

# Influence of extracorporeal membrane oxygenation on serum microRNA expression

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## Abstract

**Objective:** To date, no biomarkers have been established to predict haematological complications and outcomes of extracorporeal membrane oxygenation (ECMO). The aim of this study was to investigate the expression of a panel of microRNAs (miRNAs), which are promising biomarkers in many clinical fields, in patients before and after initiating ECMO.

**Methods:** Serum miRNA levels from 14 patients hospitalized for acute respiratory failure and supported with ECMO in our medical intensive care unit were analysed before and 24 hours after ECMO. In total, 179 serum-enriched miRNAs were profiled by using a real-time PCR panel. For validation, differentially expressed miRNAs were individually quantified with conventional real-time quantitative PCR at 0, 24, and 72 hours.

**Results:** Under ECMO support, platelet count significantly decreased by  $65 \times 10^3/\mu\text{L}$  (25th percentile =  $154.3 \times 10^3/\mu\text{L}$ ; 75th percentile =  $33 \times 10^3/\mu\text{L}$ ). Expression of the 179 miRNAs investigated in this study did not change significantly throughout the observational period.

**Conclusions:** According to our data, the expression of serum miRNAs was not altered by ECMO therapy itself. We conclude that ECMO does not limit the application of miRNAs as specific clinical biomarkers for the patients' underlying disease.

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## Keywords

MicroRNA, extracorporeal membrane oxygenation, ECMO, biomarker, platelets, bleeding, thromboembolism

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## Introduction

The use of extracorporeal membrane oxygenation (ECMO) as a bridging therapy in life-threatening pulmonary failure is becoming increasingly widespread. In 2016, 2201 adult respiratory runs were reported, twice as many as in 2012 and 11 times the number of runs performed in 2008.<sup>1</sup> Although evidence of improved survival on ECMO compared with conventional treatment remains unclear,<sup>2,3</sup> it is clear that ECMO is a powerful tool that provides additional time to recovery, decision, or transplantation.

The most common and critical complications during ECMO are bleeding and thromboembolism.<sup>1</sup> Several mechanisms trigger the “precarious balance of haemostasis” observed on ECMO:<sup>4</sup> blood contact with the foreign surfaces of the extracorporeal circuit;<sup>5</sup> shear stress and turbulence, both resulting in endothelial damage; collagen exposure; and recurrent platelet adhesion, activation, and degranulation.<sup>4</sup> Management of bleeding and thrombotic risk in ECMO patients can be very challenging, and no biomarkers are currently available to monitor such common and life-threatening adverse effects.<sup>4,5</sup>

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. After maturation, they bind a specific mRNA in the cytoplasm, finely tuning its availability for translation.<sup>6</sup> Interestingly, miRNAs are present in serum in measurable concentrations as circulating miRNAs,<sup>7</sup> which are protected

from endogenous RNase activity.<sup>8</sup> Because miRNAs have demonstrated disease and tissue specificity,<sup>9</sup> circulating miRNAs might be promising biomarkers for a number of conditions, from cardiovascular disease<sup>6</sup> to tumour detection.<sup>8</sup> Nonetheless, platelets and platelet microvesicles are a major source of miRNAs.<sup>10</sup> There is growing evidence that miRNAs could serve as biomarkers for platelet activation<sup>11</sup> and function,<sup>12</sup> and for platelet-related disorders.<sup>13</sup> Moreover, miRNAs are rapidly released into the circulation after a cellular insult, making them exceptional molecules for monitoring a patient’s clinical status.<sup>14</sup>

We hypothesized that expression of serum miRNAs could be altered by ECMO therapy itself, preventing them from being used as specific biomarkers for the patient’s underlying disease. We therefore screened the expression of 179 circulating miRNAs in serum before the initiation of ECMO and 24 hours afterwards using high-throughput quantitative PCR to identify differentially expressed miRNAs. Thereafter, results of miRNAs with significant fold changes ( $\geq|1.9|$  or expressed only in pre- or post-treatment groups) were individually validated with quantitative real-time PCR (RT-qPCR).

## Material and methods

### Patients

The study included 14 patients supported with veno-venous (VV) ECMO for

high-grade respiratory failure in Freiburg University Hospital between April 2013 and March 2015. Patient characteristics and disease types are summarised in Table 1. Most of our patients had multiple organ failure and 71% had a diagnosed sepsis. Moreover, acute respiratory distress syndrome (ARDS) was found in more than two-thirds of our study participants. Acute kidney disease was, at least at the time of ECMO implantation, also very common, occurring in 79% of our cohort. In 11 of 14 patients, ECMO was used after invasive ventilation showed no clinical improvement.

The inclusion criterion for study subjects was VV ECMO support for high-grade respiratory failure [ratio of fraction of inspired O<sub>2</sub> to partial pressure of O<sub>2</sub> (FiO<sub>2</sub>:PaO<sub>2</sub>) ≤100 mm Hg] for at least 24 hours. Patients <18 or >75 years of age or having known platelet and coagulation disorders or metastatic malignancies were excluded from the study, as these are thought to display factors that may bias the expression of circulating miRNAs.

All runs (ECMO cycles) were performed with Maquet Cardiohelp systems (Maquet Holding B.V. & Co. KG, Rastatt, Germany); Avalon Elite bicaval dual-lumen cannulae (Avalon Laboratories, Rancho Dominguez, CA, USA) were used for cannulation.

Blood samples were collected at three time points: before the start of ECMO (during cannulation; 0 hours) and 24 and 72 hours after ECMO. After centrifugation at 2000 × g for 10 minutes, serum was transferred to a new collection tube and stored at -20°C.

The study protocol and data analysis were approved by the Ethics Committee University of Freiburg (EK-Freiburg 151/14\_161396). Informed consent could not be obtained from the patients because they were unconscious, intubated, or in a comatose state.

### Real-time PCR panel analysis of miRNAs

Exiqon's Serum/Plasma Focus microRNA PCR panels (Exiqon, Vedbaek, Denmark) were used to identify differentially expressed serum miRNAs between pre-ECMO and 24-hour post-ECMO groups. Patients were randomly assigned to three different serum pools. Each pool was assayed before ECMO and 24 hours afterwards.

First, total RNA was extracted from serum using the miRCURY RNA isolation kit-biofluids (Exiqon). Second, cDNA synthesis was performed by using miRCURY LNA Universal RT miRNA PCR (Exiqon). Spike-in controls UniSp2, UniSp4, and UniSp6 were added to control for RNA extraction efficiency and possible cDNA synthesis inhibitors. Spike-in controls UniSp2 and UniSp4 were added before isolating total RNA, and UniSp6 was added before reverse transcription (RT)-PCR to control for RT inhibitors. A total of 179 serum-enriched miRNAs, pre-loaded in panels, were screened by qPCR on the miRNA Ready-to-Use PCR, Serum/Plasma Focus Panel. Real-time PCR was performed in a LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany) in 384-well plates. Raw quantification cycles (Cq) and melting points were determined and analysed using the Roche LC software. Data were normalized to the average of assays detected in all samples (n=6), as this value represented the most stable across all samples as measured by NormFinder software (Molecular Diagnostic Laboratory, Aarhus University Hospital, Denmark, 2004).

Normalized Cq (dCq) from raw data was calculated using the following equation:

$$dCq = \text{global mean in all samples } (n = 6) \\ - \text{assay Cq (sample)}$$

**Table 1.** Main patient characteristics.

No. of patients	14 (100.0%)
Age (years)	58.50 [19.00, 74.00]
Male sex	13 (93%)
Body mass index (kg/m <sup>2</sup> )	24.5 (21.00, 77.00)
SAPS2 Score at 0 hours	48.50 [41.00, 68.00]
SAPS2 Score at 24 hours	48.50 [33.00, 59.00]
SAPS2 Score at 72 hours	44.00 [34.00, 72.00]
CPR	2 (14%)
Known CAD	1 (7%)
COPD	1 (7%)
Liver disease	0
Kidney disease	11 (79%)
Arterial hypertension	6 (43%)
Diabetes	0
Smoker	6 (43%)
Depression	3 (21%)
Cancer	3 (21%)
Haematological malignancies	1 (7%)
Solid tumours	2 (14%)
Urinary tract infections	1 (7%)
Adiposity	3 (21%)
Sepsis	9 (64%)
Hypothyroidism	4 (29%)
Hyperthyroidism	2 (14%)
DIC	2 (14%)
Pneumonia	10 (71%)
ARDS	10 (71%)
Severe hypoxia (FiO <sub>2</sub> :PaO <sub>2</sub> ≤ 100 mm Hg) at 0 hours	10 (71%)
Severe hypoxia (FiO <sub>2</sub> :PaO <sub>2</sub> ≤ 100 mm Hg) at 24 hours	6 (43%)
Severe hypoxia (FiO <sub>2</sub> :PaO <sub>2</sub> ≤ 100 mm Hg) at 72 hours	4 (29%)
WBC count at 0 hours	11.25 [6.41, 18.93]
WBC count at 24 hours	10.38 [9.60, 14.56]
WBC count at 72 hours	15.30 [11.55, 18.24]
Time on ECMO (days)	6.00 [2.00, 13.00]
Invasive ventilation	11 (79%)
Mortality at 168 hours	4 (29%)
Mortality at 30 days	9 (64%)

Continuous variables are displayed as median value (25th to 75th percentile range). Nominal variables are displayed as absolute quantity (percentage).

CPR, cardiopulmonary resuscitation; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; DIC, disseminated intravascular coagulation; ARDS, acute respiratory distress syndrome; ECMO, extracorporeal membrane oxygenation; FiO<sub>2</sub>:PaO<sub>2</sub>, ratio of fraction of inspired O<sub>2</sub> to partial pressure of O<sub>2</sub>; WBC, white blood cells; SAPS2 score, Simplified Acute Physiology 2 score (a clinical marker of disease severity).

A higher dCq value indicates that the assayed miRNA is more abundant in that sample.

Fold change (FC 24-0), reflecting the ratio of expression in the 24-hour post-ECMO group relative to the pre-ECMO group, was obtained using the following formula:  $FC(24-0) = 2^{-ddCq}$ , where  $ddCq = dCq(24 \text{ hours}) - dCq(0 \text{ hours})$ .

### Validation process

The miRNAs that showed significant ( $\alpha = 0.05$ ) differential expression in the profiling stage ( $FC \geq |1.9|$ ) or that were expressed only in pre- or post-treatment pools were individually validated with RT-qPCR.

**RNA extraction.** RNA extraction from serum was performed using the miRNeasy Serum/Plasma Kit (#217184; Qiagen, Hilden, Germany), according to the instructions provided by the manufacturer. The miRNeasy Serum/Plasma Spike-In Control (3.5  $\mu\text{L}$ ) ( $1.6 \times 10^8$  copies of cel-miR-39/ $\mu\text{L}$  working solution, 5'-UCACCGGGUGUAAAU CAGCUUG-3', #219610; Qiagen) was added to normalize data to an exogenous reference gene. Total RNA was eluted with 50  $\mu\text{L}$  of RNase-free water and stored at  $-80^\circ\text{C}$ .

**RT-qPCR.** Total RNA was reverse-transcribed in 15- $\mu\text{L}$  reactions containing 5  $\mu\text{L}$  of extracted serum RNA and 4.16  $\mu\text{L}$  of nuclease-free water, 1.50  $\mu\text{L}$  of  $10\times$  RT buffer, 0.15  $\mu\text{L}$  of 100 nM dNTPs, 0.19  $\mu\text{L}$  of RNase inhibitor, 1.00  $\mu\text{L}$  of multi-scribe reverse transcriptase 50 U/L (TaqMan MicroRNA Reverse Transcription Kit, #4366596; Applied Biosystems Inc., Foster City, CA), and 3.00  $\mu\text{L}$  of specific RT primer (#000200, #002304, #000389, #002216, #002173, #002435, #002380; Applied Biosystems Inc.). The RT cycles were performed on a Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules,

CA, USA) under the following thermal protocol: 30 minutes at  $16^\circ\text{C}$ , 30 minutes at  $42^\circ\text{C}$ , 5 min at  $85^\circ\text{C}$ , and hold at  $4^\circ\text{C}$ .

Real-time quantitative PCR was performed in a 20- $\mu\text{L}$  reaction mix, containing 1.33  $\mu\text{L}$  of RT product, 7.67  $\mu\text{L}$  of nuclease-free water, 10.00  $\mu\text{L}$  of TaqMan  $2\times$  Universal Master-Mix, and 1.00  $\mu\text{L}$  of specific  $20\times$  TaqMan miRNA primer (Applied Biosystems Inc.). Each sample was assayed in duplicates. The following thermal protocol was used: 10 minutes at  $95^\circ\text{C}$  and 40 cycles of 15 s at  $95^\circ\text{C}$ , 60 s at  $60^\circ\text{C}$ , and hold at  $4^\circ\text{C}$ .

The dCq values were calculated with the following formula:

$$dCq = \text{mean } Cq \text{ sample} - (\text{SpikeIn\_Average\_Cq} - \text{SpikeIn\_Median\_Cq})$$

where mean Cq sample represents the average Cq between duplicates of miRNAs in that sample; mean SpikeIn\_Average\_Cq represents the mean between the Cq values obtained in two independent quantifications of spike-in control in that sample; and SpikeIn\_Median\_Cq refers to the median of SpikeIn\_Average\_Cq values obtained from all the samples.

FC (24-0) and FC (72-0) were calculated as described previously for the profiling stage.

### Statistical analysis

In the profiling stage, data were normally distributed (graphically and after Shapiro-Wilk normality test). Thus, Student's paired *t*-tests were performed. In the validation stage, because the sample number was contained and the frequency distributions of data did not resemble Gaussian curves, non-parametrical approaches were preferred. Hence, we performed a repeated-measurement analysis of variance using the Friedman test and expressed measures as median  $\pm$  range. When the Friedman test

**Table 2.** Validated microRNAs.

MiRNA Name	FC 24 hours/0 hours (Exiqon Gene Array)	FC 24 hours/0 hours (RTqPCR)	FC 72 hours/0 hours (RTqPCR)
hsa-let-7e-5p	2.3 ( $P = 0.04$ )*	-1.9 ( $P = 0.63$ )	-2.0 ( $P = 0.63$ )
hsa-miR-199a-3p	-2.4 ( $P = 0.03$ )*	-2.4 ( $P = 0.16$ )	-2.1 ( $P = 0.16$ )
hsa-miR-15a-5p	-1.9 ( $P = 0.04$ )*	-1.6 ( $P = 0.73$ )	-1.4 ( $P = 0.73$ )
hsa-miR-106b-5p	On at 0 hours <sup>†</sup>	-2.2 ( $P = 0.47$ )	-3.1 ( $P = 0.47$ )
hsa-miR-15b-5p	On at 0 hours <sup>†</sup>	-1.4 ( $P > 0.99$ )	-1.6 ( $P > 0.99$ )
hsa-miR-501-3p	On at 0 hours <sup>†</sup>	-1.9 ( $P = 0.84$ )	-2.1 ( $P = 0.84$ )
hsa-miR-128-3p	On at 24 hours <sup>†</sup>	-1.7 ( $P = 0.73$ )	-2.0 ( $P = 0.73$ )

Comparison between profiled fold change (FC) (high-throughput qPCR) and validation RT-qPCR fold change.

\*No value passed the Benjamini-Hochberg correction at a significance level of  $\alpha = 0.05$ .

<sup>†</sup>MicroRNAs hsa-miR-106b-5p, hsa-miR-15b-5p, hsa-miR-501-3p, hsa-miR-128-3p were detected by gene array either before (on at 0h) or after (on at 24h) extracorporeal membrane oxygenation.

showed significance, Dunn's multiple comparison post-test was used. As this was an explorative study, accurate descriptions of quantitative data using medians and FCs were preferred over statistical tests and  $P$ -values. The chosen level of significance was  $\alpha = 0.05$ .

As shown in Table 2, the  $P$ -values of Exiqon analyses were calculated through a paired  $t$ -test, whereas the  $P$ -values of the validation stage were calculated through a Friedman test. All analyses were performed using GraphPad Prism (v7.0, GraphPad Software Inc., La Jolla, CA, USA).

## Results

### Patient characteristics

The main characteristics of the study participants are summarised in Table 1. Data are shown as median [25th, 75th percentiles]. Median age was 58.5 years [19.00, 74.00]. Thirteen of the 14 participants were men. Patients received extracorporeal support for severe respiratory failure (71% of patients had  $\text{FiO}_2\text{:PaO}_2 \leq 100$  mm Hg before ECMO); and 71% of patients received ECMO because of ARDS, mostly due to pneumonia. Sepsis was diagnosed in 9 patients (64%). Median ECMO

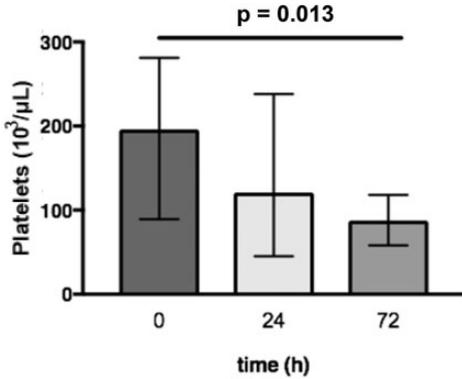
duration was 6 days [2.00, 13.00] and 30-day mortality was 64%. Four patients died within 168 hours and 3 died before 72 hours (serum sampling at 72 hours after ECMO was not possible in these patients).

### Platelets are significantly reduced during ECMO

Median platelet counts at different time points were significantly different in our patients ( $P = 0.01$ ). Dunn's multiple comparison post-test, used to compare each time point, showed a significant decrease between the time points 0 hours ( $196 \times 10^3/\mu\text{L}$ , 25th to 75th percentile range:  $110 \times 10^3/\mu\text{L}$ ,  $263 \times 10^3/\mu\text{L}$ ) and 72 hours ( $87.5 \times 10^3/\mu\text{L}$ , 25th to 75th percentile range:  $60.25 \times 10^3/\mu\text{L}$ ,  $114.3 \times 10^3/\mu\text{L}$ ) (Figure 1). A reduction in platelet number occurred in 12 of 14 (86%) and 8 of 12 (67%) patients between time points 0 and 24 hours and 24 and 72 hours, respectively.

### Screening of miRNAs with high-throughput qPCR

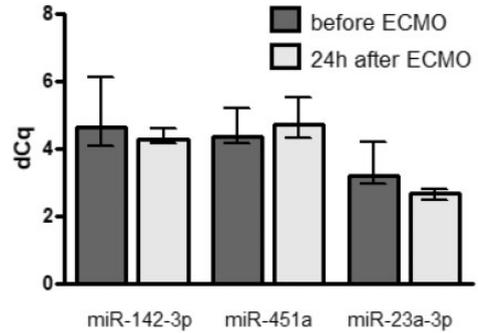
**Haemolysis and quality control.** Quality control and haemolysis-related miRNAs remained stable throughout ECMO. The spike-ins controlling for RNA isolation (UniSp2



**Figure 1.** Platelet count (thousands of cells/μL) during ECMO. Median with range of platelet counts at 0, 24, and 72 hours after ECMO began. During ECMO, the platelet count decreased (*P*-value of Friedman test = 0.01). Dunn’s multiple comparison test showed significance between timepoints 0 and 72 hours (*P* = 0.013). ECMO, extracorporeal membrane oxygenation.

and UniSp4) and for cDNA synthesis (UniSp6) were expressed at steady levels in all pools, indicating that performance of RNA extraction and cDNA synthesis were comparable among different groups. The known stable serum miRNAs (miR-30c and miR-103a), used to evaluate samples’ miRNAs content, showed constant Cq values across all samples (data not shown).

To measure the degree of haemolysis, two miRNAs were quantified and their dCq calculated: miR-23a, which is relatively stable in serum and not affected by haemolysis, and miR-451, which is highly expressed in red blood cells and whose expression is positively correlated with their mechanical rupture. The dCq value was calculated as Cq of miR-23a minus Cq of miR-451. Values >7 indicate that samples are affected by haemolysis.<sup>14</sup> As shown in Figure 2, in our screening samples, miRNAs associated with haemolysis were not significantly altered; the dCq (miR-23a – miR-451) for each pool never exceeded the threshold (data not shown).



**Figure 2.** Median ± range of serum microRNAs highly expressed in thrombocytes (miR-142-3p), erythrocytes (miR-451a), and stable in serum (miR-23a-3p). ECMO, extracorporeal membrane oxygenation; dCq, normalized quantification cycle.

miR-142-3p, which is highly expressed in platelets, did not show differential expression among different pools (Figure 2).

**Expression of miRNAs during ECMO.** On the panel analysis, 179 serum-enriched miRNAs were screened. On average, 135 miRNAs were detected per sample. Heat map and unsupervised hierarchical clustering analyses of all profiled miRNAs did not show clustering in agreement with the groups (pre- vs. post-ECMO) (Appendix Figure 1).

Three miRNAs (hsa-let-7e-5p, hsa-miR-199a-3p, hsa-miR-15a-5p) had FC values beyond threshold (≥|1.9|) and were significantly changed in post-treatment groups (*P* < 0.05). Four miRNAs (hsa-miR-106b-3p, hsa-miR-128-3p, hsa-miR-15b-3p, hsa-miR-501-3p) were found to be expressed only in pre- or post-treatment groups (on/off analysis) and passed a Student’s paired *t*-test at significance level of α = 0.05. None of these 7 miRNAs passed the Benjamini–Hochberg false discovery rate (FDR) test at α = 0.05. The FC values and *P*-values of Student’s paired *t*-tests are shown in Table 2.

### Validation analysis with RT-qPCR

The expression of the seven most interesting miRNAs to this study (hsa-let-7e-5p, hsa-miR-199a-3p, hsa-miR-15a-5p, hsa-miR-106b-3p, hsa-miR-128-3p, hsa-miR-15b-3p, hsa-miR-501-3p) was individually validated in all subjects. Furthermore, to better quantify possible alterations to miRNA expression due to ECMO, observations were extended to a third time point not included in the initial screening (72 hours after ECMO initiation). In Table 2, FC values found after validation are compared with those at the profiling stage. FC (72-0) and Friedman test *P*-values are also reported. Measured FCs at the validation stage ranged between 1.4 (miR-15a-5p) and 3.1 (miR-106-5p). None of the seven validated miRNAs showed significantly different expression between the timepoints evaluated in this study at a confidence level of 95%.

### Discussion

In this pilot study, we screened the expression of 179 serum miRNAs before and after ECMO initiation. To the best of our knowledge, no data regarding miRNA expression under the influence of ECMO can be found in the literature.

ECMO is a powerful bridging therapy that can be used to support both respiratory and haemodynamic functions. Despite ongoing development in the field of ECMO, adverse effects, mostly thrombotic and bleeding complications, remain common.<sup>1</sup> Although anticoagulant prophylaxis is mandatory, it has no ideal monitoring parameter.<sup>15,16</sup> A lack of association between coagulation parameters (partial thromboplastin time, Quicktime, D-dimer, fibrinogen, and antithrombin III) and risk of bleeding during ECMO has been reported.<sup>17</sup> Haemolysis is also a common complication of extracorporeal support,

which can lead to dire clinical outcomes, including renal and multiorgan failure.<sup>5</sup> There is a need for precise and reliable biomarkers by which to monitor ECMO.

MiRNAs are altered in several physiological states and pathological conditions.<sup>18</sup> Circulating miRNAs are highly stable molecules found in peripheral blood and are promising biomarkers in many clinical fields, including cardiovascular disease<sup>6</sup> and tumour detection.<sup>8</sup> Platelets are a major source of miRNAs<sup>10</sup> and there is increasing evidence that a number of miRNAs (e.g., miR-142-3p) take part in many platelet-related processes.<sup>10,12</sup>

In this study, we wanted to investigate whether ECMO directly influenced the expression of circulating miRNAs. We first screened the 179 most-expressed miRNAs in human serum in 14 patients before ECMO and 24 hours after ECMO using high-throughput qPCR. Then, we validated our results by conventional RT-qPCR, adding a third time point (72 hours after ECMO start) to better quantify differences in miRNA expression.

We report four major findings. First, we detected a significant decrease in platelet count during ECMO in study participants (i.e., ECMO-induced thrombocytopenia). Second, when quantifying the 179 most-expressed miRNAs in human serum with high-throughput qPCR before and 24 hours after ECMO, 2 miRNAs were significantly reduced (hsa-miR-199a-3p, hsa-miR-15a-5p) and 1 (hsa-let-7e-5p) was significantly increased. Furthermore, 4 miRNAs (hsa-miR-106b-5p, hsa-miR-15b-5p, hsa-miR-501-3p) were expressed only before ECMO and 1 (hsa-miR-128-3p) only 24 hours after ECMO. However, no screened miRNA passed the Benjamini–Hochberg FDR test for multiple comparisons at a significance level of  $\alpha=0.05$ . Third, after validation with conventional RT-qPCR, including at 72 hours after ECMO start, we detected no significant

differential expression between the time points analysed ( $\alpha=0.05$ ). Finally, platelet- and haemolysis-associated miRNAs (miR-142-3p and miR-451a, respectively) were not altered in our samples.

In our cohort, platelets were significantly decreased during extracorporeal support. ECMO-induced thrombocytopenia is a common and well-described condition, which enhances bleeding risk and raises questions about prophylactic platelet transfusion.<sup>5</sup> Possible causes of thrombocytopenia are consumption within the circuit, consumption microangiopathy, or the patient's underlying disease.<sup>17</sup>

Our major finding is that expression of the miRNAs analysed remained stable during ECMO. The 7 most interesting miRNAs resulting from high-throughput analysis, when individually quantified with RT-qPCR, did not change significantly ( $\alpha=0.05$ ) and demonstrated minimal fold changes (the highest FC was 3.1 for miR-106b-5p at 72 hours). According to the present study, the first to investigate miRNA expression before and after ECMO, extracorporeal treatment appeared not to interfere with miRNA expression. Our results open new perspectives in the application of circulating miRNAs as disease biomarkers in patients on ECMO. A large body of evidence now indicates that miRNAs are attractive candidates by which to monitor clinical conditions that may require extracorporeal treatment (e.g., myocardial infarction and ARDS).<sup>19-21</sup> Thus, miRNAs may serve as valuable prognostic indicators of disease progression or resolution.

MiR-451 is a miRNA contained in red blood cells and its level is correlated with haemolysis;<sup>14,22</sup> it was not significantly altered by ECMO in our samples. Monitoring haemolysis, which is frequently observed during ECMO,<sup>5</sup> is critical when dealing with miRNAs because it is reported to be a significant source of variation in

miRNA profiling.<sup>23</sup> Furthermore, miR-142-3p, highly expressed in platelets and a promising biomarker in coronary artery disease,<sup>10</sup> was not differentially expressed in the two cohorts (pre- and post-treatment groups). There seems to be no relationship between circulatory miR-142-3p expression in serum and the observed thrombocytopenia.

### Limitations

Several limitations must be considered when interpreting our findings. First, the study population was small and within a heterogeneous spectrum of diseases. Furthermore, three patients died during the observation period, meaning that a third blood sample could not be collected at 72 hours from these patients. Moreover, the high-throughput RT-qPCR-based methods used for miRNA profiling are powerful techniques for discovery-phase research, but have a number of limitations; conventional RT-qPCR remains the gold-standard.<sup>24</sup> In this study, we screened a large number of potentially interesting miRNAs; however, to control for sample variance, the data were pooled at the screening stage, a process that makes the analysis less sensitive. Finally, we evaluated miRNA expression in a heterogeneous pool of subjects treated with ECMO without correcting for the adverse effects rate. Analysis of the differential expression of miRNAs in patients with different clinical outcomes, especially thrombosis and bleeding rates, is required.

### Conclusions

In our study, ECMO itself did not influence the expression of the 179 screened miRNAs. We conclude that the application of circulating miRNAs as clinical biomarkers is not limited by ECMO. MiRNAs represent potentially useful biomarkers in patients

on ECMO, both to monitor the underlying conditions that required extracorporeal treatment (especially ARDS, pneumonia, heart disease and shock) and to control ECMO adverse effects. More studies are needed on the expression of specific miRNAs in patients with homogeneous diseases undergoing ECMO and in cohorts with similar ECMO-related adverse effects.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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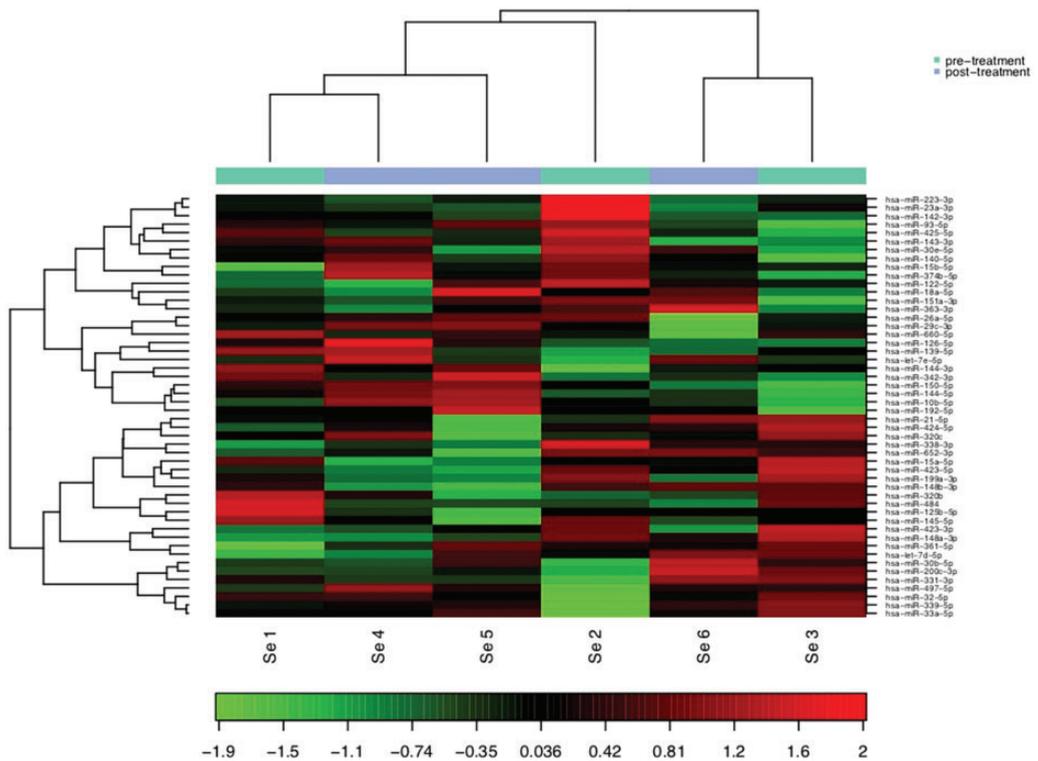
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**Appendix Figure I.** Heat map and unsupervised hierarchical clustering. The top 50 microRNAs with highest standard deviation are listed on the right. Hierarchical clustering was performed in each pool: pre-treatment pools represent pre-ECMO groups (above, green); post-treatment pools represent post-ECMO groups (above, violet). ECMO, extracorporeal membrane oxygenation. Se1 to Se6 represent randomly assigned serum pools 1, 2, and 3 pre- and post-ECMO, respectively.