Impact of intravenous heparin on quantification of circulating microRNAs in patients with coronary artery disease

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Summary

MicroRNAs are small non-coding RNAs that are detectable in plasma and serum. Circulating levels of microRNAs have been measured in various studies related to cardiovascular disease. Heparin is a potential confounder of microRNA measurements due to its known interference with polymerase chain reactions. In this study, platelet-poor plasma was obtained from patients undergoing cardiac catheterisation for diagnostic coronary angiography, or for percutaneous coronary intervention, both before and after heparin administration. Heparin had pronounced effects on the assessment of the exogenous *C. elegans* spike-in control (decrease by approx. 3 cycles), which disappeared 6 hours after the heparin bolus. Measurements of endogenous microRNAs were less sensitive to heparin medication. Normalisation of individual microRNAs with the average cycle threshold value of all microRNAs provided a suitable alternative to normalisation with exogenous *C. elegans* spike-in control in this setting. Thus, both the timing of blood sampling relative to heparin dosing and the normalisation procedure are critical for reliable microRNA measurements in patients receiving intravenous heparin. This has to be taken into account when designing studies to investigate the relation of circulating microRNAs to acute cardiovascular events or coronary intervention.

Keywords

Cardiology, coronary syndrome, clinical trials, heparins

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Introduction

MicroRNAs (miRNAs) are small (19~24 nucleotide) non-coding RNAs, which negatively regulate the expression of target mRNAs at the post-transcriptional level (1). It is estimated that miRNAs control the expression of more than 60% of protein-coding genes (2). Recently, miRNAs have been detected in plasma and serum, and show remarkable resistance to degradation (3). This may be due to their presence in microvesicles, which are impermeable to RNases (4, 5). Other findings suggest that miRNAs may be associated with lipoproteins (6) and proteins of the Argonaute family (7).

Several studies have explored the potential of miRNAs as biomarkers in cardiovascular disease (8). We have previously shown that a cluster of miRNAs identified in plasma of healthy volunteers who were subjected to ischaemia-reperfusion injury, included all miRNAs associated with risk of myocardial infarction in a population-based prospective study (9). Microarray screening revealed that miRNAs in this cluster (miR-126, miR-197, miR-223, miR-24 and miR-21) were highly expressed in platelets as well as platelet microparticles (9, 10).

Others (11, 12) and we (13, 14) have recently demonstrated that anti-platelet therapy is a confounding factor for miRNA measurement. Intravenous heparin is commonly administered in the management of patients with acute cardiovascular disease and percutaneous arterial interventions such as coronary angioplasty. Since heparin is known for its interference with qPCR reactions, we sought to investigate how miRNA measurements can be performed accurately in plasma from patients who have received heparin.

Materials and methods

Study cohort

Written informed consent was obtained from subjects planned for coronary angiography with the view, when appropriate, to proceed to percutaneous coronary intervention (PCI) at the John Radcliffe Hospital, Oxford. Baseline venous blood was collected at the beginning of the angiography procedure [time point Pre (TP-pre)]. In subjects found to have suitable coronary lesions for stenting, an intravenous heparin bolus (75 IU/kg) was administered immediately after baseline blood sampling. Repeat venous blood samples were collected 5 minutes (min) after heparin administration, prior to stent deployment (TP-0), 30 min (TP-30), and 360 min (TP-360) after stent deployment. Subjects who underwent diagnostic angiography but did not proceed to coronary stenting were included as controls and underwent serial blood sampling at the same time points. The Regional ethics committee reviewed and approved the study procedure (Ethics reference: 08/H0603/41).

Anti-platelet therapy

All patients received antiplatelet agents before the angiogram or PCI procedure. Unless patients were already established on long-term aspirin/clopidogrel therapy, they received a loading dose of aspirin (300 mg) and clopidogrel (300 mg) [for elective stable angina (SA) patients - after the pre-assessment clinic, usually within a week of procedure; for acute coronary syndrome (ACS) inpatients - at the time of diagnosis of non-ST elevation myocardial infarction (NSTEMI) during hospital admission]. After the initial loading dose, patients received 75 mg daily doses of aspirin and clopidogrel until the time of PCI procedure. However, in the cases where patients were referred to have PCI soon after hospital admission, they received a loading of aspirin 300 mg + clopidogrel 600 mg right before the PCI procedure. The same immediate loading doses were also given to elective outpatients who have not been compliant with the loading regimen. All diagnostic control patients were scheduled for angiography with the intention to proceed to PCI, therefore they received the same anti-platelet treatments as described above.

Plasma sampling

Blood samples were prepared immediately, in the cardiac catheter laboratory, at room temperature to obtain platelet-poor plasma with the following protocol: BD Vacutainer® containing K_2EDTA was used for the first spin of blood (12 min x 1,300 g). The plasma

	PCI + heparin	Dx + heparin	Dx no heparin	P-value
Number (male)	20(14)	7(5)	10(5)	0.14
Age, years (SD)#	67(10)	72(6)	67(12)	0.50
Acute coronary syndrome, n (%)	8(40)	3(43)	6(60)	0.57
Smoking status, n (%)				
Current smoker	2(10)	1(14)	1(10)	0.94
Past history of smoking (>1 month)	12(60)	5(71)	4(40)	0.39
Never smoked	6(30)	0(0)	5(50)	0.09
Past history of IHD, n (%)				
MI/ACS	6(30)	2(29)	5(50)	0.51
Stable angina	8(40)	3(43)	0(0)	0.05
Hypertension, n (%)	12(60)	6(86)	8(80)	0.32
Hypercholesterolaemia, n (%)	18(90)	4(57)	6(60)	0.09
Diabetes mellitus, n (%)	0(0)	1(14)	0(0)	0.11
Family history of IHD, n (%)	9(45)	4(57)	5(50)	0.85
Regular medication, n (%)				
Aspirin	13(65)	5(71)	8(80)	0.70
Thienopyridine	6(30)	4(57)	4(40)	0.49
Statin	16(80)	6(86)	9(90)	0.77
Beta-blocker	11(55)	4(57)	6(60)	0.97
ACE inhibitor / ARB	11(55)	7(100)	7(70)	0.09

#Comparison of age between groups was performed by one-way ANOVA. All other variables were compared as categorical data using the Fisher's exact test. Dx, diagnostic angiography; IHD, ischaemic heart disease; MI, myocardial infarction.

Table 1: Demographic data of the study cohort.

portion was then transferred to a second BD Vacutainer® without additive for the second spin (15 min x 2,500 g). Confirmation of platelet-poor plasma was established by platelet counts (15). Plasma aliquots were stored at −80°C for subsequent analysis.

Taqman qPCR assay

miRNAs were extracted using the miRNeasy kit (Qiagen). A fixed volume of 3 µl of the 25 µl RNA eluate was used as input for reverse transcription reactions as described previously (5, 16). In brief, miRNAs were reverse transcribed using Megaplex Primers Pools (Human Pool A v2.1, Applied Biosystems, Foster City, CA, USA). Reverse transcription reaction products were further amplified using the Megaplex PreAmp Primers (Human Pool A v2.1). Both reverse transcription and PreAmp products were stored at -80°C. Taqman miRNA assays were used to assess the expression of individual miRNAs. 4.5 µl diluted pre-amplification product were combined with 0.5 µl Taqman miRNA Assay (20x) (Applied Biosystems) and 5 µl Taqman Universal PCR Master Mix No AmpErase® UNG (2x) to a final volume of 10 µl. qPCR was performed on an Applied Biosystems® ViiA™ 7 Real-Time PCR System at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Relative quantification was performed using the ViiA™ 7 Software v1.1 (Applied Biosystems). Exogenous *C. elegans* miRNA (*cel*-miR-39) was used as spike-in normalization control.

Statistical analysis

Given the skewed distribution of plasma miRNA concentrations, we performed analyses using log-transformed values. Changes in miRNA concentration over time were summarised as fold changes and 95% confidence intervals (CI) compared to the earliest available measurement. For each group, we tested for differences across time points using linear mixed models. P-values were corrected for multiple testing using the Bonferroni method.

Results
Study cohort

Twenty subjects had suitable coronary lesions and proceeded to have coronary stenting [ACS: n=8, SA: n=12], 17 subjects underwent diagnostic angiography only. These diagnostic angiography control subjects all had angiographic evidence of coronary atherosclerosis, but were either recommended for coronary bypass surgery or medical therapy only. Ten of these subjects did not receive heparin (ACS: n=6, SA: n=4), while 7 did (ACS: n=5, SA: n=2). In our serial sampling design, timing related to stent deployment in the PCI cohort. In subjects who did not receive intravenous heparin, TP-0 min related to the beginning of the angiography procedure. There was no difference between subjects of the three pro-
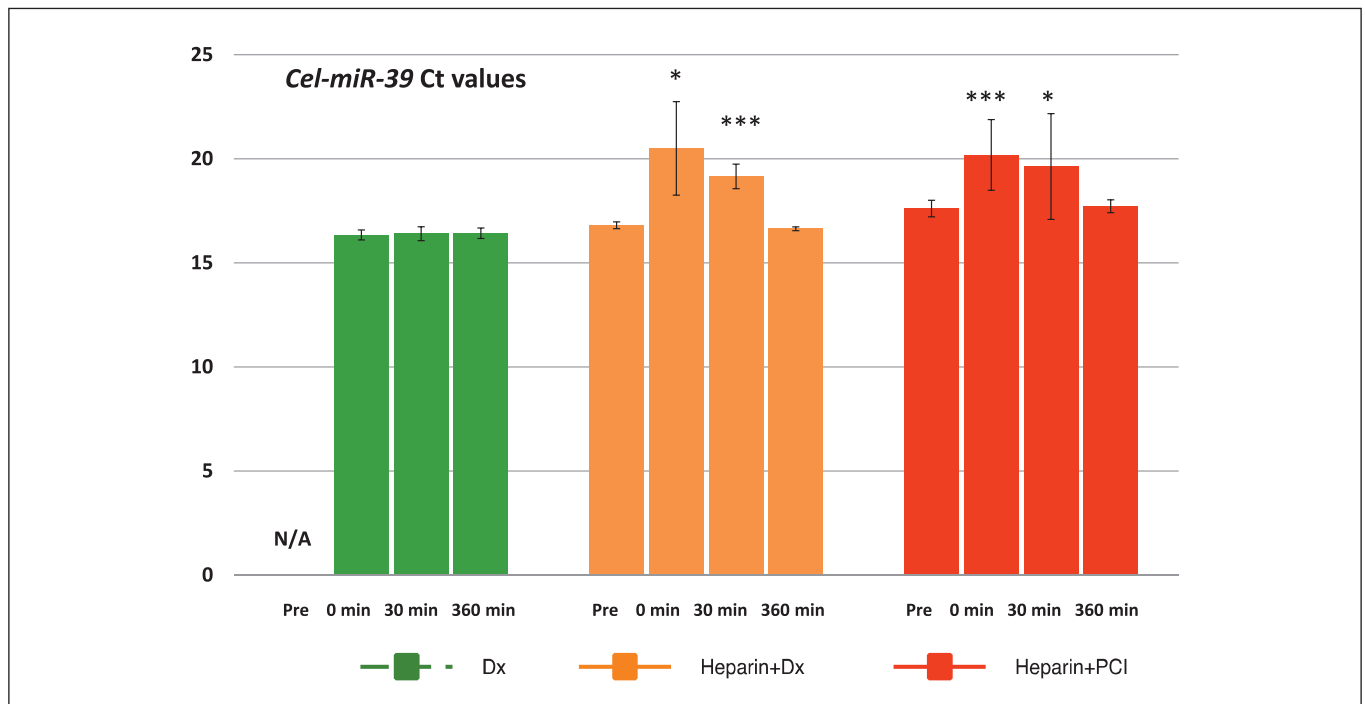

Figure 1: Effect of heparin on *C. elegans* spike-in control (Cel-miR 39). Platelet-poor EDTA plasma was collected from patients undergoing PCI (n=20) at four time points: before heparin administration (TP-pre), 5 min after heparin administration but just prior to stent deployment (TP-0 min), and 30 and 360 min after stent deployment (TP-30 min and TP- 360 min). Additional samples were obtained from patients undergoing cardiac catheterisation for diagnostic purposes (Dx) with (n=7) and without (n=10) heparin administration. Values are means ± SD. P-values were derived from paired Student's t-test: * denotes P<0.05; *** denotes P<0.001. N/A denotes "not applicable".

cedural groups. The demographic data for the study cohort is presented in ►Table 1.

miRNA measurements

A total of 14 miRNAs (miR-122, miR-125b, miR-126, miR-150, miR-191, miR-195, miR-197, miR-20a, miR-20b, miR-223, miR-24, miR-28-3p, miR-320, miR-454) plus U6 and the *C. elegans* spike-in control (*Cel*-miR-39) were assessed in platelet-poor plasma. At 5 min after heparin administration (TP-0 min), the detectability of *Cel*-miR-39 decreased by approx. 3 cycles (►Figure 1). In comparison, the effect on endogenous miRNAs was less pronounced (17).

Normalisation procedures

MiRNA levels were first normalised to exogenous *Cel*-miR-39 (►Figure 2). In a second round, the average cycle threshold (Ct) value of all endogenous miRNAs was applied as normalisation control (►Figure 3). Prominent effects of heparin on the normalisation with exogenous spike-in control were apparent in samples collected shortly after heparin administration (at TP-0 min and TP-30 min). At TP-360 min, both normalisation methods provided similar data. The alternative of normalising miRNA levels to the average Ct value of all miRNAs was capable of overcoming heparin effects within the first hour post dose.

Discussion

Administration of intravenous heparin has been shown to cause acute derangements of protein biomarkers (18, 19). Heparin also interferes with qPCR measurements, and therefore may confound

What is known about this topic?

- MicroRNAs are small non-coding RNAs that are implicated as novel biomarkers for acute cardiovascular disease.
- Heparin is known for its interference with measurements based on real-time polymerase chain reactions.
- In many microRNA studies to date, samples were taken during coronary angiography. Timing to heparin administration was not taken into consideration.

What does this paper add?

- Even systemic heparin administration in a single bolus results in derangements of microRNA measurements, including the normalisation controls.
- The effect of heparin is temporary, but has direct implications on the interpretation of microRNA measurements in cardiovascular patients.
- Following heparin administration, alternative ways of normalisation are required to ensure appropriate evaluation of temporal changes in plasma microRNA levels.
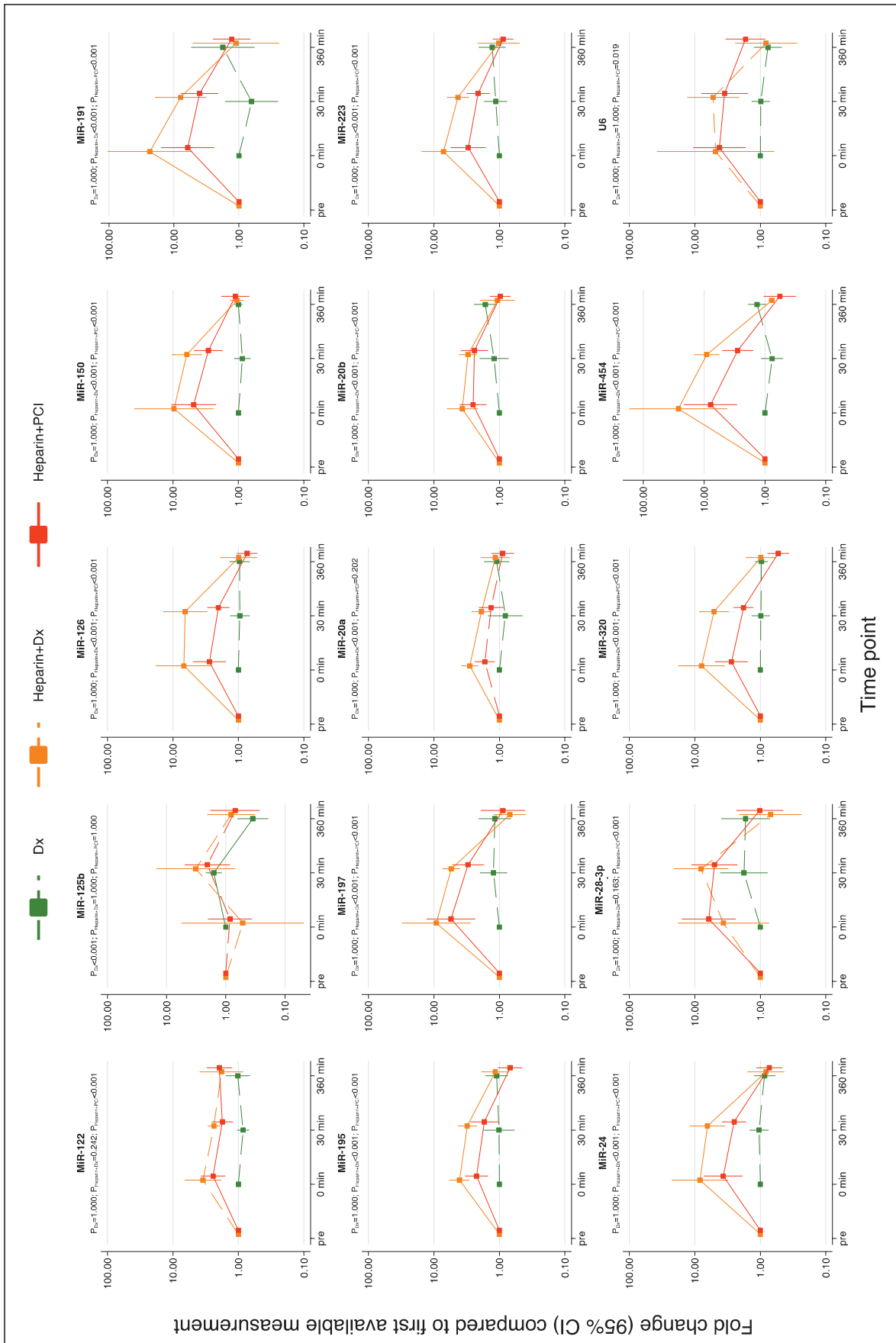
the results of clinical studies investigating miRNAs. Effects of heparin, in particular on the spike-in *C. elegans* control, were observed within the first hour post administration but not at 6 hours post dose. Normalisation to endogenous miRNAs (20) provided a more reliable means of assessing miRNAs in patients receiving intravenous heparin therapy.

Heparin effect on PCR reactions

The anticoagulant properties of heparin are primarily attributed to its interaction with anti-thrombin III, a member of the serine protease inhibitor superfamily of proteins that reacts with serine proteases to form inactive complexes. Heparin induces a conformational change that greatly enhances the inhibitory activity of anti-thrombin III. With the exception of factor VIIa, all coagulation factors are serine proteases and inhibited by anti-thrombin III, forming a covalent 1:1 complex (21). Similar to its *in vivo* interaction with anti-thrombin III, *in vitro* conjugation between heparin and DNA polymerases is known to occur (22, 23). Besides, heparin is highly negatively charged and binds magnesium ions required for the qPCR reaction (23).

Heparin effect on miRNAs

The heparin effect on small RNAs cannot be fully explained by interference with polymerases or magnesium ions as the exogenous *C. elegans* spike-in control was more affected than endogenous miRNAs. Heparin is able to disrupt already formed enzyme-template complexes, and this displacement of the template could be sequence-dependent (24, 25). Another possibility is that the strength of the interference is dependent on the compartmentalisation of miRNAs (20). Different ways to minimise the effect of heparin on PCR reactions have been proposed: 1) Inhibition of DNA polymerase activity by heparin can be overcome by addition of excess DNA polymerase (26). 2) By careful titration of the starting material PCR-inhibitors may be diluted below a threshold of interference to yield more accurate miRNA quantitation (27). 3) Samples can be treated with antibodies against heparin or with heparinase to overcome inhibitory effects of heparin (28, 29), but these additional steps may introduce sample-to-sample variation in the qPCR reactions (30). 4) In the current study, we demonstrate that interference of heparin with miRNA measurements is confined to the first hours post dose and that miRNA assessment in patients on heparin can be performed more reliably when using the Ct average of endogenous miRNAs as normalisation control. Nonetheless, this approach is expected to result in weaker associations because levels of individual miRNAs and their clusters contribute to the Ct average.

Conclusion

There is increasing interest in applying circulating miRNAs as outcome predictors in cardiovascular patients (31-34). Quantification of plasma miRNAs, however, is affected by administration of heparin used in interventional cardiology. The effect of heparin is tem-

Figure 2: Normalisation using C. elegans spike-in control (Cel-miR-39). Patient cohorts as described in legend to Figure 1. In total, 14 miRNAs plus U6 were assessed in all samples. MiRNA levels were normalised to Cel-miR 39. Note that TP-0 min refers to the stent deployment in the PCI cohort but plasma was sampled 5 min after heparin administration in all patients.
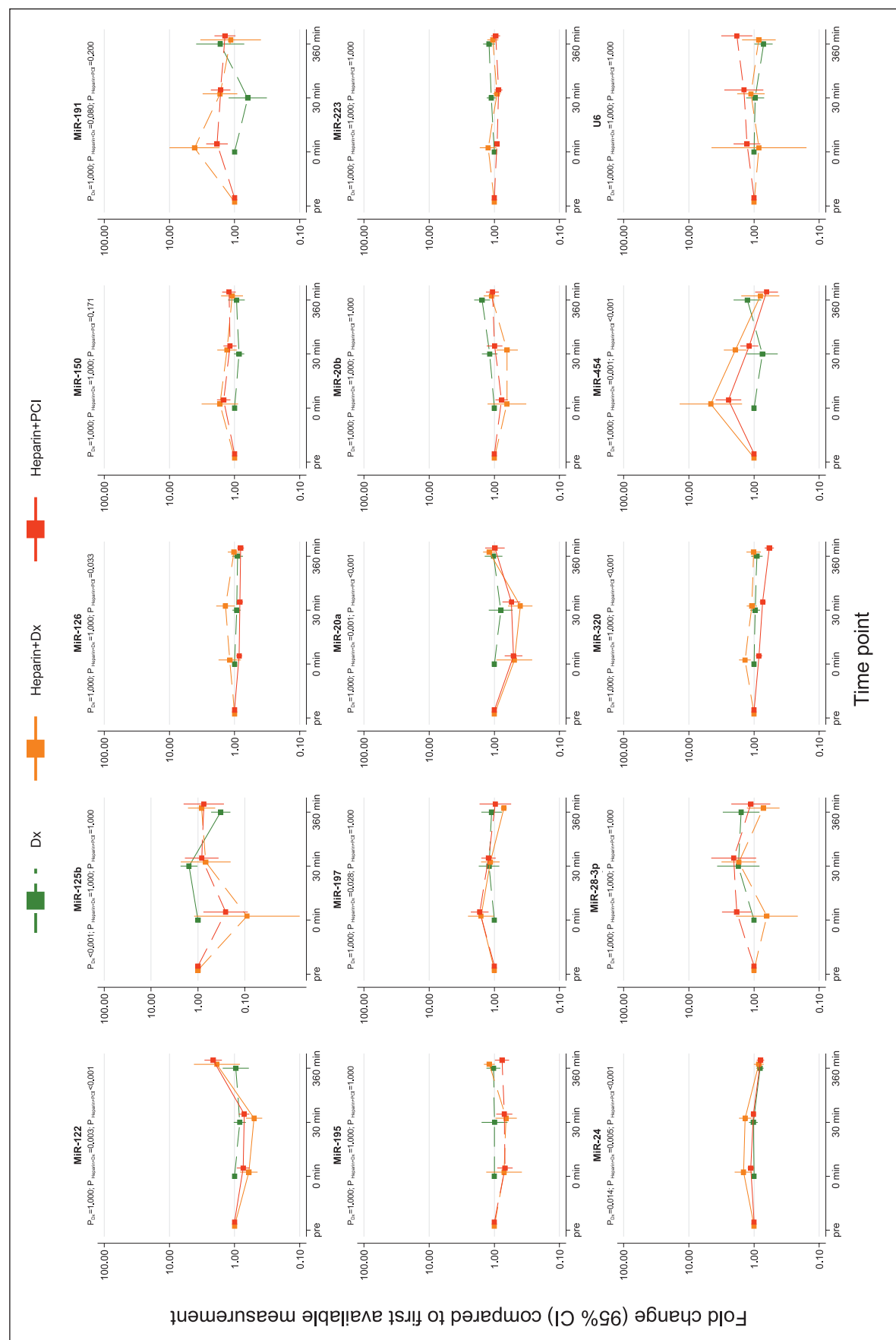
Figure 3: Normalisation using the average cycle threshold (Ct) value. Patient cohorts as described in legend to Figure 1. MiRNA levels were normalised to the average Ct value of all endogenous miRNAs. Note the discrepancies at TP-0 min and TP-30 min compared to the normalisation of miRNA measurements with exogenous *Cel*-miR-39.

Abbreviations

ACS, acute coronary syndrome; Dx, diagnostic angiography; miRNA, microRNA; PCI, percutaneous coronary intervention; qPCR, real-time quantitative polymerase chain reaction; SA, stable angina; TP, time point.

porary, but has direct implications on the interpretation of miRNA measurements in cardiovascular patients. Following heparin administration, alternative ways of qPCR normalisation are required to ensure appropriate evaluation of temporal changes in plasma miRNA levels (20).

Conflicts of interest

The authors (P.W., A.Z., S.K., M.M.) filed patent applications related to circulating miRNAs as cardiovascular biomarkers. R.F.S. reports receiving research grants, honoraria and/or consultancy fees from AstraZeneca, Eli Lilly/Daiichi Sankyo, Merck, Accumetrics, Novartis, Iroko, Sanofi-Aventis/BMS, Medscape, Roche and Esai.

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