-143-3p, miR-542-3p, and miR-542-5p predicted 12-month response with 73% accuracy (**Table 1**). Using an optimized miR-542-5p cut-off value of 5.5, patients with elevated PLD miR-542-5p levels showed inferior PFS, TTP, and OS (**Supplementary Figure 1**), and these levels were positively correlated with LDH (r=0.37, p=0.006), circulating cell-free DNA (r=0.29, p=0.04), and PMN-MDSCs (r=0.34, p=0.08).

To our knowledge, this is the first comprehensive analysis of circulating plasma miRNAs in patients receiving anti-CD19 CAR T-cell therapy. Our findings show that PLD miRNA profiles are associated with both treatment-related toxicity and therapeutic efficacy.

Specifically, low miR-136-5p and high miR-4511 identified patients at increased risk of grade ≥2 CRS. We also identified miR-1290 as a predictor of ICANS together with CRP and PMBCL histology, supporting the role of inflammatory and endothelial pathways in neurotoxicity. These findings further suggest a broader relevance of circulating miRNA signatures across CAR T-cell–related toxicities, including hematologic toxicities, although this requires further investigation.

The most clinically relevant observation of our study is the identification of miRNA-based models able to predict response before lymphodepletion, and thus before CAR T-cell infusion. In both the 3- and 12-month models, miR-542-5p emerged as the most consistent adverse biomarker. Elevated PLD miR-542-5p identified patients with inferior response and survival and negative prognostic markers, including PMN-MDSCs, ccfDNA, and LDH, suggesting that this circulating miRNA captures both tumor burden and myeloid-driven immunosuppression [13-15], both of which critically affect CAR T-cell expansion and persistence.

Overall, our data suggest that circulating miRNAs may integrate biological information on inflammation, endothelial stress, tumor burden, and immune suppression more effectively than individual clinical variables, thus supporting their potential clinical utility. These findings support the potential use of circulating miRNAs as noninvasive biomarkers for patient stratification prior to CAR T-cell therapy. In addition, these signatures may provide insights into the biological mechanisms underlying treatment response and toxicity, potentially informing future therapeutic strategies.

The main limitations of this study are the relatively small sample size and the lack of external validation. Therefore, our findings should be considered exploratory and hypothesis-generating, and require validation in larger, independent cohorts. In addition, the implementation of circulating miRNA-based biomarkers across different centers may be limited by technical challenges, including assay standardization, reproducibility, and inter-laboratory variability, which will require further harmonization before clinical adoption. Larger prospective studies will be required to validate these findings and determine the clinical utility of circulating miRNA biomarkers in patients undergoing anti-CD19 CAR T-cell therapy.

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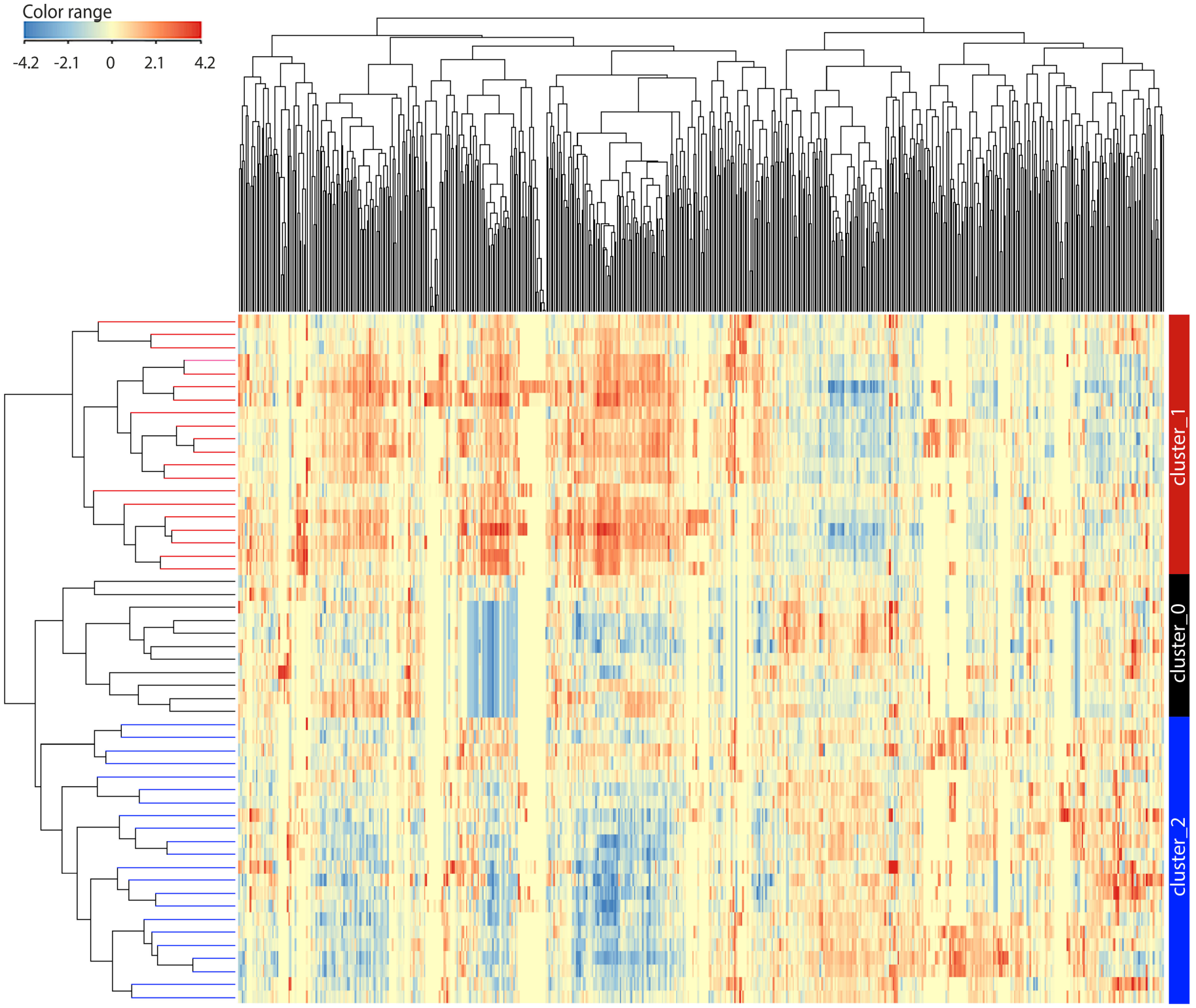
Table 1 – Response predictors

3-months follow-up prediction			
Predictors	***coefficient***	***p-value***	***accuracy***
Product	*-591.077*	*0.00473*	75%
hsa_let_7d_3p	*-0.03498*	*0.00616*	
hsa_miR_542_5p	*0.72076*	*0.01632*	
12-months follow-up prediction			
Predictors	***coefficient***	***p-value***	***accuracy***
hsa_miR_143_3p	*0.0009494*	*0.05869*	73%
hsa_miR_542_3p	*0.0201877*	*0.25701*	
hsa_miR_542_5p	*0.3405031*	*0.04141*	

Figure legends

Figure 1 - Hierarchical clustering of patients according to the pre-lymphodepletion (PLD) circulating miRNA profile. Heatmap showing unsupervised hierarchical clustering of 53 patients with relapsed/refractory non-Hodgkin lymphoma (r/r NHL) (rows) based on circulating miRNA profiles obtained by small-RNA sequencing of PLD plasma samples. A total of 573 circulating miRNAs detected across samples were included in the analysis. Clustering was performed using Pearson centered correlation and complete linkage. Colors represent normalized miRNA expression levels scaled to the mean expression across samples, ranging from blue (low expression) to red (high expression). Dendrogram branches identify three major patient clusters: cluster_1 (red), cluster_0 (gray), and cluster_2 (blue).

Figure 2 - Differentially expressed circulating miRNAs associated with toxicity after CAR T-cell therapy. A) Heatmap of the 25 differentially expressed (DE) miRNAs (rows) identified by comparing PLD circulating miRNA profiles of patients who developed grade ≥2 cytokine release syndrome (CRS) (n = 16) with those with grade 0–1 CRS (n = 37). Columns represent individual CAR T-cell–treated patients. Expression values are normalized and mean-centered across samples; blue indicates lower and red higher expression levels. Patients with grade ≥2 CRS are indicated in dark blue, whereas patients with grade 0–1 CRS are shown in light blue. **B)** Heatmap of the 33 DE miRNAs (rows) identified by comparing PLD circulating miRNA profiles of patients who developed immune effector cell-associated neurotoxicity syndrome (ICANS) (n = 18) with those who did not (n = 35). Expression values are normalized and mean-centered across samples; blue indicates lower and red higher expression levels. Patients with ICANS are indicated in dark green, whereas patients without ICANS are shown in light green.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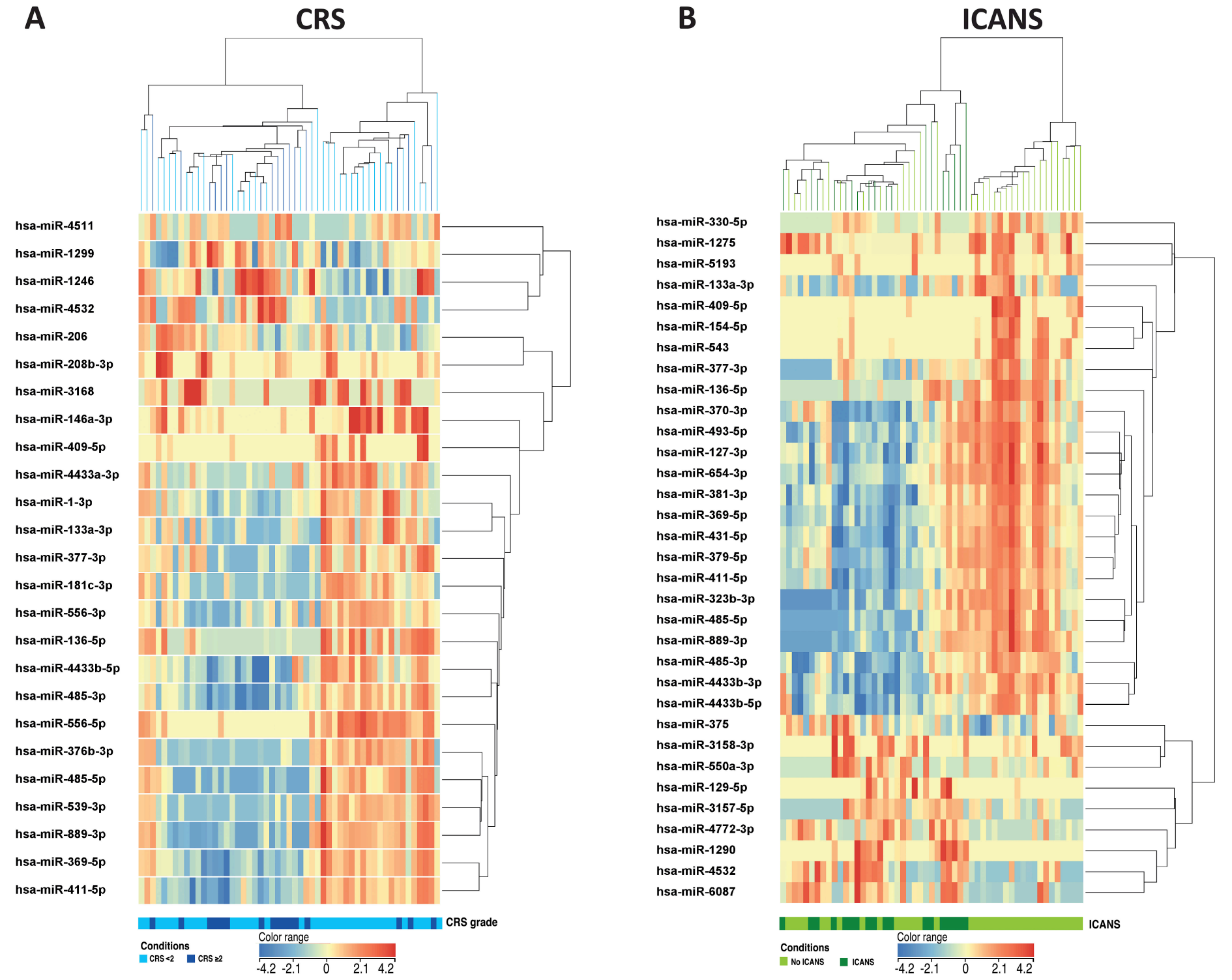
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hsa-miR-1-3p
hsa-miR-133a-3p
hsa-miR-377-3p
hsa-miR-181c-3p
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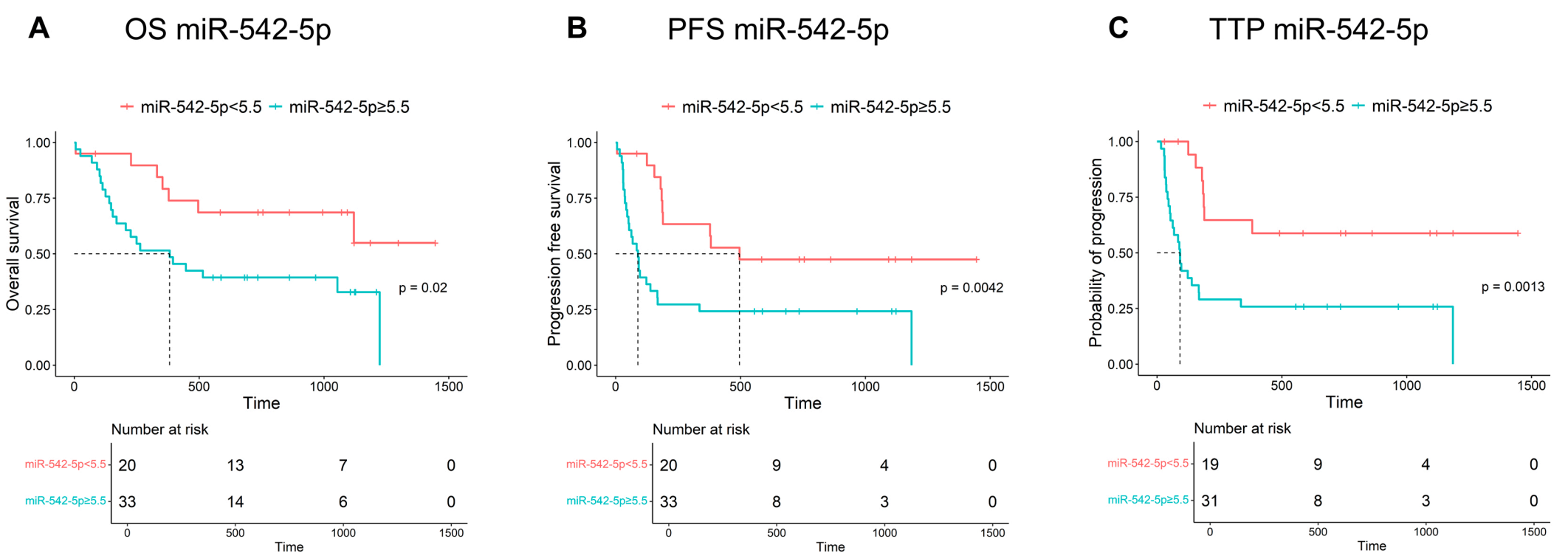
Supplementary Figure Legend

Supplementary Figure S1 - Kaplan–Meier survival analysis by PLD circulating miR-542-5p levels. Patients with high miR-542-5p levels (≥5.5 normalized counts; n = 33) showed significantly worse outcomes compared to those with low levels (<5.5 normalized counts; n = 20) in (A) overall survival (OS, p = 0.02), (B) progression-free survival (PFS, p = 0.0042), and (C) time to progression (TTP, p = 0.0013).

Supplementary Tables (see Excel file)

Supplementary Table 1 – A) Summary of patients' characteristics; B) Baseline clinical and biochemical characteristics of patients grouped according to CRS grade (0–1 vs. ≥2); C) Baseline clinical and biochemical characteristics of patients grouped according to ICANS (yes vs. no)

Supplementary Table 2 – A) MicroRNAs differentially abundant at PLD according to CRS grade (0–1 vs. ≥2); B) MicroRNAs differentially abundant at PLD in ICANS group (yes vs. no)



Supplementary figure S1.

Supplementary Figure S1. Kaplan–Meier survival analysis by PLD circulating miR-542-5p levels. Patients with high miR-542-5p levels (≥5.5 normalized counts; n = 33) showed significantly worse outcomes compared to those with low levels (<5.5 normalized counts; n = 20) in (**A**) overall survival (OS, p = 0.02), (**B**) progression-free survival (PFS, p = 0.0042), and (**C**) time to progression (TTP, p = 0.0013).