MicroRNAs as potential biomarkers in congenital heart surgery

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Glossary of Abbreviations

CPB – cardio-pulmonary bypass

CHD – congenital heart disease

CHS – congenital heart surgery

cTns – cardiac troponins

ECMO - extracorporeal membrane oxygenation

ICU – intensive care unit

ILOS – intensive care length of stay

IQR - inter-quartile range;

miR-1 – microRNA-1

miRNA - microRNA

NHS – national health system

PICU – pediatric intensive care unit

RACHS-1 - risk adjustment for CHS

SCE – severe cardiovascular event

VI – ventilation index

VIS – vasoactive-inotrope score

Central picture legend

MicroRNAs could help predicting outcomes after pediatric congenital heart surgery.

Central message

MiR-1 increases in plasma after pediatric cardiac surgery. It is associated with clinical scores indicative of post-operative outcome and predicts intensive care stay and severe cardiovascular events.

Perspective statement

miR-1 is a potential biomarker of cardiac outcomes after pediatric cardiac surgery, a field where accurate predictors are lacking. As opposed to the VIS and VI scores, changes in miR-1 appear immediately after surgery and could signal cardiopulmonary bypass-associated complications.

Abstract

Objective: Pediatric congenital heart surgery (CHS) involves intra-cardiac, valvular and vascular repairs. Accurate tools to aid short-term outcome prediction in pediatric CHS are lacking. Clinical scores, such as the vasoactive-inotrope score (VIS) and ventilation index (VI), are used to define outcome in clinical studies. MicroRNA-1-3p (miR-1) is expressed by both cardiomyocytes and vascular cells and is regulated by hypoxia. In adult patients, miR-1 increases in the circulation after open-heart cardiac surgery, suggesting its potential as a clinical biomarker. Thus, we investigated whether perioperative circulating miR-1 measurements can help predict post-CHS short-term outcomes in pediatric patients.

Methods: Plasma miR-1 was retrospectively measured in a cohort of 199 consecutive pediatric CHS patients (median age 1.2 years). Samples were taken before surgery and at the end of the operation. Plasma miR-1 concentration was measured by RT-qPCR and expressed as miR-1 copies/µl and as relative expression to spiked-in exogenous cel-miR-39.

***Results*:** Baseline plasma miR-1 did not vary across different diagnoses, increased during surgery (204-fold median relative increase, $p<0.001$) and was associated with aortic cross clamp duration post-operatively ($p<0.001$). Importantly, miR-1 levels at the end of the operation positively correlated with intensive care stay ($p<0.001$), early severe cardiovascular events ($p=0.01$), and with high VIS ($p=0.001$) and VI ($p<0.001$), suggesting that miR-1 could accelerate the identification of patients with cardiopulmonary bypass-related ischemic complications, requiring more intensive support.

Conclusions: Our study suggests miR-1 as a novel potential circulating biomarker to predict early post-operative outcome and inform clinical management in pediatric heart surgery.

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Introduction

Congenital heart disease (CHD) affects around 1% of live births.[1] Surgical results in CHD continue to improve, with varying mortality and morbidity based on case complexity.[2] Infants undergoing congenital heart surgery (CHS) with cardiopulmonary-bypass (CPB) are at particularly high risk for postoperative morbidity and mortality,[3] and few accurate predictors of outcomes are currently available beyond clinicians' expert judgment.

MicroRNAs (miRNA, miR) are short, non-coding ribonucleic acid molecules with multiple roles in regulation of cardiovascular developmental and pathological processes, including CHD.[4] miRNAs are released by cells *via* modalities, such as conjugation to lipoproteins or inclusion in extracellular vesicles, which improve their resistance to degradation while circulating in biological fluids. For this reason and for their relative cell and organ origin traceability, miRNAs are being considered as candidates for translation into clinical biomarkers.[5] However, to the best of our knowledge, only one study of a small (n=30) CHS population has so far reported expressional changes in circulating miRs and it was developed on samples harvested at 12 hours after surgery, which does not allow for early perioperative prediction of post-surgical outcomes.[6]

miR-1 is expressed prevalently by myocytes,[7–9] but also by vascular cells and smooth muscle cells.[10] Hypoxia modulates miR-1 expression,[11,12] and studies have shown changes in circulating miR-1 in subjects suffering an acute myocardial infarction and in adult patients receiving open-heart surgery and heart failure patients.[9,13–17] We have previously shown that coronary artery-bypass-graft (CABG) performed on CPB dramatically increases miR-1 trafficking from the adult human heart to the peripheral circulation and described the time-course of this phenomenon.[18] Others have showed miR-1 to be increased in the plasma after transcoronary ablation of septal hypertrophy.[19] This would suggest a diagnostic potential of miR-1 in adult cardiac surgery,

however miR-1 has not been investigated in the CHS setting and correlated with post-surgical outcome.

Currently, there are few predictors of post-operative outcomes in the pediatric intensive care unit (PICU), namely the vasoactive-inotrope score (VIS)[3], measuring cardiovascular support; the ventilation index (VI), measuring ventilatory support[20], and a composite score adding renal parameters to VIS and VI.[21] These scores, predominantly used for research purposes, have shown good correlation with the PICU length of stay, but offer little insight into the causes underlying poor outcomes and are unable to inform treatment strategy[3]. Biomarkers such as cardiac troponins (cTns) and brain natriuretic peptides are routinely used, but early measurements after surgery require repeated sampling and can be unreliable.[22,23] Therefore, there is a need for new biomarkers in pediatric CHS, where many complications are not due to pre-existing ischemic disease, but are presumably secondary to the CPB. miR-1, given its cardiovascular expression and previously documented links to ischemic injury, appears to be a promising potential perioperative predictor of post-CHS outcome.

Methods

A detailed, expanded Methods section has been included in the Online Supplement. All patients were operated in the Bristol Royal Hospital for Children, using standardized cardiopulmonary bypass and myocardial protection, and received perioperative care as per local standard. Our cardioplegia is based on St Thomas's solution and is mixed with blood in a 1:4 ratio. Maintenance doses are given every 20 minutes during mild hypothermic bypass and less frequently at hypothermia.

Study protocol

DECISION (Detection of coagulopathy in pediatric heart surgery) is a prospective observational clinical study of coagulation biomarkers and bleeding outcomes in pediatric cardiac surgery, including intra-cardiac, valvular and vascular repairs for major congenital defects.[24] Citrate plasma samples from blood were taken before the operation and at completion (before chest closure). Informed written parental consent was obtained for all participants for blood sampling and molecular biomarker analysis (Clinical study registration**:** ISRCTN55439761 (UK), National Research Ethics Service Committee reference: 13/LO/0504); all samples were obtained in accordance with the principles of the Declaration of Helsinki.

The current study included the 205 consecutive children enrolled between May 2013 and March 2015. Three patients did not consent for molecular biology analyses and 3 lacked complete clinical data, resulting in a final study group of 199 patients. All operations required CPB, but not all had aortic cross-clamping during CPB.

Clinical, demographic, procedural and outcome data were collected from electronic patient files and PICU charts (Table 1). Mean VIS values in the first 48 hours below the 25th percentile were defined as *low*, over the 75th percentile were defined as *high*, and those in between were defined as *intermediate*. We have chosen to use the mean VIS in the first 48 hours under the rationale that a continuous variable is useable in regression analysis, and it also captures both peak and prolonged support. A limitation of this metric is that it potentially has a lower predictive power. Both maximum VIS value in the first 48 hours[3] and the point value at 48 hours[25] can be used to isolate the "high VIS" group. When considering high VIS as an outcome in the logistical regression analysis, we used both the mean values above the 75th percentile and the high group as defined according to the maximum value,[3] as the two approaches reflect a different pattern of cardiovascular support. Ventilation duration and ventilation index (VI)[20,21] at 24 hours were

calculated to reflect the pulmonary impact; values were defined as *high* above the 75th percentile for both. Procedure complexity was classified using the risk adjustment for CHS (RACHS-1) score.[26]

Cross-clamp times were classified as *absent*, *short* (under 25th percentile), *intermediate* (25th-75th percentiles) and *long* (over 75th percentile). *Prolonged* intensive care length of stay (ILOS) was defined as the duration of over the 75th percentile.

Blood processing, RNA isolation, miRNA-1 measurement.

Venous blood was collected from existing intravascular lines into a citrate tube and processed within 1 hour of collection. Aliquots of citrate plasma were stored at −80°C. In preparation for reverse transcription (RT)-qPCR analyses, total RNA was isolated from whole plasma using the Qiagen miRNeasy kit. To allow for normalization of sample-to-sample variation in the RNA isolation step[27], the exogenous RNA oligonucleotide cel-miR-39 was spiked-in (5 fmol/10 µL) into each denatured sample. The RNA was eluted in RNase-free water and stored at −80°C. Because heparin reportedly inhibits both reverse transcriptase and polymerase and seems to be co-purified with the RNA[28], heparinase I treatment of RNA[18,28,29] was carried out before RT, which was performed using TaqMan miRNA RT kit with miRNA-specific stem loop primers. Standard curves of chemically synthesized RNA oligonucleotides corresponding to miR-1 and cel-miR-39 were created. The RT-qPCR reactions were carried out in duplicate. The raw Cts for miR-1 and cel-miR-39 were first normalized for inter-plate variability by calculating a normalization factor from the calibrator sample run along with the biological samples in each RT-qPCR experiment. Plasma expression of miR-1 was then analyzed in two ways: a) absolute quantification, i.e. plotting Ct values vs copies/µL of the synthetic miRNA in a standard curve obtaining miR-1 expression in copies/µL, b) relative expression to cel-miR-39 using the ΔΔCt method.

Statistical analyses

Continuous variables are presented as median and interquartile range (IQR) as appropriate. Normal distribution of continuous variables was evaluated with the Shapiro-Wilk test. Wilcoxon paired-samples test was used to compare pre- and post-operation miR-1 levels. Relative miR-1 increase is expressed as "n-fold", where *n* is equal to post-operative miR-1 level divided by pre-operative miR-1 level. Differences in miR-1 concentrations and clinical parameters were tested with Mann-Whitney U test or Kruskal-Wallis test, as appropriate, with Dunn's test and Bonferroni adjustment for multiple testing if needed. Linear logistic regression with log transformation of non-normal variables and heteroskedasticity-robust standard errors was used to assess the relationship between pre-operative miR-1 values, post-operative miR-1 values, miR-1 increase, age, cross clamp duration and binomial outcomes based on: prolonged ILOS and severe cardiovascular events (SCE) defined as death in the ICU, cardiac arrest, need for extracorporeal membrane oxygenation or cardio-pulmonary resuscitation, high mean VIS, high maximum VIS, high ventilation duration, high VI. A p value <0.05 indicated statistical significance. Analyses were performed in STATA/IC 11.2 (StataCorp LP, College Station, TX).

Results

Demographic and clinical data are reported in Table 1. The miR-1 values obtained from the two normalization methods correlated well, with $R^2=0.91$ for pre-operative values (Supplemental Figure 1A) and 0.89 for post-operative values (Supplemental Figure 1B). Because of this very good correlation level, the miR-1 level expressed in copies/µL were used for the whole set of analyses.

No significant differences in baseline miR-1 were found with respect to gender or cardiac morphology in subgroups with over 10 patients each (subgroups with fewer than 10 patients were not included in the comparison) (data not shown). Average baseline miR-1 levels were significantly higher in infants when compared to children aged >1 year (1,249 vs 766 copies/µL, $p<0.001$).

Plasma miR-1 increased dramatically during the operation, from a median of 992 (506-2,096) copies/µl at baseline to 224,277 (91,078-571,023) copies/µL ($p<0.001$) post-operatively, resulting in a median relative increase of 204 (89-489)-fold (Figure 1).

Post-operative and perioperative increase in miR-1 inversely correlated with age at operation (coefficient -0.18 and -0.12 respectively, after log transformation; Supplemental Figures 2A and 2B). Supplemental Table 1 shows the post-operative miR-1 levels and the relative miR-1 increase in patients receiving different surgical procedure types. The Fontan operation (rarely performed with cross-clamping), pulmonary valve replacements and subaortic stenosis relief (usually short cross-clamp times) were associated with both a lower miR-1 post-operative level and reduced miR-1 perioperative changes. We did not find significant associations between post-operative miR-1 levels and surgical complexity by RACHS-1 score ($p=0.6$).

Median post-operative miR-1 level (Figure 2A) and miR-1 perioperative expressional changes (fold-increase, Supplemental Figure 3A) were higher in patients with longer cross-clamp ($p<0.001$ for each comparison), with a linear correlation observed between miR-1 levels and cross-clamp duration (Figure 2B and fold-increase in Supplemental Figure 3B), suggesting that the miR-1 release in the peripheral circulation was induced by myocardial or vascular ischemia rather than by the time the patient was connected to the CPB. Numerical values for post-operative miR-1 levels and perioperative changes by cross-clamp duration are shown in Supplemental Table 2.

We found a correlation between muscular incision or resection and post-operative miR-1 levels ($p<0.001$). Moreover, muscular incision/resection correlated with the length of cross-clamping ($p<0.001$). Sensitivity analysis shows that the same trend seen in figures 2A and 2B is maintained when tested in subgroups with and without muscular incision/resection. We did not find significant associations between post-operative miR-1 levels and bypass temperature ($p=0.3$), pre-operative oxygen saturation <95% ($p=0.1$) or post-operative oxygen saturation <95% ($p=0.6$). We did find significantly higher postoperative miR-1 levels in patients with abnormal preoperative saturation versus normal saturation, but only in the subgroup with long cross-clamp duration (194,292 miR-1 copies/nL vs 544,682 miR-1 copies/nL, $p=0.01$). We did not observe higher values in those with postoperative cyanosis, either by relation to the oxygen saturation or surgical procedure (e.g. shunt, pulmonary artery band). No correlation was found between postoperative miR-1 and the need for circulatory arrest ($p=0.6$) or overall duration of CPB ($p=0.7$, Supplemental Table 3). Median post-operative miR-1 was higher in those patients with prolonged ILOS (Figure 3A) and with post-operative severe cardiovascular events (SCE, Figure 3B). The miR-1 post-operative increase from baseline was higher in patients with prolonged ILOS (Supplemental Figure 4A) but not in those with SCE (Supplemental Figure 4B). There were 4 deaths; two neonates and two infants. Two had single ventricle physiology, one had AVSD-ToF and one had common arterial trunk. Three of them had urgent interventions and all four cases were complicated by postoperative organ failure. Patients with similar diagnoses and complications were also found among those who did not die.

We finally analyzed how miR-1 compares with current predictors of acute outcome in pediatric congenital heart surgery, to address whether perioperative miR-1 measurements can accelerate the prediction of outcome, informing patient care. Median post-operative miR-1 was higher in patients with high and intermediate VIS compared to low VIS (Figure 4A). Moreover, post-operative miR-

1 was higher in the high VI group compared to the intermediate and low VI groups (Figure 4B). In line with that, the perioperative changes in circulating miR-1 were higher in patients with high and intermediate VIS and high VI in comparisons with the other groups (Supplemental Figure 4C and 4D). Table 2 reports the numerical values detailing changes in miR-1 by ILOS and SCE groups and by VIS and VI groups.

Details on univariable and multivariable analyses are presented in Table 3. In brief, in univariable analyses, post-operative miR-1, relative miR-1 increase, age and cross-clamp duration were all predictors of prolonged ILOS, VIS and VI. Only post-operative miR-1 and cross-clamp duration were predictors of severe cardiovascular events. In multivariable analysis only age and cross-clamp duration were associated with prolonged ILOS, VIS and VI. We had only 8 SCE events, therefore, we could not perform multivariable analysis for SCE.

Discussion

This study measured miR-1 levels in 199 pediatric patients undergoing CHS and correlated miR-1 to post-CHS outcomes. A dramatic increase in circulating miR-1 was observed immediately after CHS, when patients are still in the operating theatre, with higher post-operative miR-1 values in patients with prolonged cross-clamp times. Perioperative changes in miR-1 positively correlate with PICU length-of-stay and the post-operative occurrence of severe cardiac events. Circulating miR-1 levels at the end of the operation correlated with the VIS and VI, further suggesting the potential of miR-1 to be developed into a novel, minimally invasive laboratory biomarker suitable for early prediction of post-CHS complications related to ischemia due to CPB, if validated in further *ad hoc* designed studies, aiding in risk stratification and targeting interventions immediately after CHS completion. The value of miRs as peri-operative predictive biomarkers to

guide clinical management of surgical patients would require a rapid turnaround of laboratory results. Recent advances in molecular biology techniques show much promise in obtaining faster miRNA results, which is vital for clinical translation. In addition to digital PCR, an established technique known to be highly accurate, further simplifications of the traditional RT-qPCR approach abound, obviating the need for laborious RNA purification, reverse transcription and even PCR amplification itself. Alternative techniques such as flow cytometry are also showing promising development.[30–32] The identification of the value of miRs as early prognostic biomarkers after surgical interventions could attract investment, further fueling the progression of technological advancements to obtain very fast results for miR expression analysis. CPB-related ischemic injury is an important cause of intensive care unit deaths and severe complications in pediatric cardiac surgery, with reduction of CPB ischemic times being advised.[22] Thus, identifying potential markers and new pathogenic pathways could lead to better post-operative outcome through identifying patients at risk. Circulating miR-1 was higher after procedures requiring cross-clamping of the aorta and it correlated with cross-clamp duration and age at operation, but not with total CPB duration or the need for circulatory arrest. This suggests that plasma miR-1 could increase as a result of either myocardial ischemia or ischemia/reperfusion injury (which is itself dependent on the ischemic time).[33]

In patients undergoing ventriculotomy, the higher circulating level of miR-1 could derive from additional release from myocytes affected by the incision, but could also be a simple reflection of higher miR-1 release from the overall myocardium affected by ischemia/reperfusion response due to increased cross-clamp time, as indicated by *ad hoc* sensitivity analyses. This is of relevance because CPB can damage the heart not only *via* inflicting hypoxia/reoxygenation stress to the cardiomyocytes, but also by eliciting inflammatory responses.[34] Indeed, one of the strengths of our

study is that patients undergoing various CHS procedures were included, allowing a comparison between subgroups based on cross-clamping length, including a group of patients not requiring cross-clamping. We propose that a circulating biomarker that can be of use in a variety of procedures has a significant potential to be adopted into clinical practice. Therefore, the correlation between miR-1 and clinical outcome observed in a diversity of operations increases our confidence that miRNA biomarkers could progress to clinical translation in CHS. Cross-clamp time itself provides a prognostic indication that is of value for the clinical team. Nonetheless, a laboratory biomarker enabling summing up of the information derived from considering multiple clinical parameters could significantly simplify evidence-based medical decisions, offering advantages in terms of precision and ease of standardization. Additionally, miR-1 is very highly enriched in cardiac muscle, while it is undetectable in platelets[35] and is dramatically reduced in blood cells following myocardial ischemia.[36] Taken together, the above suggest that circulating miR-1 increases independently of inflammatory responses to surgery. Therefore, post-operative miR-1 levels could exquisitely indicate the level of myocardial injury and the possibility for post-operative severe cardiovascular events. Due to the limited number of SCE in our study population, we could not determine if miR-1 was an independent predictor for cardiac events. Nonetheless, we found post-operative miR-1 significantly increased in the SCE cases.

Early neonatal mortality after CHS is still significant (5-10%).[22] Laboratory-based biomarkers could improve both clinical care and clinical research in the CHS setting. Pediatric intensive care practitioners have developed "inotropic scores" to identify and quantify clinical factors from the early post-operative period indicative of illness severity and short-term outcome. However, inotropic scores present limitations: selection and titration of inotropic support is largely driven by institutional and even individual experience and practice algorithms[22]; VIS is calculated in the first

24-48 hours from arrival to PICU, which is quite a long period of time in the acute post-operative setting, and inotropic scores do little to discern whether the poor outcome is cardiac or extra-cardiac in nature. Consequently, although possibly the best currently available predictor, VIS is difficult to translate into a biomarker able to assist clinical management. Other biomarker candidates, such as cTns and brain natriuretic peptides have been associated with early post-CHS outcomes 24 hours after surgery completion. Early cTn increase is common, but it is better associated with surgical gestures, especially intraoperative myocardial incisions, rather than with future complications. Additionally, sustained high values in repeated measurements are required to offer any clinical insight. Moreover, circulating levels of cTns after CHS can be affected by the anesthetic regimen, thus complicating the interpretation of laboratory results.[37] An additional issue in using cTns to predict outcome in CHS relates to the fact that some CHD cases have significant circulating level of anti-troponin auto-antibodies already before surgery.[38] Indeed, autoantibodies vs cTns have been shown to interfere with cTn detection.[39]

The physio-pathological roles of miR-1, particularly in ischemic cardiovascular disease are only partly known.[11,12,15,18] It is possible that the early circulating changes in miR-1 observed after CHS are associated with increased release of miR-1 from the producing cells. This could impact on the gene expression and hence the biology of the parent cells, but also on potential recipient cells at neighboring or more distant sites. Should this be the case, miR-1 could represent a functional biomarker and a potential therapeutic target alike. As is the case with any new potential biomarker, further research is required to confirm the value and precise role of miR-1 in practice. At the level of clinical research, miR-1 could be useful to assess novel myocardial protection and perfusion strategies. This could indirectly reflect in improving the clinical practice. This will have to be further investigated through translational studies, which are beyond the scope of this report.

Limitations

This is a retrospective study and the original prospective observational clinical study protocol did not include the direct measurement of post-operative cardiac function, such as by echocardiography, the collection of serial blood samples and measurement of cTn. Consequently, we were prevented from studying late post-operative dynamics of circulating miR-1 and correlating miR-1 levels with functional cardiac endpoints or an established cardiac injury marker. However, we would not expect cTns to be elevated at the very early post-operative time point analyzed here. Additionally, the mechanism for organ failure can be multifactorial and lead to poor correlation with just one biological marker.

Both ventilation and vasoactive-inotropic support are dependent on clinical state and clinical decision at that time, resulting in significant expected variability not directly related to actual myocardial, vascular or pulmonary injury.

The study group was not large enough to use multivariate analyses to validate the correlation between circulating miR-1 and significant cardiac adverse events. The data observed here require further validation in different CHS cohorts. We have not measured additional cardiovascular-enriched miRs, which, when combined with miR-1, could improve the predictive value.

Conclusions

Translational research in the cardiac surgery setting is still under-developed.[40] This is particularly true in pediatric CHS, due to intrinsic difficulties linked to patient consent and to the limited amount of blood and tissue that can be sampled. Investigations into minimally invasive circulating molecular biomarkers measurable in small volumes of biological fluids through standardized protocols are of utmost importance. We have provided the first evidence that miR-1 could serve as a novel circulating biomarker and is linked to cardiopulmonary bypass-related injury. The

correlation of miR-1 with the need for more cardiac and circulatory support, longer and more intensive ventilation and severe cardiovascular events after pediatric open-heart surgery demonstrates it has the potential to become a new biomarker in the early post-operative period.

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Figures

Figure 1

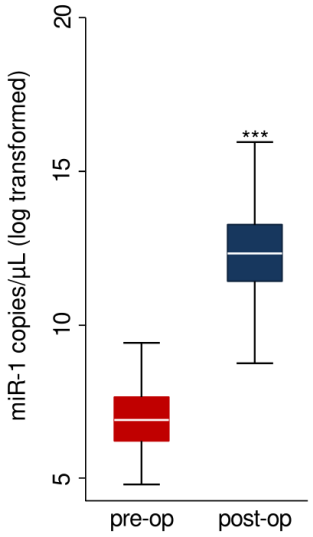

Figure 2

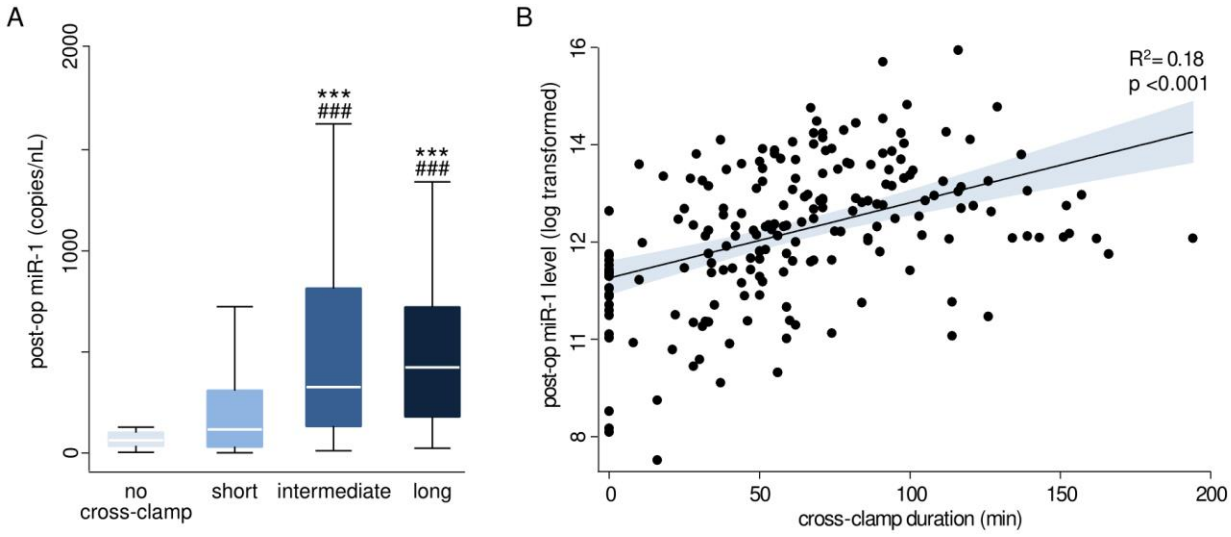

Figure 3

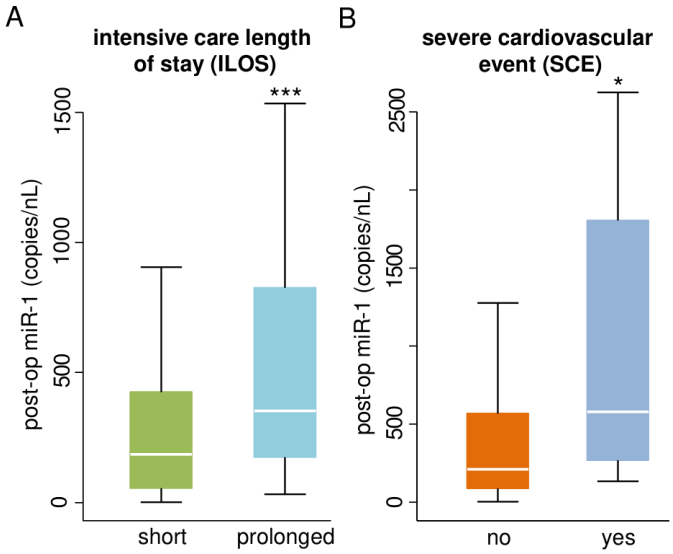

Figure 4

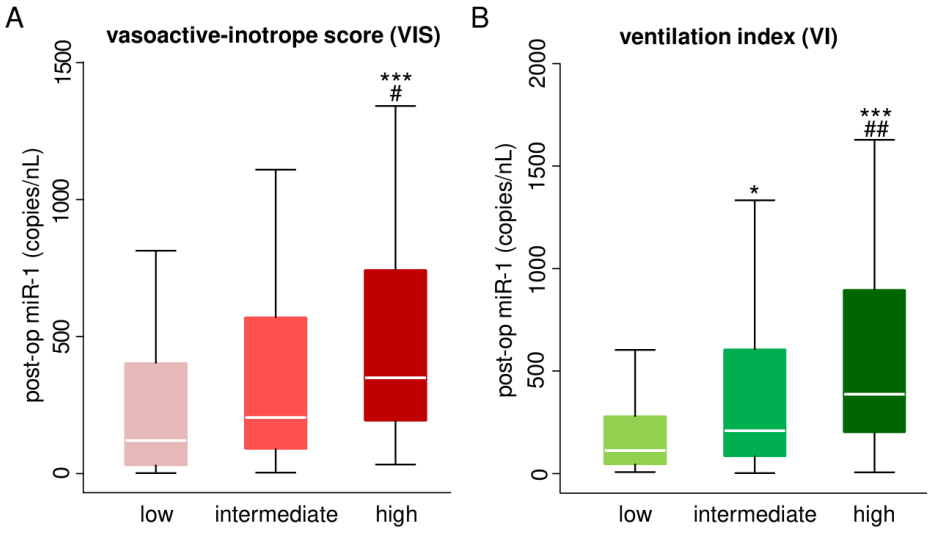

Figure legends

Figure 1. Box plot showing pre-operative and post-operative miR-1 levels (copies/µL) after congenital heart surgery. Plasma miR-1 median relative increase of 204 fold. Log transformation was used to allow for large differences to be showed graphically. ***p<0.001 (n=199), Wilcoxon matched pairs test. Middle line represents the median value. The upper and lower borders of the

box represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values of non-outliers. Outliers not shown.

Figure 2. A. Box plot showing the post-operative miR-1 (copies/nL) by cross-clamp duration after congenital heart surgery. Patients with long (>92 min) and intermediate (45-92 min) cross-clamp duration had higher post-operative miR-1 levels when compared to low duration (<45 min) and absent cross-clamp.***p<0.001 vs no cross-clamp, ###p<0.001 vs short cross-clamp, p=0.1 for comparison between short cross-clamp vs no cross-clamp, Kruskal-Wallis test with Dunn *post hoc* test. Figure elements as described in figure 1 legend. **B.** Scatter plot with linear regression line showing the correlation between duration of cross-clamping and the post-operative miR-1 relative increase (vs baseline).

Figure 3. A. Post-operative miR-1 (copies/nL) after congenital heart disease surgery in 199 children, by intensive care length of stay (ILOS): prolonged ILOS (≥4 days) compared to short ILOS (<4 days). ***p<0.001, Dunn multiple comparison test with Bonferroni adjustment for multiple testing. **B.** Post-operative miR-1 (copies/nL) in patients with severe cardiovascular events (SCE, n=8) and those without SCE (n=191) during the intensive care stay. *p<0.05, Dunn multiple comparison test with Bonferroni adjustment for multiple testing. Figure elements as described in figure 1 legend.

Figure 4. A. Post-operative miR-1 (copies/nL) after congenital heart disease surgery in 199 children, by mean vasoactive inotrope score (VIS) group: low (<2.2), intermediate (2.2-6.7) and high (>6.7) mean VIS. ***p<0.001 vs low VIS, $^{\#}$p<0.05 vs intermediate VIS, Dunn multiple

comparison test with Bonferroni adjustment for multiple testing. **B:** Post-operative miR-1 copies/nL by ventilator index (VI) group: low (<2.97), intermediate (2.97-9.85) and high (>9.85) VI. *p<0.05 and ***p<0.001 vs low VI; $^{\#\#}$p <0.01 vs intermediate VI, Dunn multiple comparison test with Bonferroni adjustment for multiple testing. Figure elements as described in figure 1 legend.

Table 1. Demographic, clinical, procedure-related and outcomes in the PICU data

	Total n=199
Age, years (median, IQR)	1.2 (0.4-5.7)
Weight, kg (median, IQR)	8.5 (5.7-19.4)
Male (n, %)	103 (51.8)
Main diagnoses	
Ventricular septal defect	40 (20.1)
Tetralogy of Fallot	31 (15.6)
Double outlet right ventricle	13 (6.5)
Atrioventricular septal defect – complete	12 (6)
Subaortic stenosis	12 (6)
Pulmonary atresia	11 (5.5)
Transposition with septal defect	10 (5)
Non-cardiac comorbidities	88 (44.2)
Main procedures	
Ventricular septal defect closure	38 (19.1)
Tetralogy of Fallot repair	22 (11.6)
Pulmonary valve replacement	17 (8.5)
Fontan operation	14 (7.0)
Subaortic stenosis relief	12 (6.0)
Glenn operation	11 (5.5)
Atrioventricular septal defect complete repair	10 (5)

Redo operation (n, %)	89 (44.7)
Operation complexity by RACHS-1 score (n, %)	
1 (least complex)	7 (3.6)
2	128 (65.3)
3	42 (21.4)
4	15 (7.7)
5	0 (0)
6 (most complex)	4 (2.0)
Unclassifiable	3 (1.0)
Pre-operative oxygen saturation <95%	68 (34)
Bypass duration, min (median, IQR)	91 (68-122)
Cross clamp used (n, %)	173 (86.9)
Cross clamp time, min (median, IQR)	65 (45-92)
Circulatory arrest needed	6 (3)
Bypass temperature*	34 (32-35)
Muscular incision/resection	64 (32)
Intubation, hours (median, IQR)	16 (5-30)
ILOS, days (median, IQR)	2 (1-4)
Intubation time, hours (median, IQR)	16 (5-31)
Mean 48 hours VIS (median, IQR)	3.8 (2.3-6.5)
VI at 24 hours (median, IQR)	6.3 (2.7-9.8)
Post-operative oxygen saturation <95%	16 (8)
Cardiovascular event	8 (5.0)

Death (n, %)	4 (2.0)
ECMO (n, %)	2 (1.0)
Resuscitated arrest (n, %)	4 (2.0)
Dialysis (n, %)	14 (7.0)

ECMO, extracorporeal membrane oxygenation; IQR, inter-quartile range; *due to anonymized data from a randomized trial, temperature data were missing in 90 patients (45%).

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Table 2. Post-operative miR-1 absolute values and relative increase by mean 48 hours VIS, VI at 24 hours and intensive care outcomes

	Post-op miR-1 (copies/nL)		Fold miR-1 increase	
Mean 48 hours VIS				
Low mean VIS	120.4 (31.6- 400.8)		106 (20-261)	
Intermediate mean VIS	204.6 (91.8- 568.1)	p<0.001	236 (116-504)	p=0.001
High mean VIS	349.6 (194.2- 740.8)		272 (136-479)	
VI at 24 hours				
Low VI	112 (47.6- 276.7)		124 (27-204)	
Intermediate VI	209.1 (86.9- 602.3)	p<0.001	217 (98-503)	p<0.001
High VI	386.4 (203.5- 893)		312 (165-651)	
Prolonged ILOS				
Yes	352.5 (174.6-825.7)		302 (158-568)	
No	185.4 (54.8-424.2)	p<0.001	171 (51-401)	p=0.003
Severe cardiovascular event				
Yes (n=8)	578.2 (267.1- 1804.2)	p=0.02	335 (143-529)	p=0.3
No (n=191)	211.2 (88-567.2)		201 (88-488)	

All values are presented as median (interquartile range). Shown are overall comparison p values from the Kruskal-Wallis test.

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Table 3. Univariable and multivariable regression of predictors for pediatric intensive care outcomes

	High mean VIS		High maximum VIS		Long ventilation		High VI		Long ILOS		Severe cardiovascular event	
	OR	p value	OR	p value	OR	p value	OR	p value	OR	p value	OR	p value
Univariable analysis												
Relative miR-1 increase (log)	1.25	0.061	1.29	0.21	1.69	<0.001	1.46	0.003	1.42	0.002	1.46	0.15
Post-op miR-1 value (log)	1.59	0.001	1.53	0.06	2.19	<0.001	1.70	<0.001	1.68	<0.001	2.18	0.01
Age (years)	0.8	0.001	0.92	0.32	0.76	<0.001	0.83	0.002	0.85	0.001	0.84	0.23
Cross-clamp duration (minutes)	1.01	<0.001	1.03	<0.001	1.02	<0.001	1.01	0.003	1.01	<0.001	1.02	0.01
Multivariable analysis												
Relative miR-1 increase (log)	0.77	0.19	0.74	0.39	1.08	0.7	1.0	0.96	0.99	0.97		

Post-op miR-1 value (log)	1.14	0.55	1.23	0.61	1.39	0.17	1.25	0.31	1.26	0.25
Age (years)	0.75	0.002	0.73	0.05	0.76	0.005	0.84	0.03	0.88	0.03
Cross-clamp duration (minutes)	1.02	<0.01	1.04	<0.01	1.02	0.001	1.01	0.05	1.01	0.01

OR, odds ratio;

Outcome was defined as VIS, ventilation length, VI or ILOS above the 75th percentile.

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SUPPLEMENTAL MATERIAL

Detailed methods

Blood collection and plasma storage

Venous blood was collected from existing intravascular lines into a citrate tube and processed within 1 hour of collection by centrifugation (1,300 *g* at 4°C) for 10 min. Plasma was aliquoted in RNase-free 1.5 mL tubes and stored at -80°C.

RNA extraction and heparinase I treatment

In preparation for RT-qPCR analyses, total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations for biological fluids. For RNA extraction from plasma, 0.5 mL of QIAzol was added to 100 µL of plasma or 100 µL purified EVs. To allow for normalization of sample-to-sample variation in the RNA isolation step,[1] the synthetic RNA oligonucleotide cel-miR-39 (Qiagen, Valencia, CA), which is identical to the mature *Caenorhabditis elegans* miRNA cel-miR-39-3p, was spiked-in to each denatured sample at 10 µL (from a 5 fmol/µL stock). The RNA was eluted in 25 µL of RNase-free water and stored at −80°C. Heparin is known to inhibit both reverse transcriptase and polymerase[2] and seems to be co-purified with the RNA. Heparinase I treatment of RNA preparations from plasma was therefore carried out before reverse transcription: 1.67 µL of purified RNA was transferred to a microtube containing 0.063 µL RNAse inhibitor, 0.5 µL 10X buffer and 0.075 µL Heparinase I (Sigma H2519, 0.02 U/µL)[3] After incubation for 1 hour at room temperature, the solution was used without further treatment for the RT and qPCR reactions. We have shown previously that heparinase treatment does not affect results of miR PCR analyses in samples not contaminated by heparin.[4]

Measurement of miRNA-1 in plasma by TaqMan RT-qPCR assays

Reverse transcriptions reactions were performed using TaqMan miRNA reverse transcription kit with miRNA-specific stem loop primers (Life Technologies, Paisley, UK) in a small scale 5 µL RT reaction. For generation of standard curves of chemically synthesized RNA, oligonucleotides

corresponding to miRNA-1 and cel-miR-39 (Qiagen, Valencia, CA) were used; varying dilutions of each oligonucleotide were made in RNase free water such that the final input into the RT reaction had a volume of 1.67 µL. RT reactions were carried out on the thermocycler using the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and then hold at 4°C.

The RT-qPCR reactions were performed in duplicate in 10 µL reaction volumes using 0.5 µL of miRNA specific primer (Life Technologies, Paisley, UK), 5 µL of TaqMan gene expression master mix (Life Technologies, Paisley, UK), 3.84 µL nuclease-free H_2O and 0.67 µL of RT product. The RT-qPCR reactions were carried out in duplicate using the following conditions: 95°C for 10 minutes, followed by 95°C for 10 seconds, 60°C for 30 sec and optical read at 70°C for 1 second.

The raw Cts for miR-1 and cel-miR-39 were first normalized for the inter-plate variability by calculating a normalization factor from the calibrator sample run along with the biological samples in each RT-qPCR experiment. Plasma miR-1 expression level were then calculated in two ways: by both absolute quantification, performed by plotting the Ct values vs copies/µL of the synthetic miRNA in a standard curve obtaining the miR-1 expression in copies/µL, and as relative expression to cel-miR-39, by using the ΔΔCt method.

577 **Supplemental tables**

Supplemental Table 1. Post-operative (post-op) miR-1 level and relative increase by main procedure types

	Post-op miR-1 (copies/nL)	Post-op miR-1 relative increase (ratio)
Ventricular septal defect closure n=38	389.7 (225.7- 855.1)	264 (127-761)
Tetralogy of Fallot repair n=22	864.2 (480- 1886.1)	366 (260-819)
Pulmonary valve replacement n=17	54.1 (23.7- 88.6)	96 (22-181)
Fontan operation n=14	72.2 (40071- 92.6)	48 (17-142)
Subaortic stenosis relief n=12	34.1 (16.2- 185.6)	67 (14-235)
Glenn operation n=11	124.2 (55.2- 308.9)	115 (35-407)
Atrioventricular septal defect complete repair n=10	505.9 (304.8- 1113.1)	315 (165-798)

Values are presented as median (interquartile range)

Glenn operation and Fontan operation are 2nd and 3rd stages for single ventricle palliation

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Supplemental Table 2. Post-operative (Post-op) plasma miR-1 level (expressed as copies/nL) and miR-1 changes vs baseline by cross-clamp duration

	Post-op miR-1 level (copies/nL)	p value	Post-op relative miR-1 increase (ratio)	p value
No cross-clamp	63.7 (36.1-101.3)		35 (17-116)	
Short cross-clamp <45 min	117.3 (31.7- 308.8)		122 (33-223)	
Intermediate cross-clamp 45- 92 min	325.9 (134140- 813.3)	p<0.001	300 (129-636)	p<0.001
Long cross-clamp >92 min	424.2 (180.5- 721.3)		307 (162-671)	

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Supplemental Table 3. Multivariable regression model of post-operative (post-op) miR-1 level and relative increase in relation to age, cross-clamp and bypass duration

Post-op miR-1 level			
	Coefficient	Robust SE	p value
Age	-0.17	0.02	<0.001
Cross-clamp duration	0.01	0.003	<0.001
Total bypass duration	0.001	0.002	0.7
constant	11.99	0.2	
R-squared: 0.44 Root MSE: 1.08			
Post-op relative increase of miR-1 (ratio)			
	Coefficient	Robust SE	p value
Age	-0.11	0.02	<0.001
Cross-clamp duration	0.01	0.003	0.003
Total bypass duration	0.002	0.003	0.46
constant	4.71	0.24	
R-squared:0.27 Root MSE: 1.24			

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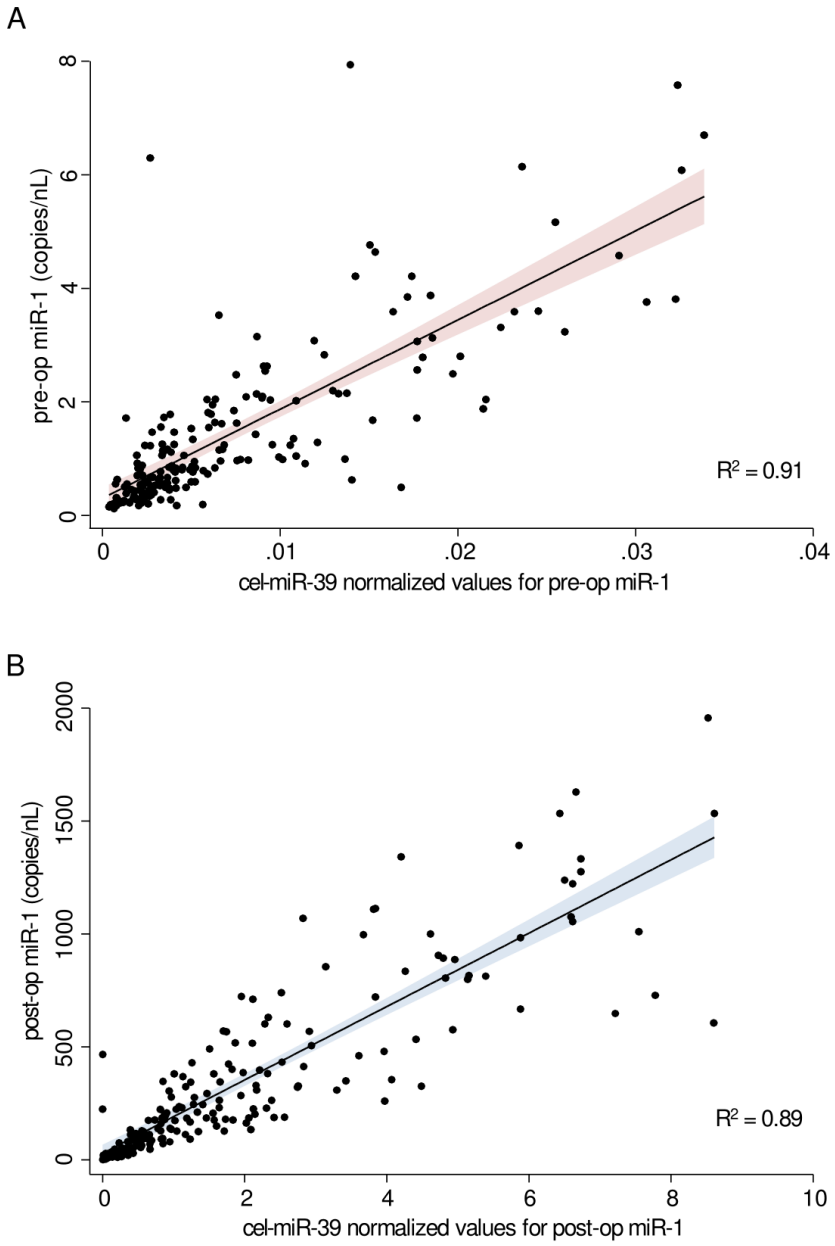
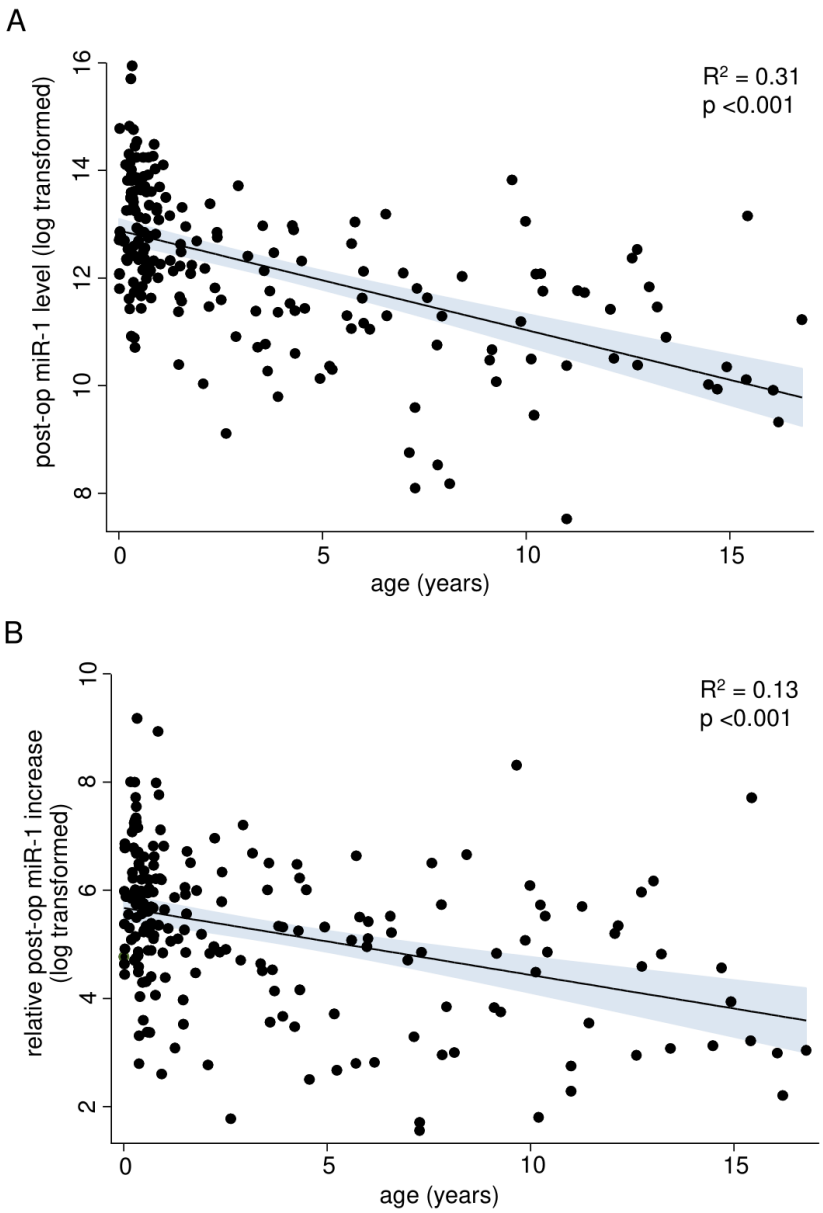
Figures and figure legends



Supplemental Figure 1. Correlation of standardized (expressed as copies/nl) versus normalized (to cel-miR-39) values, showing very good linear correlations before (**A**) and after (**B**) surgery. Values exceeding the 95% percentile were not included in the figure to avoid clutter. Post-operative normalized miR-1 values were significantly higher than the pre-operative values (median of 1.33 vs 0.004, $p<0.001$), with a median fold increase of 271 (103-591).



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593 **Supplemental Figure 2. A.** Scatter plot with linear regression line showing the inverse correlation
594 between the post-operative miR-1 level (expressed as log transformed miR-1 copies/µL) and age.
595 **B**. Scatter plot with linear regression line showing the inverse correlation between the miR-1
596 relative increase and age.

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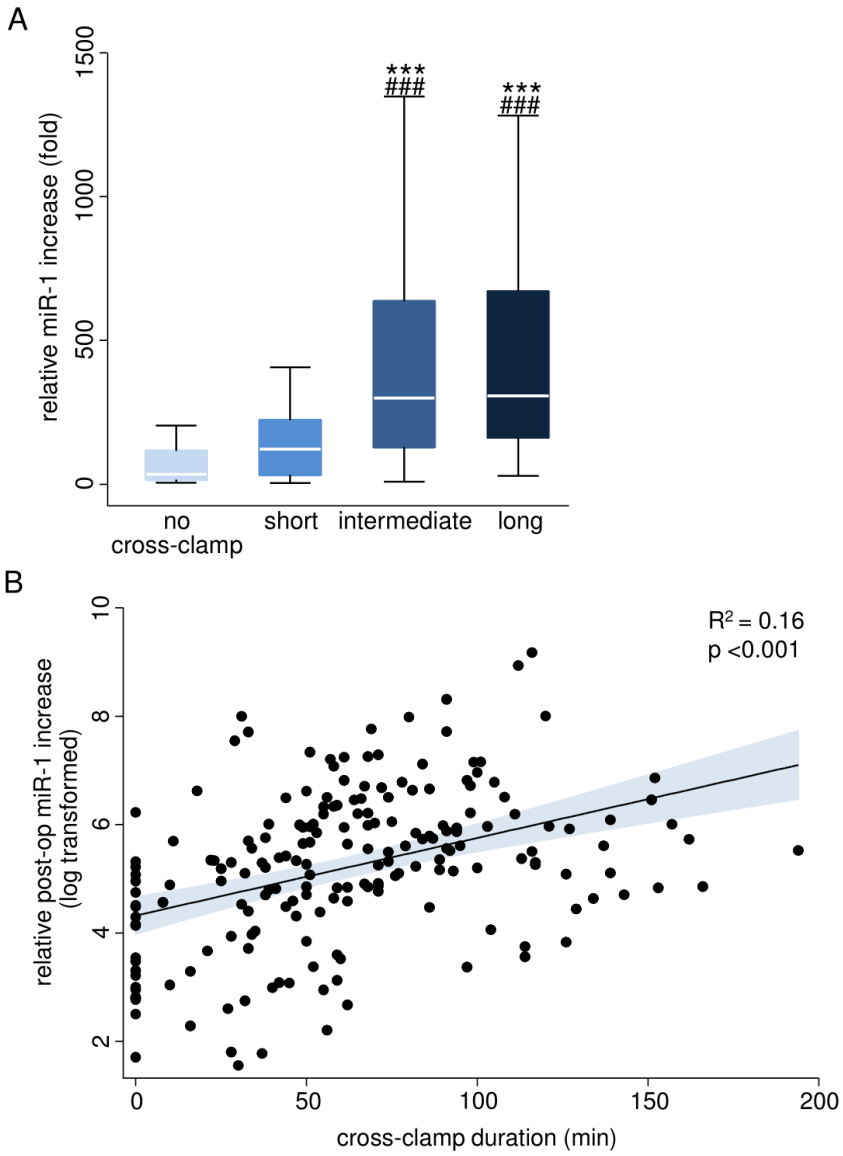

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599 **Supplemental Figure 3. A.** Box plot graph showing post-operative relative miR-1 level increase
600 by cross-clamp duration. Short (duration <45 min), Intermediate (45-92 min) and Long (>92 min)
601 cross-clamp duration. P values are from Kruskal-Wallis with Dunn *post hoc* test, ***p<0.001 vs
602 no cross-clamp, ###p<0.001 vs short cross-clamp, p=0.1 for comparison between short cross-clamp
603 vs no cross-clamp. **B.** Scatter plot with linear regression line showing the correlation between the
604 duration of the cross-clamping and the post-operative miR-1 relative increase (vs baseline). Middle
605 line represents the median value. The upper and lower borders of the box represent the 75th and

606 25th percentiles, respectively. The upper and lower whiskers represent the maximum and
607 minimum values of non-outliers. Outliers not shown.

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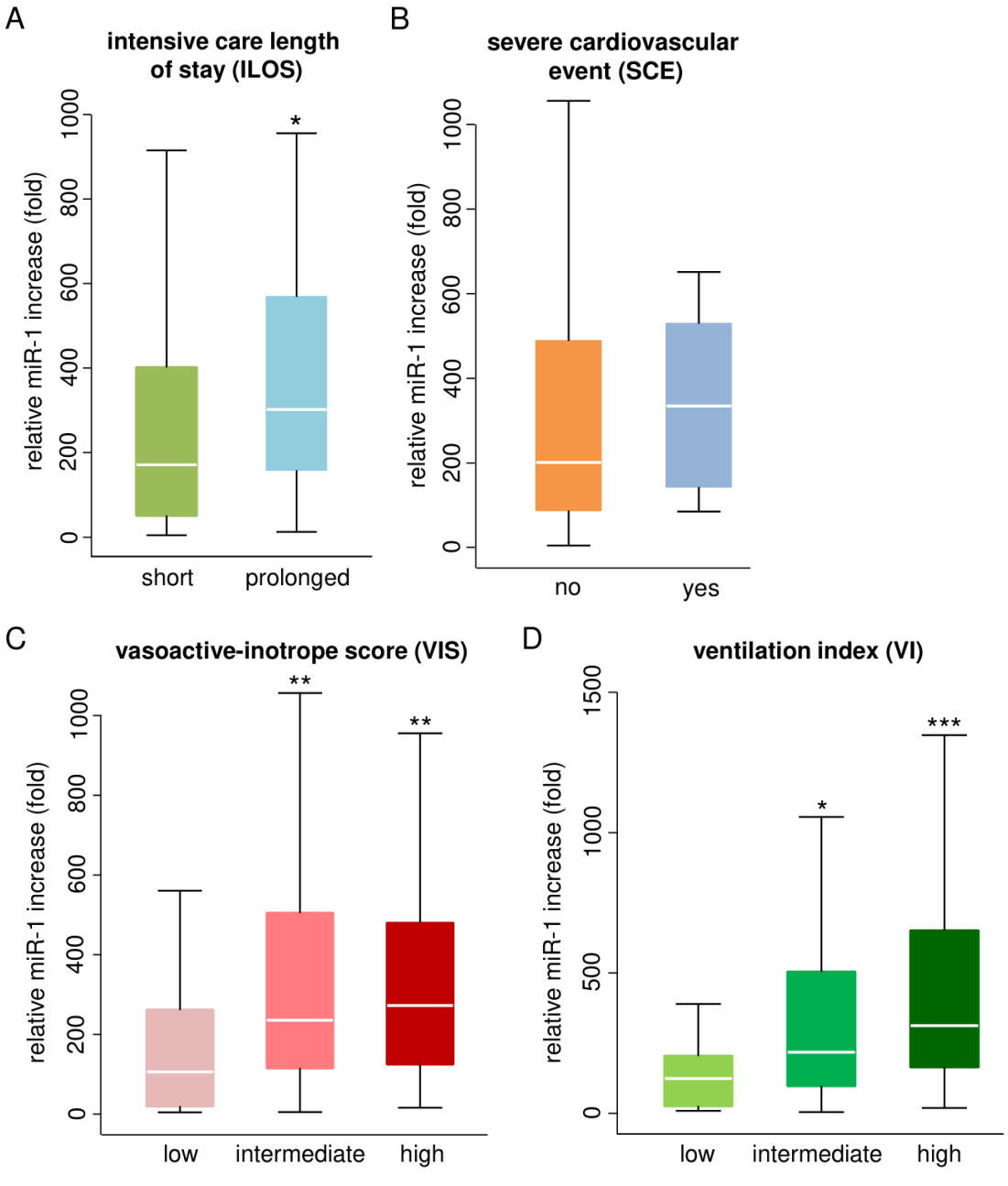

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Supplemental Figure 4. **A.** Box plot graph showing post-operative miR-1 relative increase by prolonged intensive care length of stay (ILOS). Short and Prolonged ILOS are <4 days and ≥4 days, respectively. *p<0.05, Mann-Whitney U test. **B.** Box plot graph showing post-operative miR-1 relative increase in patients with severe cardiovascular events (SCE) during the intensive care stay (n=8) and those without (n=191). Mann-Whitney U test showed no significant difference. **C.** Box plot graph showing post-operative miR-1 relative increase in the 3 mean vasoactive-inotrope score (VIS) groups: Low (<2.2), Intermediate (2.2-6.7) and High (>6.7) mean VIS. **p<0.01 vs low VIS, Kruskal-Wallis with Dunn *post hoc* test. **D.** Box plot graph showing post-operative miR-1 relative increase in the 3 mean ventilation index (VI) groups: Low (<2.97), Intermediate (2.97-9.85) and High (>9.85) VIS. *p<0.05 and ***p<0.001 vs low VI, Kruskal-Wallis with Dunn *post hoc* test.

Middle line represents the median value. The upper and lower borders of the box represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values of non-outliers. Outliers not shown.

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