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Study on the diagnostic role of exosome-derived miRNAs in postoperative septic shock and non-septic shock patients

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Abstract

Background Diagnosing septic shock promptly is essential but challenging, especially due to its clinical similarity to non-septic shock. Extracellular vesicle-derived miRNAs may serve as biomarkers to distinguish septic shock from non-septic shock, providing a more accurate diagnostic tool for postsurgical patients. This study aims to identify extracellular vesicle-derived miRNA signatures that differentiate septic shock from non-septic shock in postsurgical patients, potentially improving diagnostic accuracy and clinical decision-making.

Methods A multicentre, prospective study was conducted on miRNA profiles in shock patients. Two cohorts were recruited from the Intensive Care Units of two Spanish hospitals: a discovery cohort with 109 patients and a validation cohort with 52 patients. Plasma samples were collected within 24 h of shock diagnosis and subjected to miRNA sequencing. High-throughput sequencing data from the discovery cohort were analysed to identify differentially expressed miRNAs. These findings were validated via qPCR in the validation cohort.

Results Thirty miRNAs were identified as significantly differentially expressed between septic and non-septic shock patients. Among these, six miRNAs—miR-100-5p, miR-484, miR-10a-5p, miR-148a-3p, miR-342-3p, and miR-451a—demonstrated strong diagnostic capabilities for septic shock. A combination of miR-100-5p, miR-148a-3p, and miR-451a achieved an area under the curve of 0.894, with qPCR validation in the validation cohort yielding an area under the curve of 0.960.

Conclusions This study highlights extracellular vesicle-derived miRNAs as promising biomarkers for differentiating septic from non-septic shock. The identified three-miRNA signature has significant potential to enhance septic shock diagnosis, thereby aiding in timely and appropriate treatment for postsurgical patients.

Keywords Sepsis, Shock, MicroRNAs, Extracellular vesicles, Biomarkers

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Introduction

Sepsis is a severe condition characterised by life-threatening organ dysfunction due to a dysregulated host response to infection. Septic shock represents an even more critical form, characterized by hemodynamic instability [1]. A global study published in 2020 reported that in 2017 alone, 48.9 million cases of sepsis were diagnosed, resulting in 11 million sepsis-related deaths, which accounted for approximately 20% of all deaths worldwide that year [2]. Among all cases of sepsis, postoperative sepsis is independently associated with higher mortality rates, showing rates of 13.5% compared to 3.8% for non-operative cases, with elevated risk persisting up to one year following hospital discharge [3].

Sepsis and septic shock are considered time-sensitive conditions where prompt administration of antibiotics is crucial, as it significantly improves survival rates and reduces the risk of death in patients with septic shock [4]. However, septic shock and other non-infectious types of shock (non-septic shock) have a similar clinical presentation (with symptoms such as fever, tachycardia, and leucocytosis) [5], and due to the non-specificity of the symptoms and the lack of a definitive diagnostic test it is estimated that more than a third of patients initially diagnosed with sepsis are later found to have non-infectious conditions [6]. This leads to unnecessary antibiotic use, potentially causing adverse effects and contributing to the development of antibiotic resistance [7, 8]. Furthermore, this misinterpretation can also delay the diagnosis and treatment of other underlying causes of shock, such as bleeding, which can result in clinical deterioration and increased mortality. More than 250 biomarkers have been proposed over the last decades, yet we still lack a quick and accurate gold standard for diagnosing sepsis in post-surgical patients, as many of these biomarkers have not demonstrated consistent reliability or reproducibility stemming from the heterogeneous and multifactorial nature of sepsis [9].

Considering these challenges, the search for new sepsis biomarkers has intensified, with genome-wide expression studies aimed at improving diagnostic accuracy [10, 11]. Extracellular vesicles (EVs), which carry mRNA and miRNA, play a crucial role in intercellular communication and have emerged as valuable tools in biomarker research because they can reflect the state of their source cells [12]. The miRNAs released by EVs can propagate between cells, influencing gene expression and protein synthesis in the recipient cells, thereby significantly affecting processes such as inflammation and immune responses [13, 14]. Recent research has focused on the role of miRNA in EVs in sepsis, using the miRNA contained within to distinguish sepsis from non-infectious systemic inflammation, highlighting their potential as diagnostic biomarkers [15]. More broadly, EVs play crucial roles in intercellular communication during infection, with their ability to carry nucleic acids, lipids, and proteins contributing to the mediation of infection, immune responses, and pathogen transmission, offering potential pathways for new therapeutic strategies [16].

The aim of this study is to enhance the diagnostic accuracy of distinguishing septic shock from non-septic shock in postsurgical patients by identifying miRNAs derived from EVs that serve as specific biomarkers and validating these biomarkers in a separate patient cohort to assess their diagnostic potential.

Methods

Design and study population

This multicentre, prospective study included two cohorts of adult patients (≥18 years old) who underwent any type of surgery, recruited from the Intensive Care Units of the Hospital Clínico Universitario de Valladolid (Spain) and the Hospital Universitario de Toledo (Spain The analysis was based on effect size estimates (Cohen's d = 0.5–0.8) derived from prior studies investigating the differential expression of miRNAs in sepsis and septic shock [17–19]. The analysis indicated the need for 100 patients in the discovery cohort and 50 patients in the validation cohort to detect moderate-to-large effect sizes. The discovery cohort consisted of 109 patients (58 with septic shock and 51 with non-septic shock), while the validation cohort included 52 patients (25 with septic shock and 27 with non-septic shock). In our non-septic shock patients, haemorrhagic hypovolemic shock accounted for 36%, while the remainder corresponded to non-infectious distributive shock. No cases of cardiogenic or obstructive shock were included. A flowchart summarising the study design and patient selection is provided in Supplementary Fig. 1A. Patients in the discovery cohort were enrolled sequentially between January 2020 and May 2022, whereas those in the validation cohort were enrolled between January 2023 and December 2023. The study excluded non-Caucasians, pregnant women, patients in terminal stages, those under a limitation of therapeutic effort, and patients who met the clinical criteria for septic shock but had negative microbiological cultures (32 patients out of 115 collected). Ethical approval was obtained from the Scientific Committee for Clinical Research of Hospital Clínico Universitario de Valladolid (PI-18–972) and the Ethics Committee for Clinical Research of Hospital Universitario de Toledo (CEIC-466–2019). Informed written consent was obtained from each patient or their legal representative before recruitment. The study adhered to the ethical principles outlined in the World Medical Association's Declaration of Helsinki.

Clinical categories and microbiological diagnosis

Septic shock was diagnosed according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3 definition) [1]. The same clinical criteria were applied to define non-septic shock, excluding any evidence of infection. Patients meeting the clinical criteria for septic shock but with a negative microbiological culture were excluded from the study. All cases were reviewed independently by two physicians before patients were assigned to their respective groups. Microbiological diagnostics were conducted to confirm infections in suspected cases. This included Gram staining and culture on various media, as well as fungal-specific culture on Sabouraud agar with chloramphenicol for suspected fungal infections (Supplementary Fig. 1B). Infection confirmation adhered to the guidelines of the Infectious Diseases Society of America and the American Society for Microbiology [20].

Clinical data and sample collection

Epidemiological and clinical data were extracted from patients' medical records. Blood samples were collected using 3.2% sodium citrate tubes within the first 24 h following the diagnosis of shock. Subsequently, plasma was separated by centrifugation at $2000 \times g$ for 20 min at room temperature, and aliquots were stored at − 80 °C.

High throughput sequencing of extracellular vesicles miRNome

Total RNA enriched in small RNAs from EVs was performed based on size exclusion chromatography on 500 µL of plasma from the discovery cohort using the exoRNeasy Midi Kit (Qiagen) in line with manufacturer guidelines. RNA quantity and quality were assessed using the Agilent Bioanalyzer 2100 with the Agilent RNA 6000 Pico Kit. Libraries for small RNA sequencing were prepared using the NEXTFLEX Small RNA-Seq Kit v4 for Illumina Platforms (PerkinElmer) and sequenced on the Novaseq 6000 platform, achieving an average of over 10 million reads per sample. Library and sequencing were conducted by ADM-BIOPOLIS GENOMICS at the Parque Científico Universidad de Valencia (Spain). Sequence data derived from EV plasma samples are available through BioStudies (S-BSST1230).

Data processing pipeline and bioinformatics analysis

Raw sequence data from BCL files were converted to FASTA format using Illumina's bcl2fastq tool. Demultiplexing was performed to assign individual reads to their respective samples for subsequent analysis. After eliminating reads failing the Illumina chastity filter, the remaining reads were assessed for quality through FastQC (v0.11.3). Adapter sequences were removed with cutadapt (v1.13). miRDeep2 (v.0.1.3) was used for alignment to the reference human genome (GRCh38) with the Bowtie1-based mapper.pl module. Retention criteria included zero mismatches in the seed region and mapping to fewer than five genomic loci. miRNAs quantification was performed using the quantifier.pl module in two steps. First, mature miRNA sequences were mapped against the reference precursors in miRBase (v22), Second, sequencing reads were mapped against the precursor sequences and reads within a specific interval around these sequences were quantified. A prefilter step was applied using the recommended parameters from the DESeq2 R package documentation to remove miRNAs with low counts, requiring a count greater than 5 in at least 51 samples (corresponding to the smallest experimental group size). miRNA counts were normalised using the DESeq2 method. Partial Least Squares Discriminant Analysis (PLS-DA) was performed to evaluate group differentiation using the mixOmics R package (version 6.26.0). Differential expression analysis was conducted with a generalised linear model and a negative binomial distribution, adjusting for age and sex. Significance was determined by a fold change ≥ 1.5 and q-value ≤ 0.05 (False Discovery Rate-corrected p-value).

qPCR validation

The miRNAs identified in the discovery cohort were validated in the validation cohort using qPCR to assess reproducibility, following the protocol by Balcells et al. [21]. cDNA synthesis was performed as follows: a polyadenylation reaction was conducted simultaneously with the reverse transcription reaction on 20 ng of total RNA enriched in miRNAs from the validation cohort, which was extracted as previously described, using Poli-A polymerase (Invitrogen, Thermo Scientific) and M-MuLV reverse transcriptase (Merck), respectively. Absolute quantification of PCR products was performed using a standard curve with the SYBR-Green method, employing specific oligonucleotides according to the primer design guidelines provided by Balcells et al. [18]. The sequences of the primers used are provided in Supplementary Table 1. The amplification reaction was carried out in triplicates under the following conditions: an initial holding stage at 50 °C for 2 min, followed by denaturation at 95 °C for 2 min, and then forty cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 1 min. The amplification reaction was executed in a 7500 Fast Real-time PCR System using SYBR-Green Master Mix (Thermo Scientific). cel-miR-39a was added during the extraction steps according to the manufacturer's instructions and used as a calibration gene. The efficiency of the amplification was assessed, with values between 90 and 110% being accepted.

miRNA-based target prediction and functional analysis

Significant differentially expressed miRNAs underwent miRNA target interaction analysis using miRNAtap and ClusterProfiler R packages (versions 1.38.0 and 4.12.5, respectively). Gene targets with ≥ 3 validated interactions were selected for functional enrichment analysis using KEGG Pathways and GO cellular processes databases, with significance determined at q-value ≤ 0.05.

Statistical analysis

All statistical analyses were performed using R software (version 4.4.1). Categorical data were analysed using chi-squared tests, while continuous variables were assessed with the Wilcoxon test via the arsenal R package (version 3.6.3). The predictive performance of miRNAs, including sensitivity and specificity, was evaluated through area under curve values using receiver operating curves with the pROC and caret R packages (versions 1.18.4 and 6.0–94, respectively). Area under the curve (AUC) values were classified as fair (> 0.7), good (> 0.8), and excellent (> 0.9). For the follow-up analysis, only miRNAs with an AUC greater than 0.7 were considered. Adjustment variables were selected from the biochemistry data based on their clinical relevance to sepsis and their association with the clinical data, specifically focusing on variables with a p-value < 0.05 between the cohort groups. We also compared the diagnostic performance of miRNAs with traditional biomarkers, such as C-reactive protein and procalcitonin, using ROC curve analysis and AUC values. Additionally, we assessed accuracy, balanced accuracy, sensitivity, specificity, PPV, NPV, and kappa. The statistical significance of the classification performance was evaluated by comparing the p-value for accuracy against the No Information Rate (NIR) to determine whether each model provided meaningful diagnostic improvement. Correlations between miRNA expression and clinical parameters were assessed using Pearson's correlation coefficient.

Results

Patient characteristics

The characteristics of postsurgical participants from both the discovery and validation cohorts are summarised in Table 1. In the discovery cohort, significant differences were observed for several variables, including arterial hypertension, hepatopathies, and patients who had developed cancer within the previous 3 months. Significant differences were also noted for diagnostic biochemical parameters, such as procalcitonin, C-reactive protein, lactate levels, and platelet counts. Additionally, individuals in the discovery cohort showed a significant increase in the length of hospital stay. In the validation cohort, significant differences were observed in relation to age and pulmonary disease. Significant differences were also found for procalcitonin levels, C-reactive protein, haematocrit, sodium and potassium levels.

Differential miRNome profiling between septic and non-septic shock patients

A total of 2888 miRNAs from miRbase were interrogated, resulting in the detection of 728 miRNAs, of which 426 had sufficiently high counts for differential expression analysis. PLS-DA revealed a clear separation between septic and non-septic shock patients based on miRNA expression patterns (Fig. 1A). Differential expression analysis using DESeq3 identified 30 miRNAs as significantly differentially expressed (SDE). Of these, 20 miRNAs were upregulated and 10 miRNAs were downregulated in septic shock patients compared to non-septic shock patients. The most upregulated miRNA was miR-222-3p, with a log2 fold change (log2FC) of 2.24, followed by miR-3615 (log2FC = 2.09). Among the downregulated miRNAs, miR-4488 exhibited the strongest downregulation with a log2FC of −4.52, followed by miR-148a-3p (log2FC = − 2.82) and miR-100-5p (log2FC = − 2.40). The volcano plot and heatmap for these SDE miRNAs are shown in Fig. 1B and C, respectively, and the complete list of miRNAs can be found in Supplementary Table E2.

To explore the functional implications of the differentially expressed miRNAs (SDE miRNAs), we conducted pathway enrichment analyses. The Gene Ontology (GO) enrichment analysis identified several functional categories significantly enriched among the SDE miRNAs, including small GTPase-mediated signal transduction, positive regulation of cell adhesion, and positive regulation of protein localization (Fig. 2A). Additionally, the KEGG pathway enrichment analysis revealed several pathways potentially influenced by the dysregulated miRNAs, such as cancer, MAPK signalling, endocytosis, and proteoglycans in cancer (Fig. 2B).

Validation of miRNAs for the diagnostic of septic shock versus non-septic shock

From the SDE miRNAs identified in the discovery cohort, we assessed their ability to distinguish between septic shock and non-septic shock patients. miRNAs with an AUC greater than 0.7 were selected for further analysis, and their diagnostic accuracy was combined into a single diagnostic model. The combination of miR-100-5p, miR-148a-3p, and miR-451a was identified as the most effective, yielding an AUC value of 0.894. To compare our findings with the most used biomarkers for diagnosing and monitoring septic shock, we evaluated the ability of procalcitonin and C-reactive protein to differentiate between patients with septic shock and those with non-septic shock. The AUC increased

Table 1 Descriptive table of both discovery and validation cohorts

Parameter	Discovery cohort			Validation cohort		
	Non-septic shock (n = 51)	Septic shock (n = 58)	*p*	Non-septic shock (n = 27)	Septic shock (n = 25)	*p*
Characteristics						
Male [n (%)]	32 (62.7%)	37 (63.8%)	0.910	18 (69.2%)	22 (81.5%)	0.300
Age [median (IQR)]	69 (11)	73 (16.5)	0.173	69 (16)	81 (14)	**0.010**
Comorbidities [n (%)]						
Smoker	8 (15.7%)	11 (19.0%)	0.653	5 (19.2%)	5 (18.5%)	0.947
Cardiovascular disease	22 (43.1%)	31 (53.4%)	0.283	9 (34.6%)	8 (29.6%)	0.697
Diabetes mellitus	13 (25.5%)	14 (24.1%)	0.870	3 (11.5%)	6 (22.2%)	0.300
Neurological disease	8 (15.7%)	17 (29.3%)	0.091	1 (3.8%)	0 (0.0%)	0.304
Arterial hypertension	41 (80.4%)	34 (58.6%)	**0.014**	14 (53.8%)	12 (44.4%)	0.494
Hepatopathies	0 (0.0%)	5 (8.6%)	**0.032**	0 (0.0%)	1 (3.7%)	0.322
Pulmonary disease	5 (9.8%)	5 (8.6%)	0.831	2 (7.7%)	8 (29.6%)	**0.041**
Renal disease	1 (2.0%)	6 (10.3%)	0.075	4 (16.7)	1 (3.7%)	0.270
Obesity	9 (17.6%)	10 (17.2%)	0.956	9 (34.6%)	5 (18.5%)	0.184
Cancer last 3 months	3 (5.9%)	12 (20.7%)	**0.025**	2 (7.7%)	7 (25.9%)	0.077
Surgery type [n (%)]						
Abdominal	5 (9.8%)	30 (51.7%)	**< 0.001**	5 (18.5%)	17 (68.0%)	**< 0.001**
Cardiac	29 (56.9%)	19 (32.8%)	**0.006**	11 (40.7%)	4 (16.0%)	**0.027**
Urological/renal	4 (7.8%)	3 (5.2%)	0.290	3 (11.1%)	1 (4.0%)	0.170
Vascular	11 (21.6%)	5 (8.6%)	**0.029**	6 (22.2%)	1 (4.0%)	**0.030**
Other	2 (3.9%)	1 (1.7%)	0.242	2 (7.4%)	2 (8.0%)	0.467
Microbiology [n (%)]						
Gram −	0 (100%)	35 (60.3%)	**< 0.001**	0 (100%)	15 (60.0%)	**< 0.001**
Gram +	0 (100%)	30 (51.7%)	**< 0.001**	0 (100%)	15 (60.0%)	**< 0.001**
Fungi	0 (100%)	17 (29.3%)	**< 0.001**	0 (100%)	4 (16.0%)	**< 0.001**
Source of infection [n (%)]						
Abdomen	0 (100%)	19 (32.8%)	**< 0.001**	0 (100%)	11 (44.0%)	**< 0.001**
Bacteraemia	0 (100%)	8 (13.8%)	**< 0.001**	0 (100%)	3 (12.0%)	**< 0.001**
Respiratory tract	0 (100%)	15 (25.9%)	**< 0.001**	0 (100%)	3 (12.0%)	**< 0.001**
Surgical site	0 (100%)	8 (13.8%)	**< 0.001**	0 (100%)	4 (16.0%)	**< 0.001**
Urinary tract	0 (100%)	8 (13.8%)	**< 0.001**	0 (100%)	4 (16.0%)	**< 0.001**
Biochemistry at diagnosis						
Glucose (mg/dL)	170 (72.5)	158.5 (72.25)	0.130	160 (68)	162 (25)	0.999
Leukocytes (cells/mm^3)	12,180 (5205)	13,010 (9457.5)	0.542	12,310 (5050)	10,080 (2245)	0.260
Lymphocytes (cells/mm^3)	928.35 (738.7)	714.3 (755.8)	0.444	755 (478)	660 (622)	0.066
Neutrophils (cells/mm^3)	10,720 (5669.8)	11,043.8 (9090.2)	0.362	10,714 (5215)	9066 (3137)	0.716
Procalcitonin (ng/mL)	1.25 (4.285)	2.8 (16.14)	**0.029**	0.4 (1.7)	26 (36.3)	**< 0.001**
C-reactive protein (mg/L)	39.485 (150.1)	221.95 (188.03)	**< 0.001**	49.4 (34.5)	190.8 (222.84)	**< 0.001**
Lactate (mM)	3.25 (4.842)	2.05 (1.63)	**< 0.001**	2.65 (2.87)	2.57 (0.66)	0.305
Hematocrit	29.2 (6.7)	30.4 (8.725)	0.552	28.2 (7.4)	36.6 (7.6)	**0.013**
Sodium (mEq/L)	139.96 (5.625)	139.27 (6.257)	0.055	141 (3)	135 (6)	**0.003**
Potassium (mEq/L)	4.2 (0.6)	4.3 (0.9)	0.696	4.5 (1)	3.6 (0.7)	**< 0.001**
Creatinine (mg/dL)	1.26 (0.82)	1.15 (0.993)	0.998	1.46 (0.95)	1.27 (0.65)	0.808
Total bilirubin (mg/dL)	0.99 (0.89)	0.96 (1.22)	0.520	0.67 (0.52)	0.87 (1.045)	0.071
Platelets (10^3 cells/mm^3)	138 (78)	184 (151)	**0.002**	134 (102)	151 (70)	0.227
SOFA Score	8 (3)	8 (3.5)	0.943	8 (4)	7 (2)	0.474

Table 1 (continued)

Parameter	Discovery cohort			Validation cohort		
	Non-septic shock (n = 51)	Septic shock (n = 58)	*p*	Non-septic shock (n = 27)	Septic shock (n = 25)	*p*
Time course and outcome						
Resuscitation fluids (ml), median (IQR)	1655 (515)	1500 (550)	**0.048**	1500 (1500)	1500 (500)	0.883
Norepinephrin (µg/kg/min), median (IQR)	0.4 (0.245)	0.39 (0.562)	0.693	0.3 (0.237)	0.15 (0.185)	0.31
Time to shock onset (days), median (IQR)	1 (0)	1 (2)	0.068	1 (0)	1 (2.5)	0.749
Length of ICU stay (days) median (IQR)	6 (7)	8.5 (13)	0.108	5 (5)	4 (7)	0.906
Length of hospital stay (days), median (IQR)	15 (19.5)	27.5 (33.25)	**0.002**	16 (20)	23 (12)	0.191
Mortality [% at 28 days]	8 (15.7%)	14 (24.1%)	0.273	4 (15.4%)	0 (0.0%)	0.060
Mortality [% at 60 days]	10 (19.6%)	18 (31.0%)	0.173	5 (19.2%)	2 (9.5%)	0.353
Mortality [% at 90 days]	10 (19.6%)	19 (32.8%)	0.121	5 (19.2%)	3 (14.3%)	0.654

A *p*-value < 0.05 was considered to indicate significant differences (bold values)

to 0.959 when combined with C-reactive protein values. These results are presented as receiver operating characteristic (ROC) curves in Fig. 3, and the full list of the AUC values for all the SDE miRNAs can be found in Supplementary Table 3. In addition, a correlation heatmap was generated to explore the relationships between the significant biochemical variables identified in the descriptive analysis and different organ failures with the three selected SDE miRNAs (Supplementary Fig. 2). Among them, only miR-148a-3p showed a significant correlation with procalcitonin levels, while the miR-100-5p was significantly correlated with haemodynamic organ failure.

Our results demonstrate that the miRNA-based model (AUC = 0.894) outperforms both CRP (AUC = 0.878) and PCT (AUC = 0.784) alone, supporting the clinical relevance of miRNAs as diagnostic biomarkers for septic shock. Additionally, the combination of miRNAs and CRP further enhances diagnostic accuracy (AUC = 0.959, Accuracy = 87.50%), significantly improving sensitivity and specificity compared to CRP alone (Accuracy = 75.00%) or PCT alone (Accuracy = 75.00%). The *p*-value (Acc > NIR) confirms that models incorporating miRNAs (alone or in combination) provide statistically significant improvements in classification performance, while CRP and PCT alone do not reach significance. These results are summarized in Supplementary Table 4.

qPCR validation of selected SDE miRNAs

We evaluated the expression levels of miR-100-5p, miR-148a-3p and miR-451a by qPCR in a separate study cohort (validation cohort) to assess their reliability as potential biomarkers. Consistent with our RNA-seq results, miR-100-5p, miR-148a-3p and miR-451a were found to have significantly lower expression levels in septic shock patients compared to non-septic shock patients. These results are illustrated in Fig. 4. The diagnostic performance of these miRNAs was assessed using ROC curve analysis in the validation cohort, with an AUC used to quantify their accuracy. miR-100-5p exhibited the highest AUC value of 0.921, followed by miR-148a-3p and miR-451a. Remarkably, the combination of the three miRNAs achieved an AUC of 0.960, which increased to 0.976 when combined with procalcitonin. These findings are shown in the ROC curves in Fig. 5. Detailed predictive values for each miRNA, used to classify patients based on the presence or absence of infection, are provided in Supplementary Table 5.

Discussion

In this study, we evaluated the extracellular vesicle-derived miRNome of patients who developed septic shock compared to those with non-septic shock following major surgery. Our analysis resulted in two key findings: (1) extracellular vesicle-derived miRNAs differ between septic and non-septic shock patients, with 30 SDE miRNAs, and (2) three miRNAs were identified as potential biomarkers for differentiating septic shock from non-septic shock, with their validity confirmed through qPCR analysis in a distinct cohort of plasma-derived EVs.

EVs play a crucial role in intercellular communication in critically ill patients, carrying biomolecules such as miRNAs that reflect cellular origin and modulate gene

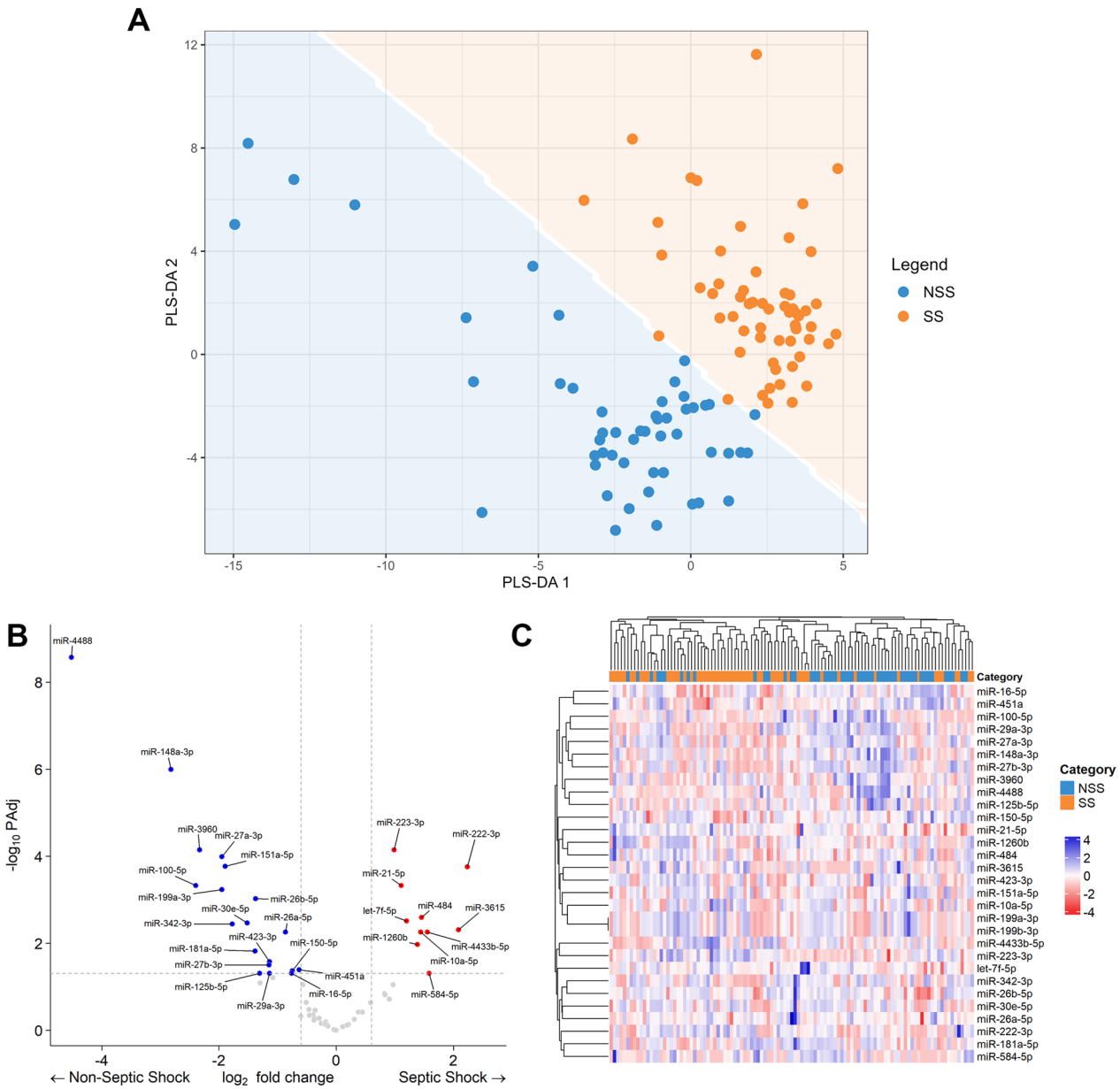

Fig. 1 **A** A multivariate analysis was carried out by partial least squares discriminant analysis from prefiltered and variance stabilizing transformation and scaled miRNA expression data (SS: septic shock; NSS: non-septic shock). **B** Volcano plots of the significantly differentially expressed miRNAs for septic shock and non-septic shock patients. Vertical dashed lines indicate the threshold value for absolute fold change ≥ 1.5 and horizontal dashed line indicates the threshold value for FDR-adjusted *p*-value ≤ 0.05, grey dots represent the miRNAs that are below the threshold and red and blue dots indicate the miRNAs that are above the threshold. **C** Heatmap and hierarchical clustering of the SDE miRNAs for septic shock and non-septic shock patients. Study subjects are represented in columns and SDE miRNAs in rows, with clustering dendrograms on the left for miRNAs. The colour scale shows the relative expression level of SDE miRNAs. Blue colour indicates a higher expression level and red indicates a lower expression level. SDE: Significantly Differentially Expressed, FDR: False Discovery Rate

expression during infection and inflammation [22–24]. These vesicle-derived miRNAs contribute to organ failure and the pathophysiology of septic shock by modulating gene expression and the immune response [25, 26]. A previous study explored the differences in the plasmatic miRNome profile between septic patients and non-septic controls [27], no previous research has specifically investigated miRNAs as diagnostic biomarkers to differentiate septic shock from non-septic shock. In this context, our analysis identified 30 SDE miRNAs between septic and

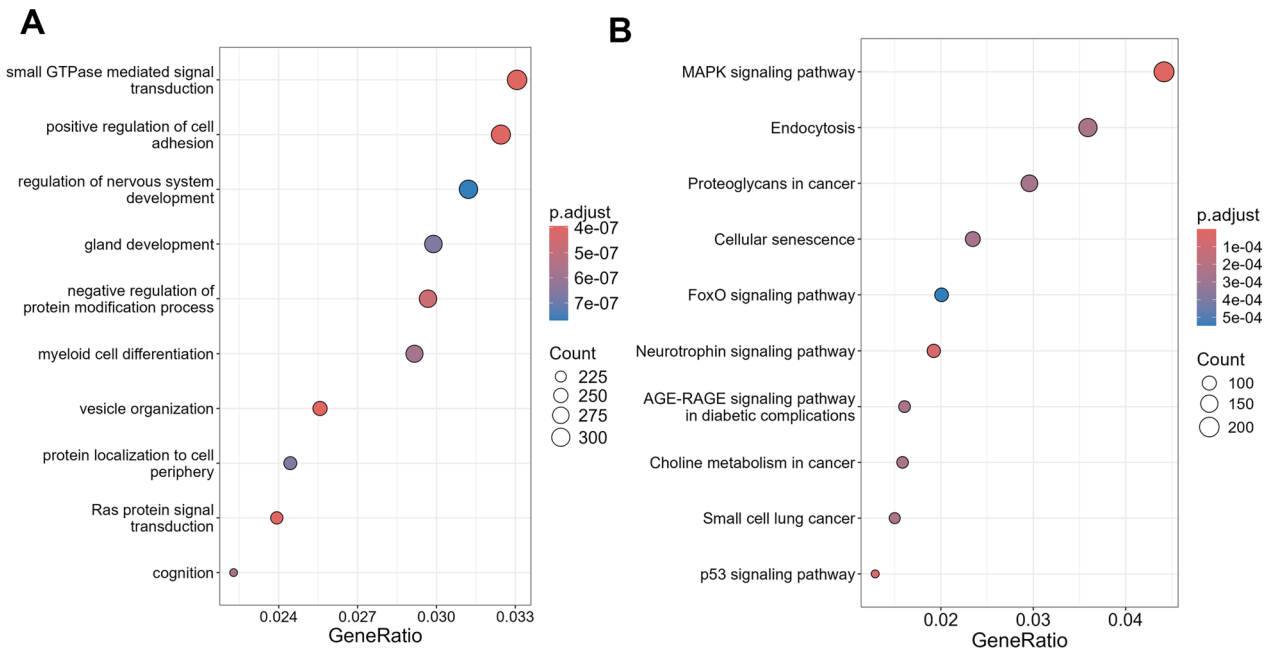

Fig. 2 **A** GO enrichment for cellular processes and **B** KEGG molecular pathways for the target genes of SDE miRNAs identified in the comparison between the two groups. The dot size represents the number of genes associated with each pathway or cellular process, while the colour indicates the *q*-value. The significance thresholds for the analysis were set at 0.05 for both *p*-value and *q*-value. SDE: Statistically Differential Expressed

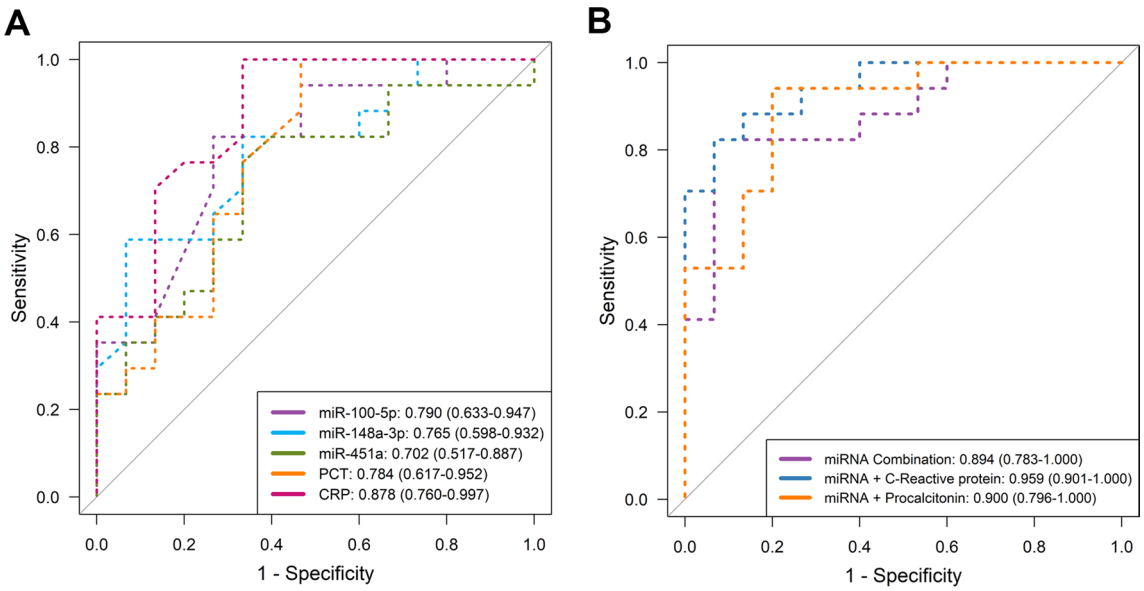

Fig. 3 **A** Receiver operating characteristic (ROC) curves to assess the diagnostic potential of the significantly expressed miRNAs on the discovery cohort. ROC curve of miR-100-5p, miR-484, miR-10a-5p, miR-148a-3p, miR-342-3p, miR-451a, procalcitonin (PCT) and C-reactive protein (CRP). **B** Combined values of miR-100-5p, miR-148a-3p and miR-451a (purple), together with C-reactive protein (blue) and procalcitonin (orange). The values shown in the legend correspond to the area under curve values plus the confidence interval

non-septic shock patients, several of which have already been implicated in sepsis progression. For example, miR-451a, released by neutrophils, can induce endothelial cell damage and inflammation by promoting apoptosis and increasing pro-inflammatory cytokine expression. We observed downregulation of miR-451a in septic shock patients, which may suggest a heightened pro-inflammatory state in non-septic shock patients [28]. Conversely,

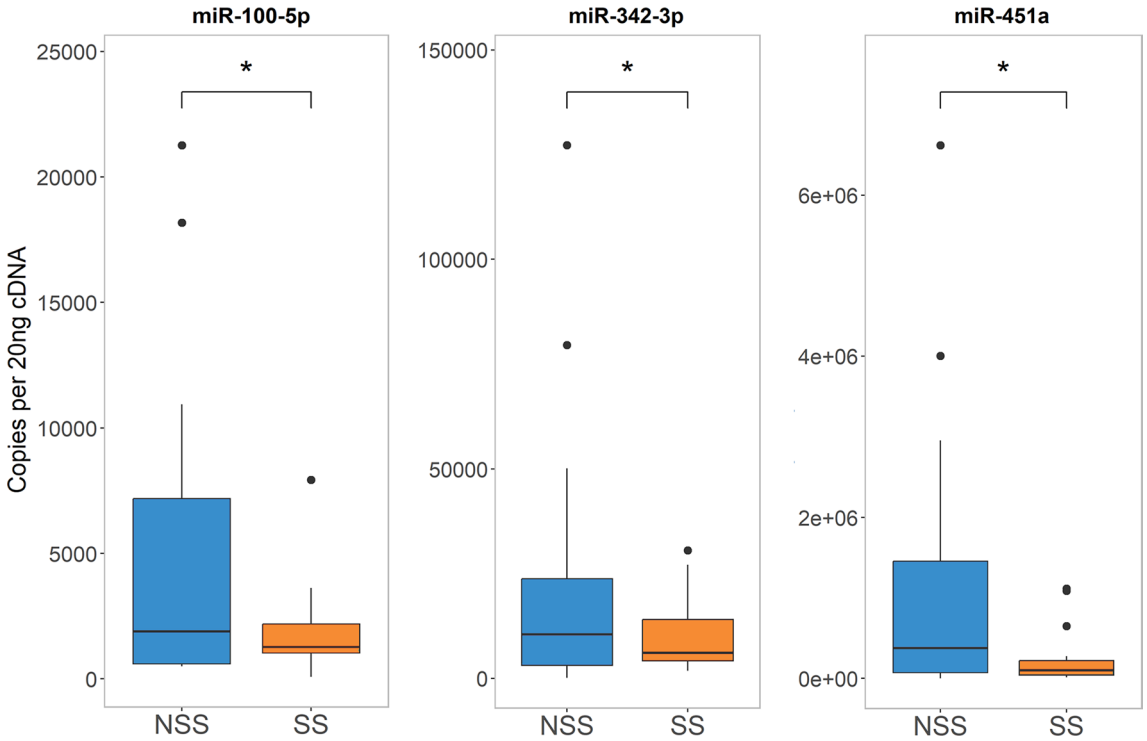

Fig. 4 Absolute expression of miR-100-5p, miR-342-3p and miR-451a on the validation cohort measured by qPCR. The values show the number of copies per 20 ng of cDNA. *p-value < 0.05

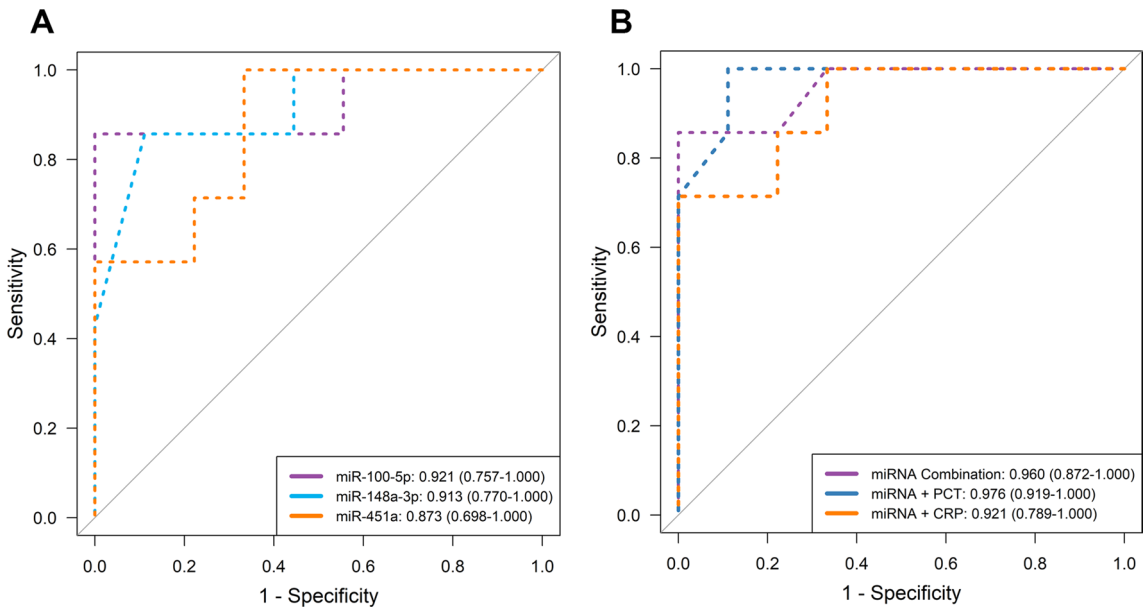

Fig. 5 **A** Receiver operating characteristic (ROC) curves to assess the prediction potential of the significantly expressed miRNAs on the validation cohort. ROC curve of miR-100-5p, miR-342-3p and miR-451a. **B** Combined values of miR-100-5p, miR-148a-3p and miR-451a (purple), together with C-reactive protein (orange) and procalcitonin (blue). The values shown in the legend correspond to the area under curve values plus the confidence interval

miR-223, known for contributing to endothelial dysfunction, was upregulated in our septic shock patients, possibly reflecting underlying endothelial damage, an early feature of septic shock that affects vascular permeability and contributes to multiple organ dysfunction syndrome [29, 30]. miR-21-5p, linked to reactive oxygen species production and inflammasome activation [31], was overexpressed in the septic shock group, suggesting that it may exacerbate endothelial damage. While miR-21-5p is known for its pro-inflammatory role, it also has a protective effect in sepsis by promoting M2 macrophage polarisation and reducing inflammation [32]. Additionally, miR-150-5p, which was downregulated in septic shock patients in our discovery cohort, has been shown to inhibit inflammation and promote macrophage polarisation through the downregulation of the PI3K/Akt/mTOR pathway [33]. Likewise, miR-342, which was also downregulated in septic shock patients, has been shown to reduce inflammation [34]. Furthermore, exosomal miR-27b-3p derived from mesenchymal stem cells has been shown to inhibit the development of sepsis by inactivating the NF-κB signalling pathway, while EVs replenishment of miR-26a-5p protected against hepatocyte death and liver injury caused by sepsis [35, 36]. Both miR-223-5p and miR-3p have been associated with cardioprotection in poly-microbial sepsis, while miR-125b-5p protects against sepsis-induced acute lung injury [37, 38]. In our study, miR-27b, miR-26a-5p, miR-223 and miR-125b-5p were downregulated in septic shock patients, suggesting a loss of their protective effects, which may correlate with the severity of the disease. Notably, miR-223 was found to be upregulated in the non-septic shock group, which had a higher proportion of patients who underwent cardiac surgery. This aligns with previous findings indicating that plasma exosomal miR-223 regulates inflammatory responses during cardiac surgery with cardiopulmonary bypass (CPB) [39]. These results suggest a potential role of miR-223 in the inflammatory response induced by CPB, contributing to the pathophysiology of non-septic shock in these patients. In addition, GO and KEGG pathway enrichment analyses were performed to explore the functional implications of the SDE miRNAs. GO enrichment analysis identified several functional categories significantly enriched among the SDE miRNAs, such as small GTPase-mediated signal transduction, positive regulation of cell adhesion, and positive regulation of protein localisation. These results suggest that the dysregulated miRNAs may be involved in regulating key cellular processes, including cell signalling, adhesion, and protein localisation, which are important for immune responses and disease progression [40, 41]. KEGG analysis revealed several enriched pathways that may be influenced by the dysregulated miRNAs, including MAPK signalling and endocytosis. These pathways are crucial in sepsis progression and may play a role in immune response and inflammation [42]. Identifying these 30 SDE miRNAs and their associated molecular pathways enhances our understanding of the molecular mechanisms underlying shock and highlights the differences between infectious and non-infectious aetiologies.

Extracellular vesicle-derived miRNAs are being prioritized over plasmatic miRNAs due to their potential as valuable diagnostic and prognostic biomarkers, providing unique insights and a more dynamic understanding of progression and therapeutic responses in various malignancies [43]. Given the diverse causes and similar clinical presentations of septic shock and non-infectious shock, there is growing consensus on the urgent need to re-evaluate existing biomarkers and develop more effective indicators for rapid and precise diagnosis. Extracellular vesicle-derived miRNAs have emerged as promising candidates in this context [44, 45]. Common biomarkers such as procalcitonin and C-reactive protein are frequently used to diagnose septic shock. However, their lack of specificity often results in misdiagnosis and inappropriate antibiotic use, which can contribute to the rise of antibiotic-resistant strains and undermine the effectiveness of current treatments [46–48]. While advances in omics sciences, including mRNA arrays, have enabled the discovery of new biomarkers for earlier detection of organ dysfunction [10, 49], our study focuses on miRNA sequencing of EVs to establish a gene expression signature in postoperative patients. Through this approach, we identified six miRNAs with an AUC above 0.7: miR-100-5p, miR-484, miR-10a-5p, miR-148a-3p, miR-342-3p and miR-451a. Our analysis revealed that miR-100-5p and miR-148a-3p were negatively correlated with C-reactive protein levels, a commonly used biomarker of infection. These correlations offer additional biological insights into their roles and strengthen their clinical relevance in the context of septic shock. Prior studies have highlighted the anti-inflammatory role of miR-100-5p in vascular responses to injury and inflammation. Specifically, miR-100-5p is a key modulator of mammalian target of rapamycin signalling and autophagy within the vascular system [50, 51]. miR-484 has been explored as a potential biomarker for sepsis due to its role in regulating inflammatory processes and cellular homeostasis [52], potentially aiding in the differentiation between septic and non-septic patients. Similarly, miR-10a-5p influences immune responses by regulating both pro-inflammatory and anti-inflammatory pathways in sepsis [53]. On the

other hand, miR-342-3p has been proposed as a specific miRNA associated with microglia and could serve as a biomarker for sepsis-associated encephalopathy [54]. Additionally, members of the miR-148 family, particularly miR-148a, have emerged as potential sepsis biomarker due to their involvement in immune regulation and inflammatory pathways [55]. Moreover, the serum levels of miR-451a have been correlated with sepsis severity, making it a promising biomarker for diagnostic and prognostic purposes in septic and septic shock patients [56]. These biomarkers, many of which are already recognised in the literature, underscore their potential value as reliable tools for enhancing the diagnosis and management of sepsis.

Our study also proposes a novel combination of three miRNAs (miR-100-5p, miR-148a-3p, and miR-451a) that shows significant promise as precise biomarkers for differentiating septic shock from non-septic shock in postsurgical patients. The altered levels of these extracellular vesicle-derived miRNAs may contribute to the dysregulated inflammatory response observed in sepsis, potentially disrupting endothelial integrity and leading to subsequent organ dysfunction and tissue damage. The diagnostic potential of our findings was further validated by qPCR in a separate cohort, underscoring the robustness of our findings. Notably, the validation cohort included patients with less severe septic shock, as indicated by lower SOFA scores and the absence of 28-day mortality. Despite this, the identified miRNAs effectively discriminated between septic and non-septic shock, demonstrating their robustness across different severity levels and highlighting their potential clinical utility as biomarkers, even in patients with varying degrees of shock severity. This three-miRNA signature could be efficiently implemented using a multiplex qPCR assay, enabling simultaneous detection of these miRNAs to facilitate streamlined and scalable diagnostic processes. Future studies should further assess the clinical applicability of the proposed cutoff values (miR-100-5p: 801.3 copies, miR-148a-3p: 11,789.5 copies, miR-451a: 215,420.8 copies). In our analysis, dichotomised miR-100-5p and miR-451a remained significant in both univariate and multivariate models, supporting their potential diagnostic value.

This approach has the potential to significantly improve clinical decision-making by enhancing the accuracy in distinguishing septic shock from non-septic shock. Furthermore, since EVs play a crucial role in the pathophysiology of septic shock, they represent a promising vehicle for therapeutic interventions, particularly through the development of extracellular vesicle-based treatments [57]. This combination of diagnostic precision and targeted therapy highlights the potential of EVs in both early detection and treatment of septic shock.

This study offers valuable insights into the dynamics of miRNA in EVs and their role in septic shock. The specificity of our cohort, consisting of post-surgical patients, provides a focused analysis that lays the groundwork for future research into miRNA roles across diverse sepsis contexts. Ultimately, this could contribute to the development of targeted therapeutic strategies for managing sepsis-related complications. However, the cohort design limits the generalizability of our findings to post-surgical sepsis. The main limitation of this study is the lack of data on temporal fluctuations in miRNA levels, as samples were only collected at a single time point following shock diagnosis. Despite this, the identification and validation of a three-miRNA combination in an additional cohort strengthen our findings. This multi-miRNA approach enhances the potential for clinical applicability, as relying on a single biomarker often proves insufficient for accurate differentiation. Thus, our findings suggest that this combination of miRNAs could significantly improve the diagnosis of septic shock, distinguishing it from non-septic shock patients.

In summary, our study uncovers a unique miRNA profile derived from EVs in post-surgical patients with septic shock compared to those with non-septic shock. Through differential analysis, we identified 30 miRNAs, including six that show potential as biomarkers for early diagnosis of septic shock. Importantly, the combination of three specific miRNAs demonstrates strong promise as complementary and reliable biomarkers for accurately diagnosing sepsis in post-surgical patients experiencing shock.

Abbreviations

AUC	Area Under the Curve
EV	Extracellular vesicle
log2FC	Log2 Fold Change
miRNA	MicroRNA
PLS-DA	Partial Least Squares Discriminant Analysis
ROC	Receiver Operating Characteristic
SDE	Significantly Differentially Expressed

Supplementary Information

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Supplementary material 1.

Supplementary material 2.

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Author contributions
Conceptualization: AG-C, ET. Methodology: AG-C, PM-P, ET. Formal Analysis: AG-C. Investigation: BS-Q, EG-S, LS-dP, AT-V, ML, EG-P, RP-Á, PMP DB, MM-F, RPU, MB-C, RL-H, MFA, RCF, FTC, TA, MH-R. Validation: MST-D, HG-B, MÁJ-S, AF-R, SR. Writing—Original Draft Preparation: AG-C. Writing—Review & Editing: PM-P, MÁJ-S, AF-R, ET. Visualization: AG-C, PM-P, ET. Supervision: PM-P, ET. Project Administration: ET.

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Availability of data and materials
Sequence data that support the findings of this study have been deposited in BioStudies with the primary accession code S-BSST1230.

Declarations

Ethics approval and consent to participate
The study was conducted in adherence to the ethical principles of the Declaration of Helsinki of the World Medical Association. It was approved by the Scientific Committee for Clinical Research of Hospital Clínico Universitario de Valladolid (PI-18-972) and the Ethics Committee for Clinical Research of Hospital Universitario de Toledo (CEIC-466-2019). Written informed consent was obtained from each patient or their legal representative before their involvement.

Consent for publication
All authors have read the manuscript and have given their consent for its publication.

Competing interests
The authors declare no competing interests.

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