Circulating microRNA kinetics analysis unveils markers of failed myocardial reperfusion

Short title: miRNAs and failed myocardial reperfusion in STEMI

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Abstract

Background: Failed myocardial reperfusion occurs in up to 50% of patients with ST elevation myocardial infarction (STEMI) treated with primary percutaneous coronary intervention (PPCI). It is strongly associated with worse clinical outcome but is not routinely screened for. Muscle-enriched microribonucleic acids (miRNAs) levels are deregulated following STEMI. Whether they inform about failed myocardial reperfusion is poorly understood. Our aim was to investigate the release kinetics of miRNAs following PPCI and their association with failed myocardial reperfusion in patient with STEMI.

Methods and results: Two candidate miRNAs (miR-1 and miR-133b) were identified following screening of 2,083 miRNAs by next generation sequencing in plasma from STEMI patients with (n=6) and without (n=6) failed myocardial reperfusion or healthy controls (n = 4). Measurements of miR-1 and miR-113b were performed at 13 time points within 3h post-reperfusion in 20 STEMI patients using TaqMan small RNA assays by RT-qPCR. This revealed that miR-1/miR-133b are rapidly released following reperfusion in a monophasic or biphasic pattern. In a ‘validation’ STEMI cohort (n = 50), miR-1 and miR-133b levels at 90min post-PPCI were approximately 3-fold (p = 0.001) and 4.4-fold (p = 0.008) higher in patients with failed myocardial reperfusion as assessed by cardiac resonance cardiac (CMR) imaging, respectively. In addition, miR-1 was particularly elevated in a subgroup of patients with worse left ventricular (LV) functional recovery 3 months post-PPCI.

Conclusion: miR-1 and miR-133b levels increase within 3h of PPCI. They are positively associated with failed myocardial reperfusion and worse LV functional recovery post-PPCI.

Keywords: STEMI; PPCI; Failed myocardial reperfusion; cardiac resonance cardiac imaging; microRNAs
1. INTRODUCTION

Primary percutaneous coronary intervention (PPCI) has substantially reduced mortality following ST-elevation myocardial infarction (STEMI) (1, 2). Nonetheless, approximately 40% and 28% of patients who survive a STEMI develop adverse left ventricular (LV) remodelling and heart failure (3, 4), respectively. This may partially result from sub-optimal myocardial reperfusion despite successful stenting of the epicardial vessel. Failed myocardial reperfusion can be detected by cardiac magnetic resonance (CMR) imaging as a hypoenhanced area within the hyperenhanced infarct core, termed microvascular obstruction (MVO) (5). MVO is strongly associated with worse clinical outcome in STEMI patients (6). Although MVO occurs in up to 50% of patients treated with PPCI (7), it usually passes undetected due to lack of sensitivity or availability of current diagnostic methods. Therefore, identification of novel, affordable approaches for detection of cardiac injury associated with MVO is warranted.

Microribonucleic acids (miRNAs) are small non-coding RNA transcripts of approximately 22 – 23 nucleotides in length that regulate gene expression at the post-transcriptional level (8, 9). MicroRNAs can be released into the extracellular space in response to cellular activation, stress, or injury (10-12). Consequently, their role as disease markers has been focus of intense investigation during the last decade (13). To date, their potential role for STEMI diagnosis has been reported by several studies, which have shown deregulated miRNA levels early after symptom onset (14). However, miRNA plasmatic kinetics following PPCI are still very poorly understood. Exploratory studies suggest that the most significant changes in miRNA levels seem to occur within 2 to 3 hours post-PPCI (15, 16), but no detailed miRNA kinetics study in this time frame has been performed thus far. The aims of this study were to investigate miRNA plasmatic kinetics in the initial 3h post-
PPCI and whether they can inform about failed myocardial reperfusion and cardiac damage as assessed by CMR.

2. METHODS

2.1. Patient cohorts and blood sampling

To study circulating miRNA kinetics following PPCI, a cohort of 20 STEMI patients (derivation cohort) was recruited at the Freeman Hospital (Newcastle upon Tyne, UK) between January and October 2017. The study was approved by the local ethics committee (REC reference: 16/NE/0405) and written consent was obtained. A second cohort of 50 STEMI patients (validation cohort) was used to validate candidate miRNAs and to assess their correlation with CMR parameters. This cohort was comprised by participants of the CAPRI trial (EudraCT number: 2014-002628-29 / REC reference: NE/14/1070). In both STEMI cohorts, blood samples were collected at the start of the PPCI procedure (pre-reperfusion) and at multiple time points within 3 hours following coronary reperfusion. Only patients who achieved TIMI flow 2 or 3 post-PPCI were included. To test a proof of concept of a coronary artery perfusion status effect on miRNA kinetics, (i) one patient undergoing transcoronary ablation of septal hypertrophy (TASH), hence with optimal coronary perfusion at the time of cardiomyocyte injury induction; (ii) one STEMI patient with TIMI flow 3 pre-PPCI; and (iii) one STEMI patient with TIMI flow 0 post-PPCI were also included. For details, see the Supplemental material online.

2.2. Standard and platelet-poor plasma isolation

Standard plasma was obtained from all blood samples for miRNA quantification whereas platelet-poor plasma was obtained for circulating microparticle (MP) isolation, as previously recommended (17, 18). For detailed protocols, see the Supplemental material online.
2.3. Microparticle isolation and quantification

To gain insight on candidate miRNAs release mechanisms, circulating microparticles (MP) were isolated from fresh platelet-poor plasma samples collected prior to and at 30 min and 90 min post-PCI from the derivation STEMI cohort (n = 10). Microparticle quantification was performed by flow cytometry using Megamix-Plus SSC calibration beads (BioCytex, France) and TruCount™ tubes (BD biosciences, USA) in a BD FACS Canto II cytometer. For details, see the Supplemental material online.

2.4. RNA isolation

Total RNA was isolated from 200μL of plasma and freshly isolated MP using the mirNeasy serum/plasma kit (Qiagen, Germany). See protocol in the Supplemental material online.

2.5. miRNA next generation sequencing and RT-qPCR screening

To identify candidate miRNA markers of MVO, a two-step miRNA screening approach was employed. First, the HTG EdgeSeq miRNA Whole Transcriptome Assay (miRNA WTA; HTG Molecular, Tucson, AZ, USA) was used to quantify 2,083 human miRNA transcripts using next-generation sequencing (NGS) in plasma collected prior to and at 90 min post-PPCI from STEMI patients with (n = 6) and without MVO (n = 6). Subsequently, screening of 179 circulating miRNAs was performed with human serum/plasma focused miRNA PCR panels (Exiqon, Denmark) in STEMI patients with MVO at 30min post-PPCI (n=5) and healthy controls (n=4). For detailed protocols, see the Supplemental material online.

2.6. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Following screening, the top 2 miRNA candidates were quantified in the derivation and validation cohorts by RT-qPCR using stem-loop specific TaqMan microRNA assays (Applied Biosystems, USA). For protocol details, see the Supplemental material online.
2.7. Index of microvascular resistance
The index of microvascular resistance (IMR) was invasively determined in 14 STEMI patients immediately after stent deployment in the infarct-related artery using a combined temperature and pressure coronary wire sensor (Certus, St. Jude Medical, USA). Hyperaemia was induced by intravenous adenosine infusion at 140 μg/kg/min. IMR was determined as the distal coronary pressure multiplied by the mean transit time of three consecutive bolus injections of room temperature saline (3 mL) during maximal coronary hyperaemia.

2.8. Cardiac magnetic resonance imaging
Cardiac magnetic resonance (CMR) imaging was performed in patients from the validation cohort at 1-7 days post-PPCI (‘baseline CMR’) as well as at 3 months post-PPCI (‘follow-up’ CMR) with a Siemens Avanto 1.5 Telsa MRI scanner using a phased array body coil combined with a spine coil. Gadobutrol (Gadovist, Bayer Schering Pharma AG, Berlin, Germany) was administered intravenously at a dose of 0.1mmol/kg and after 10 minutes short axis end-diastolic LGE images were obtained. Imaging analysis was carried out using the cvi42 software (Circle Cardiovascular Imaging Inc., Calgary, Canada). CMR was used to determine infarct size (IS), MVO, left ventricular ejection fraction (LVEF), end systolic and diastolic volumes (ESV, EDV). More information available in the Supplemental material online.

2.9. Statistical analysis
Statistical analysis was performed with the SPSS software v22.0 (IBM, New York, USA). Data normality was assessed using the Shapiro-Wilk test. Gaussian-distributed data were analyzed using parametric tests and non-Gaussian data using non-parametric tests. Correlations between variables were analyzed with the Spearman’s correlation test. Data are presented as mean and standard deviation (SD) or median and interquartile ranges (IQR).
where appropriate. All $p$ values are two-sided and $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Patient characteristics

Baseline characteristics of the derivation cohort are displayed in Supplemental table 1. Baseline characteristics of the validation cohort are summarised in Table 1. In summary, patients with MVO were more likely to have an anterior infarct ($p = 0.009$) and higher 12h post-PPCI high-sensitivity cardiac troponin T (hs-cTnT) levels ($p < 0.001$). Otherwise no differences in baseline characteristics were observed between patients with and without MVO (Table 1).

3.2. Selection of candidate miRNA markers of failed myocardial reperfusion

MicroRNA NGS and subsequent miRNA screening by RT-qPCR identified a group of 17 and 20 miRNAs with expression at least 2-fold higher in STEMI patients with MVO compared to no MVO patients and healthy controls, respectively (Figures 1B and 1C). The 3 most highly expressed miRNAs in patients with MVO by both NGS (miR-133a, miR-133b, and miR-378a) and RT-qPCR screening (miR-1, miR-133a, and miR-133b) are known to be muscle-enriched. Considering these findings and previous reports from the literature, miR-1 and miR-133b were selected for further validation.

3.3. miR-1 and miR-133b release following myocardial reperfusion

Prior to reperfusion, median miR-1 levels were approximately 4-fold higher in STEMI patients ($n = 18$) in comparison to healthy controls ($n = 6$). Following reperfusion, gradually increasing median miR-1 levels were observed until a first peak [49 (9.8 – 169.3)-fold] was
achieved at 30 min post-PCI followed by a second peak [53.1 (8.6 – 123.1)-fold] at 90 min post-PCI, returning to comparable levels of pre-reperfusion at 24h post-PCI (Figure 2A). A very similar pattern was observed for miR-133b, with the presence of 2 distinct peaks at 30min [32.6 (10.3 – 125)-fold] and 90min [28.8 (9.5 – 106)-fold] post-PCI (Figure 2B). When kinetics curves were analysed at individual level, it was possible to observed that some patients had a more prominent initial peak (n = 7) whereas others had both an early and later peak (n=10, data not shown). Indeed, by grouping these patients, two distinct miR-1 and miR-133b kinetics patterns emerged: (i) monophasic, with an initial peak at 20 – 40 min post-PPCI, followed by even higher levels at 90 – 180 min post-PPCI (Figures 2C and 2D).

In terms of miRNA transport in plasma, MP significantly high median circulating MP levels were observed in the aorta (1,400 MP/μL, p < 0.008) and culprit coronary artery (1,385 MP/μL, p = 0.024) prior to reperfusion in STEMI patients compared to controls (526 MP/μL). This was followed by a rapid decrease in MP levels as early as 5 min and at 180 min post-reperfusion (Figures 3A and 3B). Despite the decrease in circulating MP concentration post-PPCI, miR-1 and miR-133b expression in MPs isolated from STEMI patients prior to and at 30 and 90 min post-PPCI remained significantly higher than in controls (p<0.05). Prior to reperfusion, no difference in miR-1 and miR-133b expression was observed between plasma and MP from the same STEMI patients (Figures 3C and 3D). Following reperfusion, miR-1 expression in MPs was significantly lower than in plasma but no difference in miR-133b expression between these two compartments was observed (Figures 3C and 3D).

3.4. Presence of a second miR-1 peak seems to be associated with higher IMR

When patients were divided according to IMR tertiles, miR-1 kinetics seemed to differ amongst IMR tertile groups, especially regarding the magnitude and timing of peak miRNA
expression (Figure 4). All IMR tertile groups presented an initial miR-1 peak, which occurred at 30 min post-PCI for the highest tertile, at 40 min for the middle tertile, and at 60 min for the lowest tertile group (Figure 4). As for a second peak, this was absent in the first tertile group, which presented a monophasic kinetics pattern (Figure 4). A second miR-1 peak was observed at 90 min post-PCI in the middle tertile group and at 180 min post-PCI for the highest tertile group (Figure 4). Regarding miR-133b kinetics, all patient groups presented biphasic kinetics, however association with IMR values was not observed (Supplemental figure 1).

3.5. Higher post-PCI miR-1 and miR-133b levels are associated with the occurrence of MVO and larger infarct size

Baseline CMR imaging was performed at an average of 3.1 (±1.7) days post-PPCI in all patients, with no difference in time of CMR acquisition between MVO groups (p = 0.603). The ‘follow-up’ CMR imaging was performed in 45 out of the 48 patients in the validation cohort at 3 months post-PPCI. In patients with MVO, miR-1 and miR-133b levels were approximately 4.3-fold (p = 0.006) and 2.3-fold (p = 0.048) higher at 30min post-PPCI compared to patient without MVO, respectively (Figure 5). Similarly, 90min post-reperfusion levels of these miRNAs were also significantly elevated in patients with MVO [miR-1: 3-fold higher in MVO +/ve vs. MVO -/ve, p = 0.001; miR-133b: 4.4-fold higher in MVO +/ve vs. MVO -/ve, p = 0.008] (Figure 5). In addition, circulating levels of miR-1 and miR-133b at 30 and 90 min post-reperfusion positively correlated with baseline and 3-month infarct size (Supplemental table 2).

3.6. miR-1 is elevated in a subgroup of patients with worse 3-month left ventricular function recovery

When patients were divided according to baseline IS tertiles, those in the highest tertile group (IS > 13.3%; n = 16) had no improvement in LVEF after 3 months of PPCI as opposed to a
significant increase in LVEF observed in the lower baseline IS tertile groups (Supplemental figure 2). Median miR-1 levels at 90min post-PPCI were 3.3-fold and 2.7-fold higher in the highest baseline IS tertile compared to the lowest (p = 0.013) and middle (p = 0.031) tertile groups, respectively (figure 6B). Although miR-1 levels at 30min and miR-133b at 90min were also significantly elevated in the highest IS tertile group in relation to the lowest tertile, they were not significantly raised compared to the middle tertile (figures 6A and D). There was no difference in miR-133b levels at 30min post-PCI across baseline IS tertile groups (figure 6C).

4. DISCUSSION

The main findings of this study are that (i) the muscle-enriched miRNAs miR-1 and miR-133b are quickly released into the circulation in a biphasic or monophasic pattern following myocardial reperfusion in STEMI patients; (ii) the presence of a second miR-1 peak seems to be associated with higher IMR; (iii) higher miR-1 and miR-133b levels post-PPCI are associated with MVO and positively correlate with acute and 3-month IS; (v) elevated miR-1 levels are associated with worse 3-month left ventricular functional recovery.

Primary PCI successfully restores normal or near-normal coronary blood flow in more than 90% of STEMI patients (19). Despite re-establishment of coronary artery perfusion, impairment in microvascular flow occurs in approximately 50% of reperfused STEMI patients (7). This results in a failure of myocardial reperfusion which can effectively hinder the benefits of reperfusion therapy (20). Recent advancements in CMR protocols revealed that MVO substantially overlaps with areas of intramyocardial haemorrhage (IMH) and collectively represent regions of myocardial tissue with vascular damage and erythrocyte extravasation, instead of microvascular occlusion (21, 22). Therefore, the current
understanding is that failed myocardial reperfusion initially manifests as MVO in the core infarct zone followed by severe microvascular injury and IMH in 40% of the cases (22). Failed myocardial reperfusion can be detected by invasive and non-invasive methods. Electrocardiographic ST segment resolution and angiographic myocardial blush grade score lack sensitivity and reproducibility as routine tests (23). CMR imaging, the gold-standard technique for MVO detection, has prognostic importance (24, 25). However, CMR is not feasible as a routine investigation, due to high costs, contraindications (26) and lack of capacity. More recently, assessment of coronary microcirculatory function with the IMR has been shown to reliably inform about severe microvascular pathology, IMH, left ventricular remodelling (27), and mortality after STEMI (28). Nevertheless, although in principle safe, IMR is a costly, invasive procedure that adds in the time of exposure to radiation and is not available in all centres. Therefore, identification of early, affordable markers of myocardial damage associated with failed myocardial reperfusion could help in optimising clinical management in these patients.

In this study, miR-1 and miR-133b were identified as the top 2 most highly expressed candidates in STEMI patients with MVO after screening of 2,083 and 179 miRNAs by NGS and RT-qPCR, respectively. These 2 miRNAs are highly expressed in skeletal and cardiac muscle and have been shown to regulate cardiac development, cardiomyocyte proliferation and apoptosis (29). Now our meticulous study of miR-1 and miR-133b kinetics in patients in whom TIMI 2 or 3 flow was achieved post-PPCI, reveals for the first time that these miRNAs quickly increase in the circulation in the initial 3 hours post-PPCI following a biphasic pattern with peaks at 30 min and 90 min. We also demonstrate a proof of concept that this kinetic pattern may be influenced by the degree of coronary artery perfusion as assessed by TIMI flow pre- and post-PPCI. This is supported by previous experimental studies in animal
models of myocardial infarction, in which delayed cardiac-enriched miRNA peak levels occurred in rodents submitted to permanent left anterior descending (LAD) artery ligation compared to a porcine model of transient LAD occlusion followed by reperfusion (15, 30-32).

In addition, we hypothesize that, at least for miR-1, the occurrence of a second peak at 90 min post-PPCI may reflect a different myocardial injury process as that of the first peak at 30 min. Considering that (i) cardiac-enriched miRNAs are released almost immediately after cardiomyocyte injury under optimal coronary perfusion (33); (ii) all STEMI patients presented an ‘early’ peak; and (iii) the occurrence of the ‘late’ peak was associated with a surrogate marker of failed myocardial reperfusion (i.e. high IMR (27)) this study provides a hypothesis-generating concept that the ‘early’ miR-1 peak might reflect ischaemia-related myocardial injury whereas the ‘late’ miR-1 peak might be associated with cardiac injury due to failed myocardial reperfusion in STEMI patients who achieved TIMI 2 or 3 flow after PPCI. The patterns of association between miR-1 and miR-133b kinetics and IMR may be explained by the distinct mechanism of release of these miRNAs after reperfusion which might reflect different cellular injury processes, as miR-133b was predominantly released within MPs whereas miR-1 was not. Interestingly, very similar results were described by Deddens et al. (34) in a porcine model of myocardial ischaemia and reperfusion, in which miR-1 and miR-133b were raised in plasma and circulating MP at 60 min post-reperfusion but only miR-133b expression was enriched in MP in relation to plasma.

Further validating the association between miR-1 and miR-133b levels and failed myocardial reperfusion, this study is the first to show higher post-PPCI levels of such miRNAs in patients with MVO. Previously, Eitel et al. reported that occurrence of MVO was significantly higher in a group of STEMI patients with admission miR-133a concentration ≥
median (35). Considering the strong association between MVO and IS (36-38), it is not surprising that miR-1 and miR-133b post-reperfusion levels correlated with the extent of cardiac damage both in the acute (3-day) and the convalescent (3-months) phases of STEMI. Furthermore, miR-1 levels were specifically elevated in a subgroup of patients with worse left ventricular functional recovery at 3 months post-PPCI. There is discrepancy in the literature regarding the association between elevated cardiac-enriched miRNAs and reduced LVEF in patients with acute myocardial infarction or heart failure (35, 39-41). The studies that showed no association, however, performed miRNA quantification at 24 – 72h post-admission (39) or at patient discharge (41), which are late time points considering miR-1 and miR-133a kinetics. This highlights the importance of understanding the release kinetics of cardiac-enriched miRNAs following reperfusion so that optimal time points for miRNA quantification and their correlation with clinical parameters can be identified. Our findings suggest a potential role for cardiac-enriched miRNAs, especially miR-1, in the detection of MVO and monitoring of therapeutic strategies targeting failed myocardial reperfusion.

This study has some limitations such as (i) it is single-centre study; (ii) the CMR protocol did not include parameters such as IMH and area at risk; (iii) small sample size; (iv) lack of simultaneous IMR and CMR measurement in the same patients; (v) In this setting it was not possible to perform characterisation and isolation of cardiomyocyte-derived MPs due to the lack of reliable, specific surface markers.

In conclusion, this study presented the most detailed description of cardiac-enriched miRNA (miR-1 and miR-133b) release kinetics in the initial 3h following PPCI. This analysis, coupled with invasive measurements of microvascular function and cardiac imaging, revealed that miR-1 and miR-133b might be useful to understand different forms of cardiac injury.
associated with failed myocardial reperfusion. Finally, our study provided evidence for a
potential clinical role of circulating miR-1 and miR-133b as early surrogate markers of failed
myocardial reperfusion as well as cardiac function after PPCI in STEMI patients.

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Figure legends

**Figure 1. MicroRNA screening for selection of candidate markers of failed myocardial reperfusion.** (A) Screening of 179 miRNAs using PCR panels revealed a cluster of 7 miRNAs with progressive increase in expression across the initial 90 min post-reperfusion in STEMI patients. (n = 3). (B) Group of 17 miRNAs presenting copy number at least two-fold higher in patient with MVO compared to patients without MVO at 90 min post-reperfusion (MVO +ve n = 6; MVO -ve n = 6) after screening of 2,083 miRNAs. (C) The 15 most highly expressed miRNAs after screening of 179 miRNAs in 30 min post-reperfusion samples from STEMI patients with microvascular obstruction (n = 5). In both screenings, samples from age-matched patients with stable coronary artery disease were used as controls (n = 4). * p < 0.05, Kruskal-Wallis test with Dunn’s correction for multiple comparisons (A) and Mann-Whitney U test (B).

**Figure 2. miR-1 and miR-133b plasmatic kinetics following myocardial reperfusion in STEMI patients.** (A) miR-1 expression was higher than in controls at all time points and increased in relation to pre-reperfusion levels between 20 min and 180 min post-PCI. (B) Kinetics analysis revealed that 20 min up to 180 min post-PCI miR-133b levels were elevated in relation to controls. Also, 20 min up to 180 min post-reperfusion miR-133b expression was higher than pre-reperfusion levels. (C) Monophasic miR-1 and miR-133b kinetics pattern. Data presented as mean and SEM of the ratio fold change/fold max for each time point, n = 7. (D) Biphasic miR-1 and miR-133b kinetics pattern. Data presented as mean and SEM of the ratio fold change/fold max for each time point, n = 10. (A,B) Data presented as median (central line in boxes), interquartile range (limits of the boxes), and range (error bars). Dashed lines represent reference control levels. n STEMI = 18; n controls = 6. Differences between STEMI patients and control miRNA levels were determined by Kruskal-Wallis test with Dunn’s correction for multiple comparisons. Differences in miRNA expression among time points in STEMI patients were determined by Friedman test with Bonferroni’s post hoc correction for multiple comparison. *p < 0.05 vs. controls; **p < 0.01 vs. controls; ***p < 0.001 vs. controls; §§p < 0.01 vs. pre-reperfusion; §§§p < 0.001 vs. pre-reperfusion.

**Figure 3. miR-1 and miR-133b expression in circulating microparticles isolated from STEMI cohort 1 patients.** (A) Gating strategy for MP detection by flow cytometry. Megamix-Plus SSC beads were used to determine the boundaries for MP detection: four populations of beads with different sizes Small (300 – 500nm) and large (500 – 1000 nm) MP as detected by flow cytometry. (B) Microparticle concentration in STEMI patients prior to and after coronary reperfusion. Microparticle concentration is significantly raised in the aorta and culprit coronary artery of STEMI patients compared to controls. *p < 0.05 vs. controls; **p < 0.01 vs. controls; ns, non-significant. Kruskal-Wallis test with Dunn’s correction for multiple comparisons. (C) Comparative miR-1 expression between plasma and microparticles isolated from the same STEMI patients prior to and at 30 min and 90 min post-reperfusion. *p < 0.05, Wilcoxon matched-pairs signed rank test. (D) Comparative miR-133b expression between plasma and microparticles isolated from the same STEMI patients prior to and at 30 min and 90 min post-reperfusion. *p < 0.05, Wilcoxon matched-pairs signed rank test. n STEMI = 10; n controls = 3.
Figure 4. miR-1 kinetics according to index of microvascular resistance (IMR) tertiles. Data presented as median (dots). n = 14; n 1st tertile = 4; n 2nd tertile = 5; n 3rd tertile = 5.

Figure 5. Post-reperfusion miR-1 and miR-133b levels are elevated in patients with MVO. A) 30min post-reperfusion miR-1 and miR-133b levels in patients with and without MVO determined by the baseline MRI; B) 90min post-reperfusion miR-1 and miR-133b levels in patients with and without MVO. Lower panels depict examples of CMR imaging showing infarct cores with and without MVO. Differences between MVO (n = 19) and no MVO (n = 29) patients were determined by Mann-Whitney U test. * p < 0.05; ** p < 0.01. MVO, microvascular obstruction.

Figure 6. Post reperfusion miR-1 and miR-133b levels according to baseline IS tertiles. A) miR-1 levels at 30min post-PCI. Significantly higher miR-1 measurements were observed in the highest IS tertile in comparison to the lowest tertile; B) miR-1 levels at 90min post-PCI were significantly elevated in the highest tertile compared to the two lower tertile groups; C) No differences in miR-133b levels at 30min post-PCI amongst baseline IS tertile groups were observed. D) miR-133b levels at 90min post-PCI were significantly increased in the highest IS tertile in comparison to the lowest tertile. Differences between groups were determined by Kruskall-Wallis test with Dunn’s corrections for multiple comparisons. * p < 0.05; ns, non-significant. n (1st tertile) = 16; n (2nd tertile) = 16; n (3rd tertile) = 13. IS, infarct size.
Table 1. STEMI validation cohort baseline characteristics

<table>
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<th>Variable</th>
<th>Entire cohort (n = 50)</th>
<th>MVO – (n = 29)</th>
<th>MVO + (n = 21)</th>
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<td>22 (75.8)</td>
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<td>IHD</td>
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<td>2 (6.9)</td>
<td>1 (4.8)</td>
<td>0.754</td>
</tr>
<tr>
<td>Past MI</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Past Angiogram</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>1 (4.8)</td>
<td>0.235</td>
</tr>
<tr>
<td>CVA / TIA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Regular medication, n (%)</td>
<td></td>
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<tr>
<td>Aspirin</td>
<td>3 (6)</td>
<td>2 (6.9)</td>
<td>1 (4.8)</td>
<td>0.754</td>
</tr>
<tr>
<td>ACE inhibitor / ARB</td>
<td>5 (10)</td>
<td>3 (10.3)</td>
<td>2 (9.5)</td>
<td>0.924</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>2 (9.5)</td>
<td>0.090</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>4 (8)</td>
<td>2 (6.9)</td>
<td>2 (9.5)</td>
<td>0.735</td>
</tr>
<tr>
<td>Diuretic</td>
<td>4 (8)</td>
<td>3 (10.3)</td>
<td>1 (4.8)</td>
<td>0.473</td>
</tr>
<tr>
<td>Statins</td>
<td>10 (20)</td>
<td>5 (17.2)</td>
<td>5 (23.8)</td>
<td>0.567</td>
</tr>
<tr>
<td>Laboratory tests [median (IQR)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR [mL/min]</td>
<td>83 (74 – 93.7)</td>
<td>85 (74.5 – 101.5)</td>
<td>82 (72 – 90)</td>
<td>0.409</td>
</tr>
<tr>
<td>Pre-PPCI hs-cTnT [ng/L]</td>
<td>43 (27 – 80.7)</td>
<td>43 (26.5 – 80)</td>
<td>44 (27.5 – 96.5)</td>
<td>0.595</td>
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<tr>
<td>12h hs-cTnT [ng/L]</td>
<td>3447 (1184 – 6619)</td>
<td>2108 (844 – 3826)</td>
<td>5765 (3621 – 9946)</td>
<td>&lt;0.001</td>
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<tr>
<td>STEMI and PCI parameters</td>
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<td></td>
</tr>
<tr>
<td>Onset to reperfusion, min [median (IQR)]</td>
<td>173 (112 – 259)</td>
<td>195 (126 – 273)</td>
<td>146 (102 – 237)</td>
<td>0.340</td>
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<tr>
<td>Infarct location, n (%)</td>
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<tr>
<td>Anterior</td>
<td>16 (32)</td>
<td>5 (17.2)</td>
<td>11 (52.4)</td>
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<tr>
<td>Non-anterior</td>
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<td>24 (82.8)</td>
<td>10 (47.6)</td>
<td>0.009</td>
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<td>TIMI flow pre PCI, n (%)</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>42 (84)</td>
<td>23 (79.3)</td>
<td>19 (90.5)</td>
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</tr>
<tr>
<td>1</td>
<td>8 (16)</td>
<td>6 (20.7)</td>
<td>2 (9.5)</td>
<td>0.288</td>
</tr>
<tr>
<td>Thrombus aspiration, n (%)</td>
<td>10 (20)</td>
<td>5 (17.2)</td>
<td>5 (23.8)</td>
<td>0.567</td>
</tr>
<tr>
<td>TIMI flow post PCI, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>2 (9.5)</td>
<td>0.090</td>
</tr>
<tr>
<td>GPIIa/IIIb inhibitors, n (%)</td>
<td>3</td>
<td>48 (96)</td>
<td>29 (100)</td>
<td>19 (90.5)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------</td>
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</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme; MI, myocardial infarction; ARB, angiotensin receptor blocker; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; eGFR, estimated glomerular filtration rate; hs-cTnT, high-sensitivity cardiac troponin T; IHD, ischemic heart disease; IQR, interquartile range; MI, myocardial infarction; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; MVO, microvascular obstruction; PPCI, primary percutaneous coronary intervention; PVD, peripheral vein disease; RCA, right coronary artery; STEMI, ST-elevation myocardial infarction; TIA, transient ischaemic attack; TIMI, thrombolysis in myocardial infarction angiographic score
Figures

Figure 1
Figure 2

A) miR-1

B) miR-133b

C) Monophasic pattern

D) Biphasic pattern
Figure 3

A) Flow cytometry analysis with gates P1 to P4 for different beads sizes.

B) Bar graph showing microparticle concentration (MP/mL) across different conditions.

C) Box plot indicating miR-1 fold change to control at different time points.

D) Box plot showing miR-133b fold change to control at different time points.
Figure 4

A) miR-1

![Graph showing miR-1 fold change to control over time post-reperfusion (min)]

- 1st Tertile (IMR < 22.1)
- 2nd Tertile (22.1 < IMR < 33.9)
- 3rd Tertile (IMR > 33.9)

Figure 5

A) miR-1 and miR-133b

![Box plots showing 30 min post-PPCI miRNA fold change to control for No MVO and MVO groups]

B) miR-1 and miR-133b

![Box plots showing 90 min post-PPCI miRNA fold change to control for No MVO and MVO groups]

MVO -/ve

MVO +/ve
Figure 6

A) miR-1 - 30min post-PPCI

B) miR-1 - 90min post-PPCI

C) miR-133b - 30min post-PPCI

D) miR-133b - 90min post-PPCI
SUPPLEMENTAL MATERIAL

1. Supplemental methods

1.1. Patients cohorts and blood sampling

The derivation cohort (n = 20) was prospectively recruited between January 2017 and June 2017 at the Freeman Hospital. The study was approved by the local ethics committee (REC reference: 16/NE/0405). Patients presenting with chest pain suggestive of myocardial ischaemia with ST-segment elevation or new left bundle branch block on the ECG and indication of PPCI (<12h of symptom onset) were included. Exclusion criteria comprised clinically unstable patients (haemodynamically unstable, shocked, or unconscious patients) and previous myocardial infarction. As per current clinical guidelines, patients received 300 mg of aspirin and 600 mg of clopidogrel, 60 mg of prasugrel or 180 mg ticagrelor loading dose along with standard doses of heparin (70 units/kg) or bivalirudin (0.75mg/kg) at admission to the cardiac catheterization laboratory. Administration of GPIIIb/IIIa inhibitors was at the discretion of the attending interventional cardiologist during PPCI.

The validation cohort was comprised of participants of the CAPRI trial. This clinical trial randomised suitable STEMI patients admitted at the Freeman Hospital between March 2015 and November 2016 to receive a bolus injection of cyclosporine or placebo at the start of PPCI. The study included patients presenting within 6h of chest pain onset, ST-segment elevation, and with a major (at least 3mm) culprit coronary artery occluded (TIMI flow grade 0-1) at the time of admission coronary angiography. Exclusion criteria comprised clinically unstable patients, presence of immunological, neoplastic, hepatic, or kidney disorders, patients with an open (TIMI flow > 1) culprit coronary artery at the time of angiography, previous MI, or contra-indications to cardiac MRI. Patients received standard pharmacological therapy as per current international guidelines and were randomized to have
a bolus dose of cyclosporine or placebo at the start of PCI. In addition, demographic, clinical and laboratorial data were recoded. All patients underwent cardiac MRI assessment during hospitalisation and after discharge.

Patients received standard pharmacological therapy as per current international guidelines. Participants of the CAPRI trial underwent CMR assessment during hospitalisation and after discharge.

In both the derivation and validation cohorts, arterial blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes [Becton Dickinson (BD) Biosciences, USA] at the start of the PPCI procedure when the arterial sheath was inserted. These samples represented the pre-reperfusion time point. The procedure was performed as usual and the exact time of reperfusion was recorded. Reperfusion was defined as restoration of TIMI flow 2 or 3 and/or occurrence of reperfusion arrhythmias. In the derivation cohort, blood samples were collected in additional 12 time points post-reperfusion (5, 10, 20, 30, 40, 50, 60, 75, 90, 120, 180 min and 24h). Samples were drawn from a central arterial source until the 90 min time point using a 6F radial artery sheath left in since the PPCI procedure. At 120 min, 180 min, and 24h post-reperfusion, samples were collected by venepuncture from a peripheral venous source (antecubital vein). In the validation cohort, samples were collected exactly as described for STEMI cohort 1, except that blood was drawn only at pre-reperfusion and at 5, 15, 30, 90 min and 24h post-reperfusion.

1.2. Standard and platelet-poor plasma isolation

For standard plasma isolation, blood samples were centrifuged at 1,500 x g and room temperature for 15 min. The top two thirds of the supernatant were collected without disturbing the red cell pellet, aliquoted (250μL) in cryopreservation tubes and stored at -80°C until analysis.
Platelet-poor plasma was isolated from derivation cohort patients (n = 10) blood samples collected prior to and at 30 min and 90 min post-reperfusion as well as controls (n = 3). This was used for circulating microparticle quantification, as standard plasma contains residual platelets that might interfere with microparticle (MP) analysis (1, 2). Isolation of platelet-poor plasma comprised two centrifugation steps: (i) whole blood was centrifuged at 1,500 x g and room temperature for 15 min, after which the top two thirds of the supernatant (standard plasma) were collected and transferred to a new polypropylene tube; (ii) whole plasma was then centrifuged again at 1,500 x g and room temperature for 15 min, the top two thirds (platelet-poor plasma) were transferred to a fresh polypropylene tube, and stored at -80°C until analysis.

1.3. Circulating microparticle isolation

In brief, 250μL of fresh platelet-poor plasma were diluted (1:4) in 750μL of phosphate buffered saline (PBS) (Life Technologies, USA). Subsequently, samples were centrifuged at 20,000 x g and 4°C for 30 min. The supernatant was carefully aspirated leaving the MP pellet in 20μL of the solution, which were immediately processed for downstream flow cytometric analysis or RNA extraction.

1.4. Flow cytometry

1.4.1. Microparticle staining with annexin-V

Microparticle quantification was performed by flow cytometry. Microparticle pellets were resuspended in 100μL of 1X annexin-V binding buffer (10nM HEPES, pH 7.4, 140nM NaCl, 2.5nM CaCl$_2$,) (BD Biosciences, USA, cat. no. 51-66121E), which had been diluted in 1:10 with distilled water. The MP suspension was then incubated with 5μL of FITC annexin-V or 5μL of a FITC isotype control (FITC mouse IgG2b κ isotype control,
Biolegend) for 30 minutes, protected from light, at room temperature. At the end of the incubation period, MP samples were washed with 500μL of PBS and centrifuged at 20,000 x g and 4°C for 20 minutes. The supernatant was carefully aspirated and the MP pellet was resuspended in 500μL of 1X annexin-V buffer. The MP suspension was then transferred to TruCount™ tubes (BD biosciences, USA, cat. no. 340334). The tubes were gently vortexed for 5 seconds and incubated at room temperature in the dark for 20 minutes. Samples were then taken immediately for quantification by flow cytometry.

1.4.1 Microparticle gating strategy

Because of MP’s very small size, flow cytometric MP analysis requires working conditions very close to the lowest limit of detection of current flow cytometers. Therefore, adequate MP quantification relies on the optimal balance between MP detection and signal background exclusion. To achieve this, Megamix-Plus SSC (BioCytex, France, cat. no. 7803) were employed to calibrate the flow cytometer and establish a gate for MP sample analyses. Megamix-Plus SSC are fluorescent beads of varied diameters (0.16μm, 0.20μm, 0.24μm, and 0.5μm) that are equivalent to the size range of MP (0.3 to 1μm) when using side scatter (SSC) as a size-related parameter. Briefly, 500μL of the beads suspension were transferred to a cytometric tube, which was vortexed and positioned in a BD FACS Canto II cytometer for calibration using the FACSDiva software (BD Biosciences, San Jose, CA, USA). The cytometer settings were adjusted according to BioCytex’s recommendations and bead acquisition was performed in the lowest available speed. Gating strategy comprised 3 steps (Figure 1):

1. Adjustment of the FITC detector voltage so that the 0.5μm bead population is at the beginning of the 5th decade (Figure 1A left upper panel).
2. Determination of MP region boundaries. On a SSC-H count histogram, the number of events and SSC-H median parameters were calculated for each of the 4 bead populations. The lowest MP gate boundary was determined by the formula: Low SSC-H level = Md 0.16 + (0.3 x (Md 0.20 – Md 0.16)), where Md 0.16 represented the median SSC-H for the 0.16μm bead population and Md 0.20 represented the median SSC-H for the 0.20μm bead population. The highest boundary corresponded to the end of the 0.50μm SSC-H peak (Figure 1A middle upper panel). Another boundary was also set at centre of the gap between the 0.20μm and 0.24μm peaks, which corresponds to differentiate small (< 0.50μm) and large (≥ 0.50μm) MP.

3. Setting the MP gate in dual scatter. Once the MP gate boundaries were defined on the SSC (log) scale, the corresponding regions were created on a dual scatter plot (SSC-H x FSC-H), which was used for later MP analysis (Figure 1A right upper panel). This calibration procedure was repeated regularly to minimise variability across different samples.

To confirm whether the MP gate was indeed able to separate ‘true’ annexin-V events from the instrument background noise the following samples were analysed under the same settings (n = 3 for each): (i) filtered 1X annexin-V binding buffer only (Figure 1B); (ii) unstained MP (Figure 1C); (iii) MP sample with FITC isotype control (Figure 1D); (iv) annexin-V-stained MP (Figure 1E).
1.4.2. **FACS analysis and microparticle quantification**

TruCount™ tubes containing microparticle samples stained with FITC annexin-V or FITC IgG isotype control were gently vortexed prior to analysis to mix contents well. All samples were analysed at low speed until 10,000 TruCount™ beads were acquired using the BD FACS Canto II cytometer and the FACSDiva software. Microparticles were considered as annexin-V positive events detected within both the large and small MP gate. Considering that the number of TruCount™ beads in each tube is accurately determined by the manufacturer and was known in each case, the absolute MP count (MP/μl) in each 250μL
plasma sample could be determined by comparing the number of positive events in the MP gate, with the number of TruCount™ bead events. This was calculated using the formula:

$$\text{MP count (MP/µl)} = \frac{\#\text{events in MP gate}}{\#\text{TruCount bead events}} \times \frac{\#\text{beads/tube}}{\text{volume plasma/test (µl)}}$$

1.5. RNA isolation

1.5.1 RNA isolation from plasma

Plasma samples were quickly thawed at 37°C for 2 min and centrifuged at 1,900xg and room temperature for 10 min to avoid formation of cryoprecipitate and remove residual cells and debris (3). Total RNA was isolated from 200µL of plasma using the miRNeasy serum/plasma kit (Qiagen, Germany). In brief, 200µL of each plasma sample were mixed with 1000µL of the Qiazol lysis reagent in 2mL polypropylene tubes and incubated at room temperature for 5 min. During the incubation period, 3.5µL (1.6 x 10⁸ copies/µL) of synthetic cel-miR-39 (Qiagen, Germany, cat. no. 219610) were added to all samples. In samples used for miRNA screening, the cel-miR-39-3p RNA spike-in template (Exiqon, Denmark, cat. no. 203202) was added instead. Then, 200µL of chloroform were added to each tube, the mixture incubated for 3 minutes at room temperature, and centrifuged for 15 min at 12,000 x g and 4°C. The upper aqueous phase (500µL) was carefully collected without disturbing the interphase and transferred to a new 2mL polypropylene tube. Subsequently, 750µL of 100% ethanol were added to the aqueous phase, the mixture (750µL) was transferred to an RNeasy MinElute spin column, and centrifuged for 30 sec at 10,000 x g and room temperature. The flow-through was discarded and the procedure repeated with the remaining 500µL of the
mixture. Serial washes of the column were then performed by quick centrifugation with buffer RWT (700μL), buffer RPE (500μL), and 80% ethanol (500μL) for 30 sec (buffers RWT and RPE) and 2 min (80% ethanol) at 10,000 x g and room temperature. After the serial washes, the column’s membrane was dried by centrifugation for 5 min at 10,000 x g and room temperature. Finally, 60μL of RNAse-free water were added to the centre of the membrane to elute the RNA and this was collected in a fresh tube by centrifugation for 1 min at 10,000 x g and room temperature.

Following RNA extraction, assessment of total RNA concentration and integrity was performed by a 2100 Bioanalyzer instrument using the RNA 6000 Pico Kit (Agilent technologies, Germany), according to the manufacturer’s protocol. RNA samples were then stored at -80°C until analysis.

1.5.2. RNA isolation from microparticles

Immediately after microparticle isolation, the 20μL MP pellet was resuspended in 180μL of PBS. The 200μL suspension was then subjected to the same RNA extraction process described in the previous section. RNA samples were stored at -80°C until analysis.

1.6. HTG Edgeseq miRNA whole transcriptome assay (WTA)

The HTG EdgeSeq miRNA Whole Transcriptome Assay (miRNA WTA) measures the expression of 2,083 human miRNA transcripts using next-generation sequencing (NGS). For each sample, 15μl of plasma was lysed with 15μl of HTG plasma lysis buffer and 3μl proteinase K (both HTG Molecular, Tucson, AZ, USA), then incubated for 180 minutes at 50°C with orbital shaking. From each prepared sample, 25μL were added per well to a 96-well sample plate and run on an HTG EdgeSeq Processor using the HTG EdgeSeq miRNA WTA (HTG Molecular, Tucson, AZ, USA). Sample miRNAs are protected with proprietary
protection probes, while all non-hybridized probes and non-targeted RNA are degraded by S1 nuclease, resulting in a 1:1 stoichiometric ratio of probes to targeted RNA. Samples were subsequently individually barcoded by PCR with adapters and dual molecular barcodes via tailed primers. For PCR, 3ul sample was incubated with 6µl OneTaq PCR GC buffer, 2.4µl Hemo KlenTaq enzyme (both New England Biolabs), 0.2mM dNTPs, and 3µl F and R primers (HTG Molecular) in a 30µl reaction. Samples were heated at 95°C for 4 minutes, followed by 16 cycles of: 95°C for 15 seconds, 56°C for 45 seconds and 68°C for 45 seconds; then 68°C for 10 minutes. Barcoded samples were individually purified using AMPure XP beads (Beckman Coulter), quantitated using a KAPA Library Quantification kit (KAPA Biosystem, Wilmington, MA, USA) then pooled at a concentration of 4pM. The library was sequenced on an Illumina NextSeq (Illumina, Inc., San Diego, CA) using a NextSeq 500/550 High Output Kit v2.5 (75 cycles) kit with two index reads and 5% PhiX spike-in as standard. Data were returned from the sequencer in the form of demultiplexed FASTQ files, with one file per original well of the assay. The HTG EdgeSeq Parser (HTG Molecular, Tucson, AZ, USA) was used to align the FASTQ files to the probe list to provide raw sequencing reads per miRNA.

1.7. Reverse transcription

Prior to reverse transcription, RNA samples were incubated with 0.3U of heparinase I from Flavobacterium heparinum (Sigma-Aldrich, Germany) for 1 hour at room temperature, as previously described (4).

1.7.1. Universal reverse transcription

For miRNA screening using RT-qPCR panels, 4µL of total RNA were reverse transcribed using the Universal cDNA synthesis kit II (Exiqon, Denmark, cat. no. 203301). RNA samples were mixed with 1µL of heparinase (0.3U) and 4µL of the kit’s 5X reaction buffer and
incubated for 1 hour at room temperature. Subsequently, 2μL of enzyme mix, 1μL of a spike-in miRNA (UniSp6), and 8μL of nuclease-free water were added to the mixture to a total of 20μL. Reverse transcription (RT) reactions were carried out in a thermocycler with the settings: (i) incubation at 42°C for 60 min; (ii) inactivation at 95°C for 5 min.

1.7.2. Taqman-based reverse transcription

Reverse transcription for specific target miRNA in the derivation and validation cohorts was performed using 5μL of RNA, TaqMan® microRNA reverse transcription kit (Applied Biosystems, USA) and stem-loop specific 5X TaqMan microRNA assays (Applied Biosystems, USA). In brief, 5μL of RNA were mixed with 1μL of heparinase (0.3U), and 0.19μL of RNAse inhibitor for 1 hour at room temperature. Subsequently, the remaining components of the TaqMan microRNA reverse transcription kit along with 3.16μL of nuclease-free water were added to the mixture to a total volume of 15μL. Reverse transcription was carried out in a thermocycler with the settings: (i) incubation at 16°C for 30 min; (ii) incubation at 42°C for 30 min; (iii) inactivation at 85°C for 5 min.

1.8. Real-time quantitative polymerase chain reaction

1.8.1. SYBR-based qPCR

Screening of 179 circulating miRNAs was performed using human serum/plasma focused miRNA PCR panels (Exiqon, Denmark) and the Exilent SYBR Green master mix (Exiqon, Denmark). Each miRNA PCR panel comprised 2x 96-well plates pre-coated with LNA miRNA primers for target miRNAs, internal controls (cel-miR-39, UniSp6, and UniSp3) as well as blank wells. For each plate, 10μL of cDNA were mixed with 500μL of the Exilent SYBR Green master mix and 490μL of nuclease-free water in a 2mL polypropylene tube. Subsequently, 10μL the mixture was added to each well on ice. The PCR reaction was carried out in a 7500AB PCR instrument (Applied Biosystems, USA) with the following settings: (i)
polymerase activation at 95°C for 10 min; (ii) denaturation (40 cycles) at 95°C for 10 sec; (iii) annealing/extension at 60°C for 60 sec; (iv) melting curve analysis.

1.8.2. *TaqMan*-based qPCR

Target microRNA quantification using hydrolysis probes was performed with freshly synthesised cDNA only. Each PCR reaction for individual miRNAs was prepared in nuclease-free 0.2 mL polypropylene tubes on ice, by mixing 4.8 μL of cDNA sample, 3.6 μL of 20X TaqMan small RNA assay, 36 μL of the SensiFAST probe Hi-ROX master mix (Bioline, UK), and 27.6 μL of nuclease-free water to a total volume of 72 μL. Subsequently, 20 μL of the mixture were transferred to a 96-well PCR plate in triplicates. The PCR reaction was carried out in a 7500AB PCR instrument (Applied Biosystems, USA) with the following settings: (i) polymerase activation at 95°C for 10 min; (ii) denaturation (40 cycles) at 95°C for 15 sec; (iii) annealing/extension at 60°C for 60 sec.

1.8.3. Data analysis and normalisation

In the RT-qPCR miRNA panels screening, inter-plate calibration was performed to minimize PCR inter-run variability. A calibration factor for each plate was determined as the difference between the average Cq of UniSp3 triplicates in that plate and the overall UniSp3 Cq average for all plates. Inter-plate calibration was performed by correcting the target miRNA Cq in each plate according to the calibration factor. The global miRNA mean expression was calculated as the geometric mean of Cq values obtained for all miRNAs, excluding miRNAs with Cq > 35. In the TaqMan RT-qPCR, data was normalised for the endogenous miR-425-5p control, as previously described (4). Fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method.
1.9. Cardiac magnetic resonance imaging

Cardiac magnetic resonance imaging was performed with a Siemens Avanto 1.5 Telsa MRI scanner using a phased array body coil combined with a spine coil. In brief, according to the CAPRI trial protocol, cine images of the heart in 2, 3 and 4 chamber views were obtained using a steady state free precession pulse (SSFP) sequence (repetition time [TR]: set according to heart rate, image matrix 144x192, echo time (TE): 1.19ms, flip angle: 80°). T2 weighted STIR (short inversion time [TI] inversion recovery) images were obtained in the same projections, using a black-blood segmented turbo spin echo technique (TR according to heart rate, TE 47ms, flip angle 180°, TI 140ms, image matrix 208x256). Gadobutrol, a contrast agent (Gadovist, Bayer Schering Pharma AG, Berlin, Germany), was administered intravenously at a dose of 0.1mmol/kg, and after 10 minutes short axis end-diastolic LGE images (in corresponding locations to cine and STIR images) were obtained using an inversion recovery (IR) segmented gradient echo sequence (TR: according to heart rate, TE: 3.41ms, flip angle: 25°, image matrix: 196x256). Imaging analysis was carried out using the cvi42 software (Circle Cardiovascular Imaging Inc., Calgary, Canada) by a trained research fellow involved in the CAPRI trial. Epicardial and endocardial borders were traced automatically on each end-systolic and end-diastolic short axis cine frame with manual correction where necessary, allowing automated calculation of left ventricular mass, dimensions and ejection fraction (LVEF). For infarct size and MVO determination, LGE images taken at the end of diastole were used. Areas of enhancement with signal > 5 standard deviations above normal myocardial areas (infarction) were identified and quantified automatically. Regions of hypoenhancement within a hyperenhanced zone (MVO) were also identified and semi-automatically quantified. To prevent bias, miRNA measurements were performed in a blind fashion in relation to cardiac imaging data.
2. Supplemental results

**Supplemental table 1. STEMI derivation cohort baseline characteristics**

<table>
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</tr>
</thead>
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<td>Age [years, mean (SD)]</td>
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</tr>
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<td>Risk factors, n (%)</td>
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<td>Smoking status</td>
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<tr>
<td>Never smoked</td>
<td>9 (50)</td>
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<tr>
<td>Ex-smoker</td>
<td>1 (5.6)</td>
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<tr>
<td>Current smoker</td>
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<tr>
<td>Hypertension</td>
<td>7 (38.9)</td>
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<td>Hypercholesterolaemia</td>
<td>2 (11.1)</td>
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<tr>
<td>Obesity</td>
<td>4 (22.2)</td>
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<td>Laboratory tests [median (IQR)]</td>
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<tr>
<td>eGFR (mL/min)</td>
<td>83 (65.2 – 112)</td>
</tr>
<tr>
<td>Admission Troponin T (ng/L)</td>
<td>61.5 (14.5 – 156)</td>
</tr>
<tr>
<td>Peak Troponin T (ng/L)</td>
<td>2,752 (1,039–5,047)</td>
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<tr>
<td>STEMI clinical characteristics</td>
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<tr>
<td>Onset to reperfusion (min)</td>
<td>131 (108 – 223)</td>
</tr>
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<td>Infarct location, n (%)</td>
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<tr>
<td>Anterior</td>
<td>6 (33.3)</td>
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<tr>
<td>Non-anterior LCx</td>
<td>12 (66.7)</td>
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<td>TIMI flow pre PPCI, n (%)</td>
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<td>0</td>
<td>16 (88.8)</td>
</tr>
<tr>
<td>1</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>2</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TIMI flow post PPCI, n (%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>2</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>3</td>
<td>16 (88.8)</td>
</tr>
<tr>
<td>Medication during PPCI</td>
<td></td>
</tr>
<tr>
<td>Heparin, n (%)</td>
<td>15 (85)</td>
</tr>
<tr>
<td>Bivalirudin, n (%)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>gpIIb/IIIa inhibitor, n (%)</td>
<td>13 (72.2)</td>
</tr>
</tbody>
</table>

eGFR, estimated glomerular filtration rate; gpIIb/IIIa, glycoprotein IIb/IIIa; IQR, interquartile range; PPCI, primary percutaneous coronary intervention; SD, standard deviation; TIMI, thrombolysis in myocardial infarction angiographic score.
Supplemental table 2. Correlation between miR-1, miR-133b, and CMR parameters

<table>
<thead>
<tr>
<th>CMR parameters</th>
<th>miR-1 (30min)</th>
<th>miR-133b (30min)</th>
<th>miR-1 (90min)</th>
<th>miR-133b (90min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline MRI (n = 48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarct size, % of LV</td>
<td>0.420†</td>
<td>0.304*</td>
<td>0.497§</td>
<td>0.445†</td>
</tr>
<tr>
<td>3-month MRI (n = 45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarct size, % of LV</td>
<td>0.449†</td>
<td>0.357*</td>
<td>0.546§</td>
<td>0.538§</td>
</tr>
</tbody>
</table>

*p < 0.05; †p < 0.01; §p < 0.001.
Supplemental figure 1. Proof of concept of the effect of coronary artery perfusion status on miRNA release kinetics. (A) miR-1 and miR-133b kinetics following ethanol-induced myocardial injury in a patient undergoing transcoronary ablation of septal hypertrophy. (B) miR-1 and miR-133b kinetics in a STEMI patient with spontaneous recanalization of the culprit coronary artery (TIMI flow 3) prior to PCI. (C) miR-1 and miR-133b kinetics in a STEMI patient with unsuccessful re-establishment of culprit coronary artery patency post-PCI (TIMI flow 0). Dots represent miRNA expression fold-change to controls at each time point. Dashed lines represent reference control levels.
Supplemental figure 2. Final infarct size and 3-month post-PCI left ventricular functional recovery according to baseline IS tertiles. A) When patients were divided according to baseline IS tertiles, those in the highest tertile remained with the largest IS in relation to the lowest tertile at 3-months post-PPCI; B) Patients in the two lower 3-day IS tertiles had significant improvements in LVEF over the initial 3 months following PCI. No changes in LVEF were observed for the highest tertile group; C) Compared to baseline levels, a trend in ESV reduction at 3 months post-PCI was observed for the lowest IS tertile group and a significant decrease for the middle tertile. There was no significant difference in ESV between baseline and 3-month measurements in the highest IS tertile. Box plots display median (central line), 25th and 75th percentiles (limits of the box), and range (error bars). Differences between 3-day and 3-month LVEF and ESV measurements were determined by paired Wilcoxon signed rank test. * p < 0.05; ** p < 0.01; ns, non-significant. n (1st tertile) = 16; n (2nd tertile) = 16; n (3rd tertile) = 13. IS, infarct size; ESV, end systolic volume; LVEF, left ventricular ejection fraction.

