Comparative Analysis of Circulating Non-Coding RNAs Versus Protein Biomarkers in the Detection of Myocardial Injury

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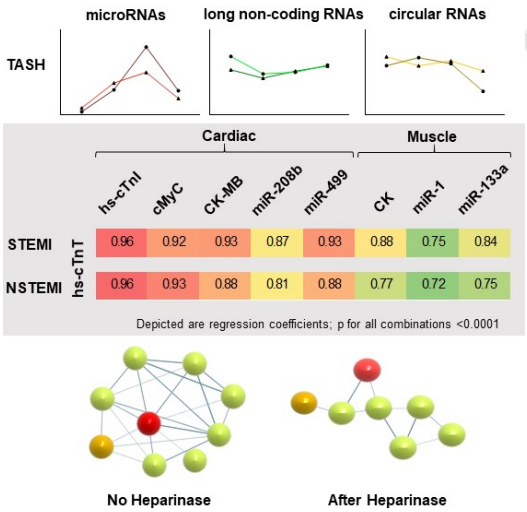
ABSTRACT

***Rationale*:** Non-coding RNAs (ncRNAs), including microRNAs (miRNAs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs), are proposed novel biomarkers of myocardial injury. Their release kinetics have not been explored without confounding by heparin, nor has their relationship to myocardial protein biomarkers.

***Objective*:** To compare ncRNA species in heparinase-treated samples with established and emerging protein biomarkers for myocardial injury.

***Methods and Results*:** Screening of 158 circRNAs and 21 lncRNAs in human cardiac tissue identified 12 circRNAs and 11 lncRNAs as potential biomarkers with cardiac origin. 11 miRNAs were included. At low spike-in concentrations of myocardial tissue, significantly higher regression coefficients were observed across ncRNA species compared with cardiac troponins and cardiac myosin-binding protein C (cMyC). Heparinase-treatment of serial plasma and serum samples of patients undergoing transcoronary ablation of septal hypertrophy (TASH) removed spurious correlations between miRNAs in non-heparinase-treated samples. After TASH, muscle-enriched miRNAs (miR-1 and miR-133a) showed a steeper and earlier increase than cardiac-enriched miRNAs (miR-499 and miR-208b). Putative cardiac lncRNAs, including LIPCAR, did not rise, refuting a predominant cardiac origin. Cardiac circRNAs remained largely undetectable. In a validation cohort of acute myocardial infarction, receiver operating characteristic curve analysis revealed noninferiority of cardiac-specific miRNAs, but miRNAs failed to identify cases presenting with low troponin values. cMyC was validated as a biomarker with highly sensitive properties, and the combination of muscle-enriched miRNAs with hs-cTnT and cMyC returned the highest area under the curve values.

***Conclusions*:** In a comparative assessment of ncRNAs and protein biomarkers for myocardial injury, cMyC showed properties as the most sensitive cardiac biomarker while miRNAs emerged as promising candidates to integrate ncRNAs with protein biomarkers. Sensitivity of current miRNA detection is inferior to cardiac proteins but a multi-biomarker combination of muscle-enriched miRNAs with cMyC and hs-cTnT could open a new path of integrating complementary characteristics of different biomarker species.



Keywords:
Non-coding RNA • myosin binding protein c • troponin • biomarkers • myocardial infarction.

Nonstandard Abbreviations and Acronyms:

AUC	area under the curve
circRNA	circular RNA
cMyC	cardiac myosin binding protein-C
LIPCAR	long intergenic non-coding RNA predicting cardiac remodeling and survival
lncRNA	long non-coding RNA
miRNA/miR	microRNA
ncRNA	non-coding RNA
PEA	proximity extension assay
ROC	receiver operating characteristic
TASH	transcoronary ablation of septal hypertrophy

INTRODUCTION

In its most recent and fourth definition of myocardial infarction (MI), the European Society of Cardiology has refined approaches to classify and differentiate MI[1]. While higher sensitivity troponin assays have improved the identification of low risk patients suitable for immediate discharge, detecting and treating minor cardiac damage may fail to result in better clinical outcomes[2]. There is still a need for biomarkers that facilitate early rule-out/rule-in of clinically relevant MI. Using proteomics, we discovered that cardiac myosin binding protein C (cMyC) is released earlier upon myocardial ischemia than cardiac troponins[3] and may contribute to a better rule-out/rule-in classification of MI[4].

Recently, non-coding RNAs (ncRNAs) have been implicated as biomarkers of MI. MicroRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) are among the ncRNAs present in the circulation. Plasma and serum levels of muscle- and cardiac-enriched miRNAs increase markedly after MI[5,6]. Besides miRNAs, lncRNAs and circRNAs have attracted interest as potential biomarkers in CVD. Levels of the Long Intergenic ncRNA Predicting CArdiac Remodeling (LIPCAR) were reported to predict adverse cardiac remodelling and death after MI[7]. On the other hand, circRNAs as a different ncRNA species are less susceptible to RNase activity and may offer tissue specificity with >15,000 circRNAs being present in the human heart[8,9].

Heparin, an anticoagulant commonly administered in the clinical setting of MI, is a major confounding factor for measurements of ncRNAs by real-time polymerase chain reaction (qPCR). Few studies on the release of ncRNAs after MI used heparinase treatment to overcome this confounding effect by heparin, a prerequisite for comparative analysis of ncRNA and protein biomarkers. Moreover, while circulating levels of muscle- and cardiac-enriched miRNAs have been shown to correlate to troponins after MI, the release of ncRNAs and novel protein biomarkers such as cMyC have not been compared in the clinically most relevant setting of MI patients presenting early with low troponin values.

The objective of this study was to use heparinase-treatment to establish the release kinetics of three different ncRNA species (miRNAs, lncRNAs, circRNAs) in serial samples from patients undergoing transcoronary ablation of septal hypertrophy (TASH) as well as in patients with acute MI presenting with a wide range of high sensitive cardiac troponin (hs-cTn) levels in the Biomarkers in Acute Cardiac Care study (BACC study, n>2500). The performance of ncRNAs is compared with hs-cTn and cMyC as established and novel protein biomarkers of cardiac injury, respectively.

METHODS

The authors declare that all supporting data are available within the article and its supplementary files. Larger data sets such as array data are available from the corresponding author upon reasonable request.

RNA extraction.
Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, with some modifications. In brief, 100µl of serum or plasma were combined with 694.75µl of Qiazol lysis reagent, 4µl of diluted synthetic *Caenorhabditis elegans* miR-39 (cel-miR-39-3p) spike-in and 1.25µl carrier RNA from bacteriophage MS2 (Roche). Following brief incubation at room temperature, 140 µl of chloroform was added and the solution was mixed vigorously. Samples were then centrifuged at 13,500 x *g* for 15 minutes at 4°C. 280 µl of the upper (aqueous) phase were transferred to a new tube and mixed with 1.5 volumes (420 µl) of 100% ethanol and applied to columns and washed according to the manufacturer's protocol. Total RNA was eluted in 35 µl of nuclease-free H_2O by centrifugation at 8500 x *g* for 1 minute at 4°C.

Heparinase treatment.
ncRNA analyses. Prior to reverse transcription, the extracted RNA was treated with heparinase 1 from Flavobacterium (Sigma) according to the following protocol: 5µl of each sample were combined with 1.25µl heparinase, 0.25µl of RNase inhibitor (Ribo Lock 40U/µl, Thermofisher) and 3.5µl of heparinase buffer (pH 7.5) and thoroughly mixed, then incubated at 25°C for 3 hrs. The samples were then immediately used for reverse transcription. For comparison, a buffer-only group was treated with heparinase buffer devoid of heparinase, which was incubated under the same conditions as the heparinase-treated samples. The untreated group received neither heparinase nor buffer, nor was it left for incubation, but instead was used for further reverse transcriptase together with the treated samples.

Proximity Extension Assay (PEA). cTnI was part of the organ damage panel offered by Olink (Uppsala, Sweden). Human plasma samples were treated by adding 0.1U (concentration: 0.2U/µl) of heparinase 1 from Flavobacterium (Sigma) per 1µl of plasma. 0.5µl of the heparinase solution was added per 1µl of plasma. The mixture was then incubated for 1h at 30°C as previously described[10].

Reverse transcription.
For reverse transcription two different platforms 1) for miRNAs (miRCURY LNA RT kit (Exiqon)) and 2) for lncRNAs and circRNAs (SuperScript VILO cDNA Synthesis Kit (Invitrogen)) were used. For further details see online-only Data Supplement.

Real-time PCR assays.
A list of primers used for qPCR detection and their sequence is provided in **Online Table I**. For further details see online-only Data Supplement.

RNA quantification.
In the analyses of raw Cq data, any value measurements beyond 35 cycles were considered undetectable. For details see online-only Data Supplement. In brief, the relative quantitation for RNAs was performed as follows:

Analysis of miRNAs. In TASH samples as well as the MI cohort the delta-delta Cq method was used for relative quantification, using cel-miR-39-3p as a normalisation control. Quantification results were calibrated against the median of three identical replicates consisting of equal volumes from all TASH or all

MI samples, respectively. Relative quantification was performed with Microsoft Excel, version 15.32 for MacOS. In the myocardial tissue *in vitro* spike-in experiment normalisation was also performed using *Cel-miR-39* spike in. Calibration was performed using the median value of all samples per individual RNA assay to remove assay-related biases.

Analysis of circRNAs and lncRNAs. Calibration of circRNAs and lncRNAs in the TASH samples was performed against the median of all samples for each assay. In the myocardial tissue spike-in experiment the same method was used. The relative quantity was calculated as described above for miRNAs.

Myocardial tissue spike-in experiment.
In order to assess the detectability of RNAs with cardiac origin, different amounts of human heart tissue were spiked into human plasma. For details see online-only Data Supplement and **Online Figure I**.

Selection of ncRNAs.
Two cardiac-enriched miRNAs (miR-208b-3p, miR-499a-5p), two muscle-enriched miRNAs (miR-1, miR-133a-3p) and 7 additional miRNAs were included in the analyses for their good detectability in human plasma as non-cardiac/non-muscle counterparts. For circRNAs we performed a microarray-based screening in 4 pooled samples per time point of the TASH cohort (n=16 pooled samples) (Arraystar Human Circular RNA Array, Arraystar INC, 9430 Key West Avenue 128, Rockville, MD 20850). For details see online-only Data Supplement. To complement this screening, a literature search was performed. circRNAs were selected from four deep sequencing datasets reporting >15,000 circRNAs, of which 158 circRNAs were RNase-R treated and validated by qPCR. These 158 circRNAs plus their linear transcripts were first tested in 12 human cardiac tissue samples. For circRNAs with more than one transcript from the same gene, the one with the best detection based on Cq values was chosen. Only circRNAs with Cq values of < 25 cycles that were derived from cardiac and/or muscle-associated genes (n=12) were included. lncRNAs were selected based on microarray screening of 33,045 lncRNAs in human plasma[7]. Of these, 768 lncRNAs showed differential plasma levels in patients developing heart failure after MI. 21 lncRNAs with high signal intensity were validated by qPCR. As for circRNAs, only lncRNAs with Cq values < 25 (n=11) were selected for further analyses. A graphical depiction of the selection process including references is shown in **Online Figure II**.

Statistical analyses.
Tissue spike-in experiment. To enable comparisons of the relative expression values for ncRNAs and the absolute concentrations for protein biomarkers, relative quantities have been calculated for all molecules by dividing their values with the median quantity for each molecule. Then linear regression curves were calculated for all miRNAs, circRNAs, lncRNAs and cardiac proteins study their release kinetics by comparing the regression curves slopes. All R^2 values were >0.9, therefore, the used linear model provides a good fit to the data. Next, the slopes of the regression curves of the three molecules with the highest scores were selected from each category: for miRNAs miR-133a, miR-208b and miR-499; for circRNAs: circALPK2, circMYBPC3, and circSLC8A1; for lncRNAs: lncDANCR, lncH19, and lncRNACOX2; for proteins: hs-cTnT, hs-cTnI, and cMyC. Mann-Whitney U-tests[12] were used to perform pairwise statistical comparisons between the slopes of proteins and ncRNAs.

TASH cohort. To study the release kinetics of proteins and ncRNAs at 1h after TASH, absolute protein measurements and relative RNA measurements were both transformed into relative values on the same scale by dividing each value by the overall maximum value of the single biomarker across all time points. The data for each molecule and each patient were curve-fitted using linear regression and slopes of the curves were calculated. Mann-Whitney U-tests were used to perform pairwise statistical comparisons between the slopes of the regression curves of protein and ncRNA molecules.

BACC study. Analyses to study release kinetics after acute MI were performed analogous to the TASH cohort for the first hour after hospital presentation. Correlation analyses of biomarkers in the acute MI cohort were performed with Graph Pad Prism 7.0d for MacOS. Nonparametric Spearman correlation was used since none of the biomarkers were normally distributed. P-values in the correlation analyses were two-tailed and approximate values were calculated.

ROC analyses. For training and testing regression models for predicting the time from onset of MI combining miRNAs and proteins we used a hybrid of a heuristic algorithm and Support Vector Regression models (details are provided in the Supplementary Material).

RESULTS

Detectability of ncRNAs in human plasma.

To compare detectability of different ncRNAs human myocardial tissue was spiked into plasma of healthy volunteers at defined concentrations of 0.25µg to 25µg/100µl plasma (**Online Figure I**). Based on published data, we selected 158 circRNAs and 21 lncRNAs that were reported as abundant in human myocardium (for details see Methods section and **Online Figure II**). circRNAs associated with cardiac-specific proteins such as cTnT, cTnI and cMyC were amongst the least well-detectable circRNAs in plasma (data not shown). Muscle- (miR-1, miR-133a) and cardiac-enriched miRNAs (miR-208b, miR-499) were chosen for comparison. These four miRNAs showed comparable regression curves to cardiac circRNAs (circSLC8A1, circMyBPC3, circALPK2) and lncRNAs (lncLIPCAR, lncH19 lncuc004.cov4) **(Figure 1A, Online Figure III, IV and V**). Levels of other miRNAs remained unaltered upon spiking human myocardial tissue into plasma (**Online Figure VI**). Next, ncRNA spike in results were compared with measurements of established and novel cardiac protein biomarkers as previously described[11] (**Online Figure VII**). Whilst ncRNAs demonstrated a continuous, linear dose-response-curve across all spike-in concentrations, measurements of cardiac proteins (hs-cTnT, hs-cTnI, cMyC) remained below their regression curve at low spike-in concentrations (0.25µg and 2.5µg/100µl plasma) (**Figure 1A,** coloured boxes). At low spike-in concentrations, ncRNA regression curves were steeper compared with cardiac protein biomarkers (**Figure 1B**). Curve fitting analyses for low spike-in concentrations returned significantly higher regression coefficients for ncRNA species (**Figure 1C**, Mann-Whitney test for comparison against cardiac protein biomarkers: miRNAs $p<0.0001$, fold-change 2.6; circRNAs $p=0.0028$, fold change 2.8; lncRNAs $p=0.0028$, fold-change 1.6).

Confounding by heparin in ncRNA analysis.

The derangements of ncRNA biomarker measurements after heparin administration can be addressed by heparinase treatment as demonstrated in two examples: First, human plasma was spiked with 10 IU of heparin per 1ml plasma. Heparin reduced the detectability of the exogenous spike-in control *Cel-miR-39* and endogenous miRNAs resulting in elevated raw Cq values, which was reversed by heparinase treatment (**Figure 2A**). Second, we assessed samples from a cohort of patients undergoing TASH[13], where the exact time point of myocardial injury and heparin administration were known and samples were obtained before myocardial injury. We evaluated plasma miRNA levels before, and 1h, 8 and 24hrs after induced myocardial injury in TASH patients (n=16). In non-heparinase-treated samples we discovered a dense miRNA correlation network, which consists of spurious correlations between miRNAs independent of their cellular origin. This observation contradicts the well-known cell- and tissue specific expression of miRNAs **(Figure 2B)**. Notably, liver-specific miR-122 and red blood cell-enriched miR-486 appeared in the same cluster area. Heparinase treatment resolved the clustering of the network removing the correlations between miRNAs in non-heparinase-treated samples. Thus, the distinct cellular origins of non-cardiac

derived plasma miRNAs became readily apparent: as visualised in **Figure 2B**, the clustering shows liver miR-122 and red blood cell miR-486 in separate cluster areas from previously reported platelet-enriched miRNAs (miR-126, miR-223, miR-191).

Release kinetics of ncRNAs after TASH.

To assess the release of ncRNAs after myocardial injury, serial samples were obtained from patients undergoing TASH[14]. Upon heparinase treatment, the release of ncRNAs was compared with hs-cTnT and cMyC at baseline, 1h, 8 hrs, and 24 hrs after induced myocardial injury. The clinical characteristics of the TASH patients were reported previously[14]. Plasma and serum from 16 patients at 4 time points were available for comparative analyses of the release of muscle- (miR-1, miR-133a, **Figure 3A**) and of cardiac-enriched miRNAs (miR-208b, miR-499, **Figure 3B**), circRNAs (circSMARCA, circPCMTDL, **Figure 3C**) and lncRNAs (lncLIPCAR, lncH19, **Figure 3D**). These circRNAs and lncRNAs were chosen for their best detectability in plasma and serum. Unlike muscle-enriched miRNAs, the two cardiac-enriched miRNAs were undetectable at baseline. MiR-208b became detectable at 1h after TASH. For miR-499, detectable levels were only reached at 8h after TASH. Neither mitochondrial lncRNA LIPCAR nor nucleus-derived lncRNA H19 changed after TASH (**Figure 3D**). Thus, LIPCAR and lncRNA H19 levels are not of cardiac origin. Unlike other ncRNA classes, cardiac circRNAs showed poor detectability at baseline and after TASH (**Figure 3C)**, despite the fact that these circRNAs were readily detectable in cardiac tissue (**Online Figure VIII)**. An additional circRNA microarray screening of 13,617 circRNAs performed at all 4 time points did not return any significantly dysregulated circRNAs after TASH (**Online Figure IX**).

Comparison of cardiac protein versus ncRNA biomarkers in TASH.

Figure 4 depicts the time course of serum levels of cardiac protein biomarkers (hs-TnT, cMyC, **Figure 4A**) and circulating muscle-enriched miRNAs after TASH (miR-1, miR-133a, **Figure 4B**). As reported previously[15], cMyC levels peaked before hs-TnT. Similarly, muscle-enriched miRNAs (miR-1, miR-133a) peaked earlier than cardiac protein biomarkers. When the measurements of cardiac proteins and the two muscle-enriched miRNAs, miR-1 and miR-133a, were expressed as proportions of the maximum detected value (**Figure 4C**), cMyC showed a significantly higher regression coefficient compared with hs-cTnT ($p<0.0001$, Mann-Whitney test, **Figure 4D**). Similarly, higher regression coefficients were observed for muscle-enriched miRNAs compared to cardiac protein biomarkers within the first hour after induced myocardial injury (Mann-Whitney test; hs-TnT vs. miR-1 $p<0.0001$, hs-TnT vs. miR-133a $p<0.0001$, cMyC vs. miR-1 $p=0.0091$, cMyC vs. miR-133a $p=0.0088$) (**Figure 4D**).

Circulating miRNAs and proteins were measured by qPCR and enzyme-linked immunosorbent assay, respectively. To rule out that the different assay methodology impacts on the observed release kinetics, cTnI was assessed using a PEA. The PEA combines dual antibody-based detection with qPCR-based quantification. While the PEA for cTnI was less sensitive compared to hs-cTnT, both assays revealed a similar temporal profile for the cTn release after TASH (**Figure 4C**). The PEA measurements of cTnI were not affected by heparin (**Online Figure XA-C**) due to the minute amount of sample input required compared to miRNA measurements (1µl of plasma for cTnI vs 100µl for miRNAs).

Comparison of miRNAs, cMyC and troponins in patients with acute MI.

To compare miRNA kinetics in patients with acute MI, we analysed plasma samples from a carefully selected subcohort (n=83) of the BACC study[16], focusing on patients with initially low hs-cTnT levels which show a steep increase within the first hour after hospital presentation (**Online Table II**). Samples were taken on admission, 1h and 3 h thereafter in 38 acute MI patients. 45 patients with non-cardiac chest pain served as controls. The plasma levels of muscle- and cardiac-enriched miRNAs strongly

correlated with concentrations of hs-cTnT, hs-cTNI and cMyC (**Figure 5)**. Correlations were stronger in patients with ST elevation myocardial infarction (STEMI, n=20) than in patients with non-ST elevation myocardial infarction (NSTEMI, n=18) (**Online Figure XI**). As expected, given their cardiac enrichment, the highest correlation with hs-cTnT was observed for the two cardiac-enriched miRNAs, miR-208b and miR-499 (r=0.81 and r=0.88, respectively, p<0.0001), which is as high a correlation level as between hs-cTnT and cMyC and hs-cTnT and hs-cTnI (r=0.87 and r=0.83, respectively, p<0.0001). The correlation was substantially weaker for the two muscle-enriched miRNAs, miR-1 and miR-133a (r=0.67 for both, p<0.0001). Correlations of miRNAs with cMyC were comparable with correlations of miRNAs with hs-cTnT.

Since the diagnosis of acute MI was adjudicated based upon hs-cTnT, hs-cTnI was measured[16]. Data for hs-cTnI were comparable with hs-cTnT **(Online Figure XII A** and **B**). At time point 0h and 1h, one sample was below the LOD for hs-cTnT for each time point, while hs-cTnT was detectable in all MI patients at 3h. In contrast, all miRNAs showed numerous undetectable values in the MI group (**Figure 6A**). Again, this was more pronounced in NSTEMI patients than in STEMI patients (**Online Figure XIII**). Hs-cTnT was above the lower limit of detection in 85% of the control patients (n=45), while cMyC levels were above the lower limit of detection in 100% of the measurements, including control patients (**Online Figures XII C and XIV**).

Next, miRNA levels were reported for defined hs-cTnT groups (**Figure 6B**). Only at high hs-cTnT concentrations (>1000ng/L), miRNAs were detectable in all MI patients. At low-positive hs-cTnT levels (comprising hs-cTnT levels between 21 and 50ng/L), miR-1, miR-133a, miR-208b and miR-499 were detectable in 47%, 87%, 7% and 13% of patients, respectively. In patients with hs-cTnT concentrations below 10ng/ml, miR-208b and miR-499 remained below the detection threshold (Cq of >35) whereas miR-1 and miR-133a were detectable in 39% and 64% of patients, respectively. To validate this finding, we included an additional group of 19 carefully selected MI patients with hs-cTnT levels of <1000ng/L at all 3 time points (n=57 samples, **Online Table III**). In an attempt to maximize detectability, we doubled the input of RNA for the RT-qPCR reaction. The rise in miR-1, miR-133a, miR-208b and miR-499, however, was mainly detectable at hs-cTnT levels >50-100 ng/L (**Online Figure XV**).

Analogous to the TASH results, miR-1 (44%) and miR-133a (63%) were also more readily detectable in the control group compared to miR-208b (0%) and miR-499 (10%) (**Online Figure XIV**). cMyC was fully detectable at 100% in all control and MI samples. Confirming the results from the TASH patients, in the MI cohort, cMyC showed a steeper increase shortly after hospital presentation than hs-cTnT with yet smaller coefficients of variation at all time points in the MI cohort (**Figure 7A)**. Curve fitting analysis revealed significantly higher regression coefficients for cMyC than for hs-cTnT and muscle- and cardiac miRNAs (**Figure 7B**).

Comparison of receiver operating characteristic analyses based on the TASH and MI cohorts.

When comparing patients before the TASH procedure with any of the time points after (1h, 8h, 24h), both cardiac miRNAs -208b and -499 showed a higher predictive value (area under the curve, AUC 0.934 and 0.948, respectively) than hs-cTnT (0.918) for the detection of myocardial injury (**Figure 8A, Online Figure XVI, Online Table IV**). The combination of hs-cTnT with cardiac miR-208b or cardiac miR-499 improved AUC values to 0.943 and 0.957, respectively. A combination of both cardiac miRNAs offered no further improvement in the predictive value. AUC values for muscle miR-1 and miR-133a were lower (0.824 and 0.790, respectively) despite their higher sensitivity. cMyC was the cardiac biomarker with the highest predictive power (0.967).

Next, the MI cohort was used as an independent validation cohort for the predictive analytics from TASH. Thus, the most promising regression models of the different combinations of proteins and miRNAs

were applied to the MI cohort. When comparing control patients with acute MI patients at any of the time points (0h, 1h, 3h), both cardiac miRNAs -208b and -499 showed similar predictive power (AUC values of 0.920 and 0.921, respectively) as hs-cTnT (0.925), for the prediction of MI. This result is comparable to the receiver operating characteristic (ROC) analyses in the TASH cohort. AUC values for muscle miR-1 and miR-133a were lower (0.825 and 0.734, respectively) despite their higher sensitivity in plasma, demonstrating the lack of cardiac specificity of muscle-enriched miRNAs (**Figure 8B, Online Figure XVII, Online Table V**). The highest AUC value in TASH was observed for cMyc (0.967), while in the MI cohort, the best performance in the ROC analysis was observed for the combination of hsTnT, cMyc and muscle-enriched miRNAs (0.969).

Finally, cases of STEMI and NSTEMI (excluding NSTEMI Type 2) were assessed separately to explore different aetiologies of myocardial injury. The ROC analyses presented in **Figure 8C** and **8D** indicate that the performance of the diagnostic models is better in STEMI patients. The performance in the NSTEMI group was inferior to both STEMI and all MI cases. Importantly, the ranking of biomarker performance was consistent in all three comparisons, independent of the overall performance of the model.

DISCUSSION

Thus far, most attention has focused on miRNAs although new classes of ncRNAs have been identified in the circulation[17, 18]. In addition to miRNAs, we assessed the potential of selected lncRNAs and circRNAs to serve as biomarkers of myocardial injury. To the best of our knowledge, no study has directly compared these classes of ncRNAs with cardiac protein biomarkers. Using heparinase-treated samples, we assessed the release of ncRNAs after TASH in a well-controlled context of cardiac injury and of miRNAs in the most relevant clinical setting of MI cases presenting with low initial troponin values.

Heparinase treatment to overcome confounding by heparin.

Heparin inhibits qPCR-based ncRNA quantitation[19]. Confounding by heparin is evidenced by decreased detectability, higher variation or spurious correlations of ncRNA measurements. Apart from endogenous miRNAs, heparin predominantly affects the quantification of the exogenous *Cel-miR-39* spike-in control. As pointed out previously[20], the inter-sample deviation of *Cel-miR-39* measurements should be less than 1 cycle. However, within the first hour after administration of the heparin bolus, the detectability of *Cel-miR-39* decreases and can span up to 4 cycles. This variability is related to the half-life of heparin in the circulation. Most publications assessing miRNAs in samples from patients with MI failed to address this issue (**Online Table VI**). If unnoticed, heparin-induced suppression of the *Cel-miR-39* normalisation control results in artificially higher levels of endogenous miRNAs, especially within the first hour after heparin administration (**Figure 2B**). Heparinase treatment can overcome the confounding introduced by heparin in samples from MI patients[21]. This is the first time that heparinise-treatment was performed on a clinical MI cohort prior to ncRNA quantification. Our analyses of miRNAs in MI patients returned substantially higher correlation coefficients with cardiac troponins than previous publications that did not use heparinase[21].

NcRNAs and protein biomarkers in myocardial injury.

Assays for cTnI and cTnT are the gold standard for detection of myocardial injury[22,23]. The excellent sensitivity of these assays is the result of decades of optimisation[24,25]. Using proteomics, we have recently identified a new cardiac biomarker, cMyC, which may allow for an earlier detection and better rule-in/rule-out of MI[3,4]. In our assessment, cMyC detected myocardial injury with a higher accuracy than hs-cTnT and hs-cTnI in the controlled TASH model. This finding is supported by a steeper rise within the

first hour after TASH and in all time intervals (0h to 1h, 1h to 3h) in the MI cohort. cMyC showed a higher detectability among control patients compared with hs-cTnT (100% vs. 85% respectively). On the other hand, ROC analysis in the validation cohort of MI revealed lower AUC for cMyC compared with hs-cTnT (0.898 vs. 0.925). This finding is most likely attributable to two factors: First, as opposed to TASH, the diagnosis of MI was adjudicated based on hs-cTnT and secondly, the selection of the MI patients from the BACC cohort was determined by initially low and then steeply rising hs-cTnT levels. Importantly, cMyC has been reported as more sensitive compared with cTn[4]. This is supported by our finding that cMyC shows a steeper rise in the first hour after onset of myocardial injury. The better detectability of cMyC in controls also suggests that cMyC might be a biomarker for cardiac disease in non-acute settings.

In addition to cardiac proteins, miRNAs offer a new opportunity for the detection of myocardial injury. The muscle-enriched miRNAs, miR-1 and miR-133a, have been implicated as markers for cardiac injury but are not specific for the heart. In contrast, miR-499 and miR-208a/b have higher cardiac specificity but are less abundant in heart and in plasma[26]. By spiking plasma with human myocardial tissue, we demonstrate that qPCR assays for ncRNAs detect the presence of smaller amounts of myocardial tissue than cardiac proteins. The regression curves for ncRNAs compared to protein biomarkers indicated a potentially higher sensitivity of qPCR-based measurements of ncRNAs. In a tightly controlled clinical setting of induced myocardial injury after TASH, muscle- and cardiac-enriched miRNAs showed an earlier rise than hs-cTn, which was similar to the release kinetics of cMyC (**Figure 3A, B** and **Figure 4A, B**). To exclude that this difference is due to the mode of measurement, we also performed additional cTnI measurements with PEA, which combines antibody-based detection with qPCR-based quantification. Another important aspect for biomarker performance is the clearance of cardiac proteins and miRNAs from the circulation. Similar to cMyC, miRNA levels peaked at 8h and declined or plateaued thereafter. In contrast, hs-cTnT concentrations were still rising at 24h after TASH. While single miRNAs failed to outperform cardiac protein biomarkers in detecting early MI, a multi-biomarker combination of two muscle-enriched miRNAs with hs-cTnT and cMyC returned the highest predictive power for the detection of MI in a subcohort of the BACC study. This was consistent across different aetiologies of myocardial injury (STEMI and NSTEMI type 1). While the biomarker selection and their ranking did not change, the overall performance of the prediction model was largely dependent on infarct severity.

Cardiac- and muscle-enriched miRNAs.

Although cardiac and muscle-enriched miRNAs have been previously studied as biomarker candidates for myocardial injury, our findings in heparinase-treated samples highlight important aspects that have, to our knowledge, not been addressed so far. First, the muscle-enriched miRNAs, miR-1 and miR-133a are more readily detectable at baseline, while miR-208 and -499 reach detectable values only at higher corresponding hs-cTnT values[27]. On the other hand, cardiac miRNAs are more specific for myocardial injury. They correlate best with hs-cTnT and predict myocardial injury better in the TASH cohort and as good as hs-cTnT in the MI cohort. This is in consistent with previous reports of miR-208 and miR-499 being elevated in plasma only in cases of MI or myocardial injury, while miR-1 and miR-133a can rise in different cardiac pathologies[28,29]. In a ROC analysis of the TASH cohort, cardiac-enriched miRNAs returned higher AUC values than muscle-enriched miRNAs. Secondly, cardiac miRNA measurements in patients with MI reached the detection limit of Cq<35 cycles only at high hs-cTnT values (>50-100ng/L). Thus, miRNAs failed to identify patients with MI that initially present with low or negative cTn values. A critical evaluation of publications is required as higher thresholds of detection may have been used in some studies and confounding by heparin was not taken into consideration (**Online Table VI**). Given their favourable release kinetics, this shortcoming of cardiac miRNA biomarkers might be attributed to the low miRNA yield from plasma, miRNA degradation after release into circulation and inadequate detection methods compared to high-sensitivity protein assays.

lncRNAs.

LncRNAs are a heterogenous group of RNAs >200 nucleotides in length[30]. Unlike miRNAs, lncRNAs are mainly located within the nucleus or in mitochondria[7,31]. Regardless, lncRNAs are readily detected in the circulation, suggesting some protection against RNase-mediated degradation similar to plasma miRNAs[30]. LIPCAR was found to predict adverse cardiac remodeling and death in the aftermath of MI[7]. LIPCAR has been proposed as a biomarker of cardiac disease. However, the cardiac origin of this lncRNA in plasma has not been confirmed. In our experiments, LIPCAR showed a comparable regression curve to cardiac miRNAs in the myocardial tissue spike-in and good detectability in plasma. LIPCAR levels, however, did not increase after TASH, refuting a cardiac origin. Since LIPCAR is of mitochondrial origin and ubiquitously expressed, its rise in plasma may be explained by a release of mitochondria from blood cells rather than cardiac injury.

circRNAs.

circRNAs are expressed in a tissue- and developmental-specific manner[10]. They can either emerge from exons or introns of pre-mRNA and are products of alternative splicing known as 'backsplicing'[8]. circRNAs have diverse functions[32,33] and are tissue-specific[34]. Sequencing data revealed the presence of more than 15,000 circRNAs in the human heart, some in high abundance[35]. circRNAs have previously been implicated in MI-related apoptosis[36]. The majority of circRNAs are located in the cytoplasm[37], which increases the probability of their early release upon tissue damage. circRNAs have been described as circulating biomarkers in the field of oncology[38]. Our study is the first to assess circRNAs as biomarkers in acute MI. circRNAs in plasma showed poor detectability despite high abundance in cardiac tissue. Also, circRNAs did not show a rise in plasma after myocardial injury. While circRNAs are supposedly less prone to degradation compared with their linear transcripts[39], this may differ in the circulation where circRNAs have been described as having a short half-life[39]. Thus, cardiac circRNAs were not well detectable in plasma and serum.

Conclusions.

In summary, heparinase treatment is essential when evaluating ncRNAs in clinical settings. Amongst ncRNAs, cardiac miRNAs remained the best predictor for the diagnosis of acute MI. In serial samples from TASH and acute MI patients, cardiac miRNAs showed comparable AUC values to hs-cTnT and the additional use of muscle-enriched miRNAs combined with hs-cTnT and cMyC returned the highest AUC in the clinical setting of MI, pointing out their potential future use in combined protein/ncRNA biomarker approaches. On the other hand, miRNA sensitivity proved to be well below hs-cTnT, arguing against their clinical application at the current stage of methodological advances. Thus, analyses in larger cohorts seem warranted once technological advances offer better sensitivity. Future miRNA assays also require faster, automated quantification if miRNAs were to be used for complementing protein biomarkers. With regards to cardiac proteins, measurements of cMyC could offer some of the benefits of miRNAs, as evidenced by an earlier rise and faster decline after myocardial injury and a better baseline detectability compared to cardiac troponins.

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DISCLOSURES
M. Mayr and M. Marber are named inventors on a licensed patent held by King's College London for the detection of cMyC as a biomarker of myocardial injury (EP2430453B1, US8546089). M. Mayr filed and licensed patent applications on miRNAs as biomarkers (EP15193448.6, EP2776580 B1, DE112013006129T5, GB2524692A, EP2576826 B, JP2013-513740). JT Neumann received honoraria from Siemens and Abbott Diagnostics. D. Westermann reports personal fees from Bayer, Boehringer-Ingelheim, Berlin Chemie, Astra Zeneca, Biotronik and Novartis. S. Blankenberg received honoraria from Abbott Diagnostics, Siemens, Thermo Fisher, and Roche Diagnostics and is a consultant for Thermo Fisher.

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FIGURE LEGENDS

Figure 1. Myocardial tissue spike-in. A) Linear regression curves of each of the three ncRNA classes with the highest coefficient of determination (R-squared) values. At low spike-in concentrations, the measured protein concentrations were markedly below the regression curve (coloured boxes). **B)** Linear regression curves of all biomarker species, combining the single biomarkers from panel A per class. ncRNAs showed steeper regression curves compared with protein biomarkers. **C)** At low spike-in concentrations, significantly higher regression coefficients, indicating higher sensitivity, were observed for all ncRNA species compared with proteins. ***: $p<0.0001$; *: $p<0.0028$ (Mann Whitney test).

Figure 2. Heparin effect on selected miRNAs and results after heparinase treatment. A) Human plasma samples were treated with heparin after blood was drawn, then miRNA expression was measured (blue). The measurements were repeated in the same samples after they were treated with heparinase (red) or with a buffer solution lacking heparinase (grey). **B)** Clustering networks of miRNAs in the TASH cohort: before heparinase treatment the analysed miRNAs showed a dense correlation network. Heparinase treatment resolved this clustering of miRNAs, removing spurious correlations between miRNAs in non-heparinase-treated TASH samples. The distinct cellular origin of miRNAs, i.e. liver-specific miR-122 versus red-blood cell derived miR-486, became more readily apparent.

Figure 3. ncRNAs after TASH. Relative plasma levels as well as raw Cq values for muscle-enriched **(A)** and cardiac-enriched **(B)** miRNAs as well as circRNAs **(C)** and lncRNAs **(D)** after TASH for time points before (0h) and after myocardial injury (1h, 8hrs, 24hrs). Dotted line indicates the detectability threshold; Cq-values above 35 were considered as undetectable. Of particular interest is the time course of the first hour after TASH with respect to biomarker sensitivity (coloured boxes). Depicted are median values, error bars indicate interquartile range.

Figure 4. ncRNAs and protein biomarkers after TASH. A) Levels of cardiac protein biomarkers and muscle miRNA (the ncRNA group with the best overall detectability and steepest increase in the first hour after onset of myocardial injury) in the TASH cohort, depicted as median plus interquartile range. **B)** Transformation of absolute protein quantification measures and relative miRNA quantification measures to the same scale by dividing each value by the maximum value of each biomarker. **C)** Slope statistics on the relative expression of miRNA species after transformation according to panel B revealed significant differences in the regression coefficients between muscle-enriched miRNAs (miR-1 and miR-133a) and protein biomarkers for the first hour after TASH (time point 0 and 1h). *** denotes $p<0.0001$; *, $p<0.01$ (Mann Whitney test) relative to the maximum value of each biomarker. Panels A-C show median values, error bars indicate interquartile range.

Figure 5. Correlation of cardiac biomarkers in AMI. All analysed biomarkers are highly correlated. Cardiac-enriched miRNAs correlate better with hs-cTnT and among each other than muscle-enriched miRNAs. Depicted are regression coefficients; p for all combinations <0.0001. CK = Creatine kinase; CK-MB = Creatine kinase muscle/brain; cMyC = Cardiac Myosin Binding Protein C; Hs-cTnI = high sensitive cardiac troponin I; hs-cTnT = high sensitive cardiac troponin T, TnI-PEA = cardiac troponin I as measured by a proximity extension assay (PEA, Olink).

Figure 6. miRNA raw expression data in the MI cohort. A) Raw Cq values of miRNAs for control patients (Ctr) and MI patients according to time of admission to hospital (0h on presentation at hospital, 1 h and 3 hrs after presentation). At every time point, there were undetectable values (Cq>35) for each miRNA. **B)** miRNA raw Cq values corresponding to different ranges of hs-cTnT concentrations (ng/L). Only for hs-cTnT levels above 1000ng/L all miRNAs showed 100% detectability. Black dotted line denotes lower limit of detection (Cq>35).

Figure 7. Release kinetics of cardiac protein biomarkers and curve fitting in MI cohort. A) cMyC rose more quickly after onset of MI compared with hs-cTnT with still a smaller coefficient of variation (values depicted are median with interquartile range). **B)** Curve fitting analysis of the first hour after hospital presentation revealed significant higher regression coefficients for cMyC than all other biomarkers. **** denotes p<0.0001; ***, p=0.0002; ns, not significant (Mann Whitney test).

Figure 8. ROC analysis comparing predictive power of protein and miRNA biomarkers. A) TASH cohort. **B)** MI cohort with subanalysis for **C)** STEMI patients. **D)** NSTEMI patients. Blue colour for proteins, red for miRNAs, white for combinations of 2 or more biomarkers. The biomarkers are ranked from left to right (highest to lowest AUC value). The intensity of the blue and red colour increases with increasing AUC values.

NOVELTY AND SIGNIFICANCE

What Is Known?

- High-sensitivity cardiac troponins are the gold standard biomarkers for myocardial infarction (MI).
- Non-coding RNAs (ncRNAs) have been reported as candidate biomarkers for MI.
- No study has compared different classes of ncRNAs with cardiac protein biomarkers.

What New Information Does This Article Contribute?

- Amongst ncRNAs, cardiac microRNAs (miRNAs) are the best biomarker candidates for MI.
- Current miRNA assays lack sensitivity for early detection of MI.
- Cardiac myosin-binding protein C (cMyC) shows promise as early biomarker for MI.

We performed the first comparative analysis of ncRNAs and protein biomarkers in serial samples from patients undergoing induced myocardial injury or admitted with MI. Heparinase treatment is essential when evaluating ncRNAs in these clinical settings. MiRNAs were the most promising ncRNA species but current miRNA assays lack sensitivity for early detection of MI. Instead, cMyC showed favourable release kinetics as early rule-in/rule-out biomarker for MI. Measurements of cMyC could offer some of the benefits of miRNAs, as evidenced by an earlier rise and faster decline after myocardial injury and a better baseline detectability compared to cardiac troponins. A combination of different cardiac protein and ncRNA biomarkers could become a new approach to improve the diagnosis of MI.

Figure 1

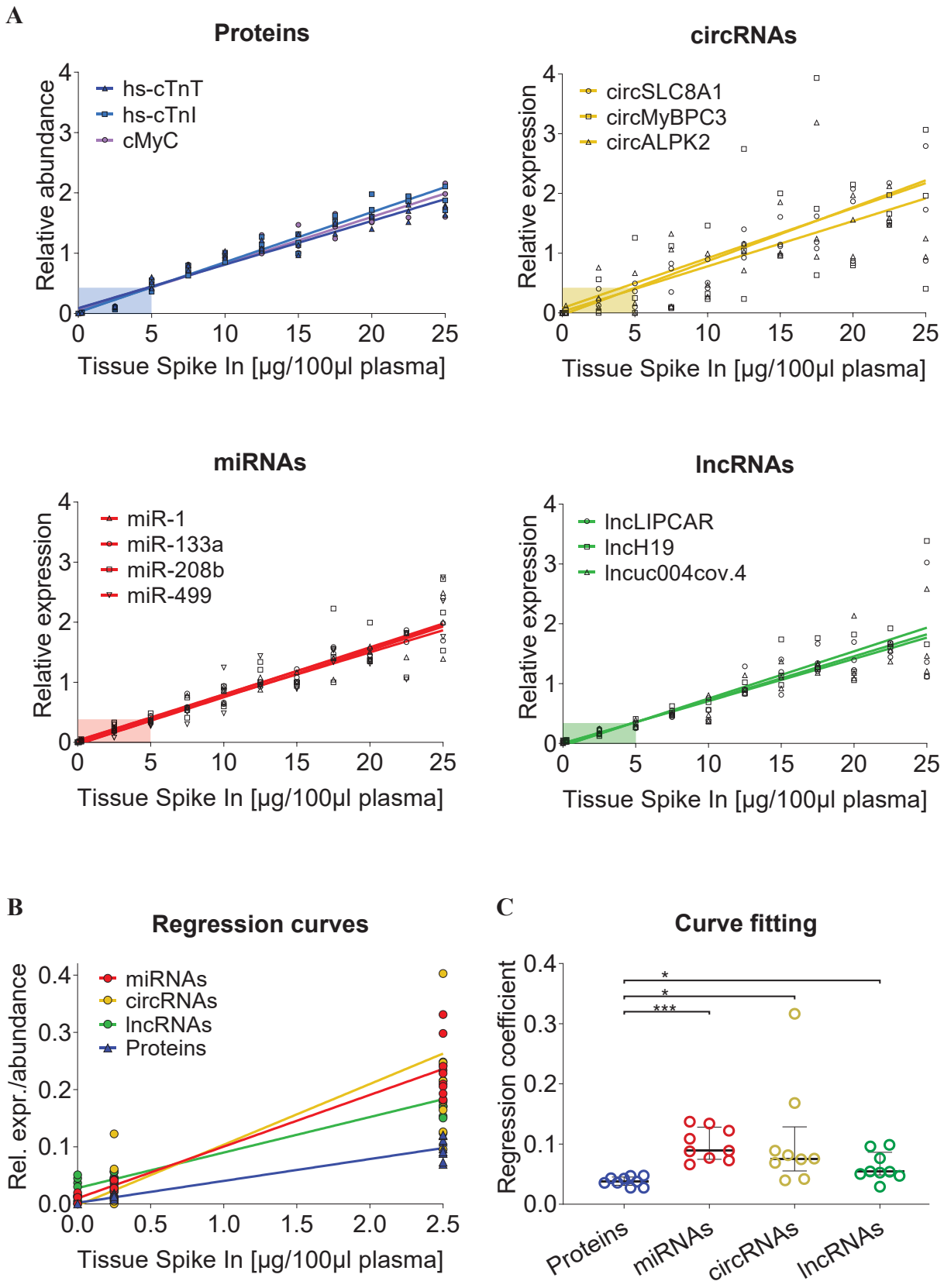


Figure 2

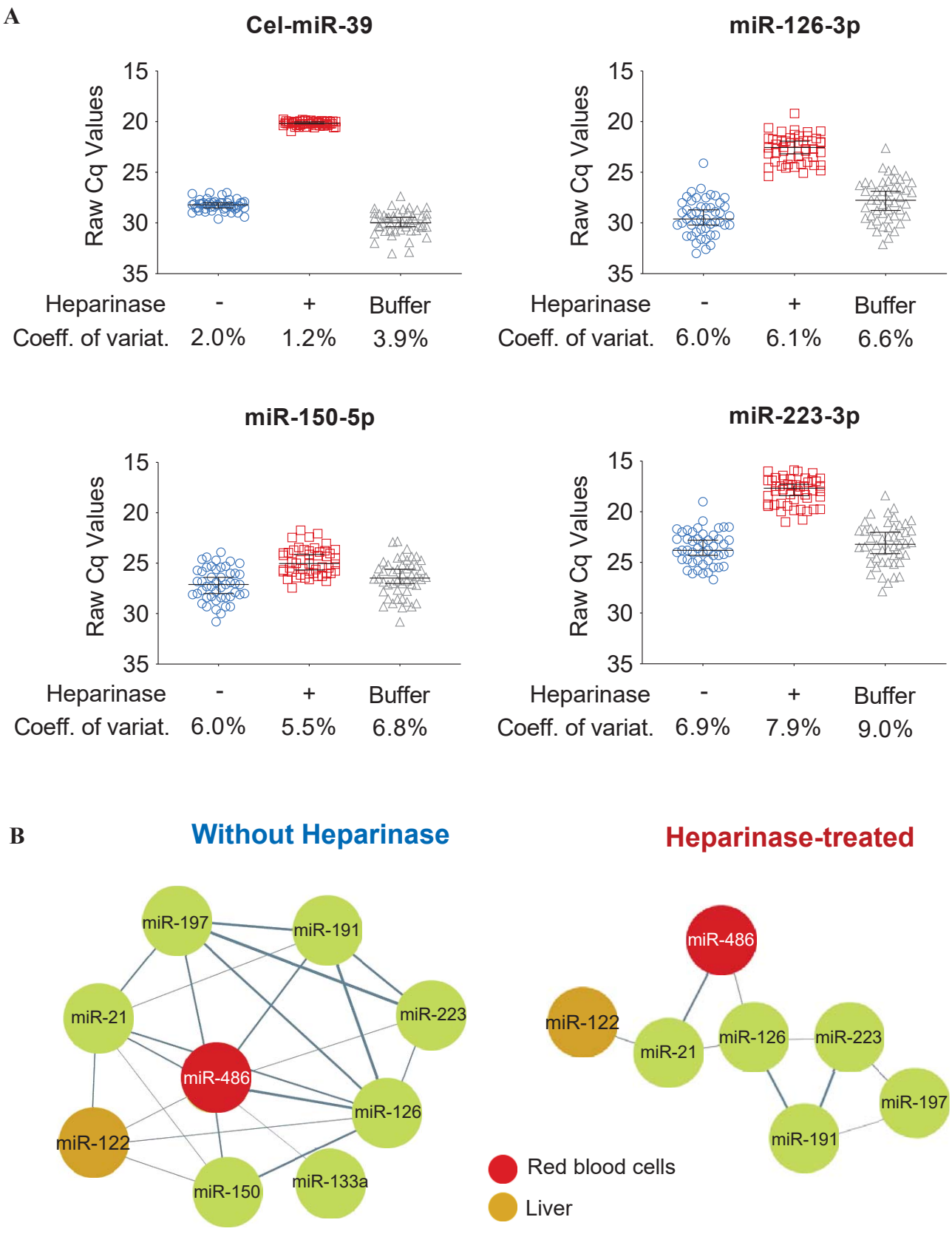


Figure 3

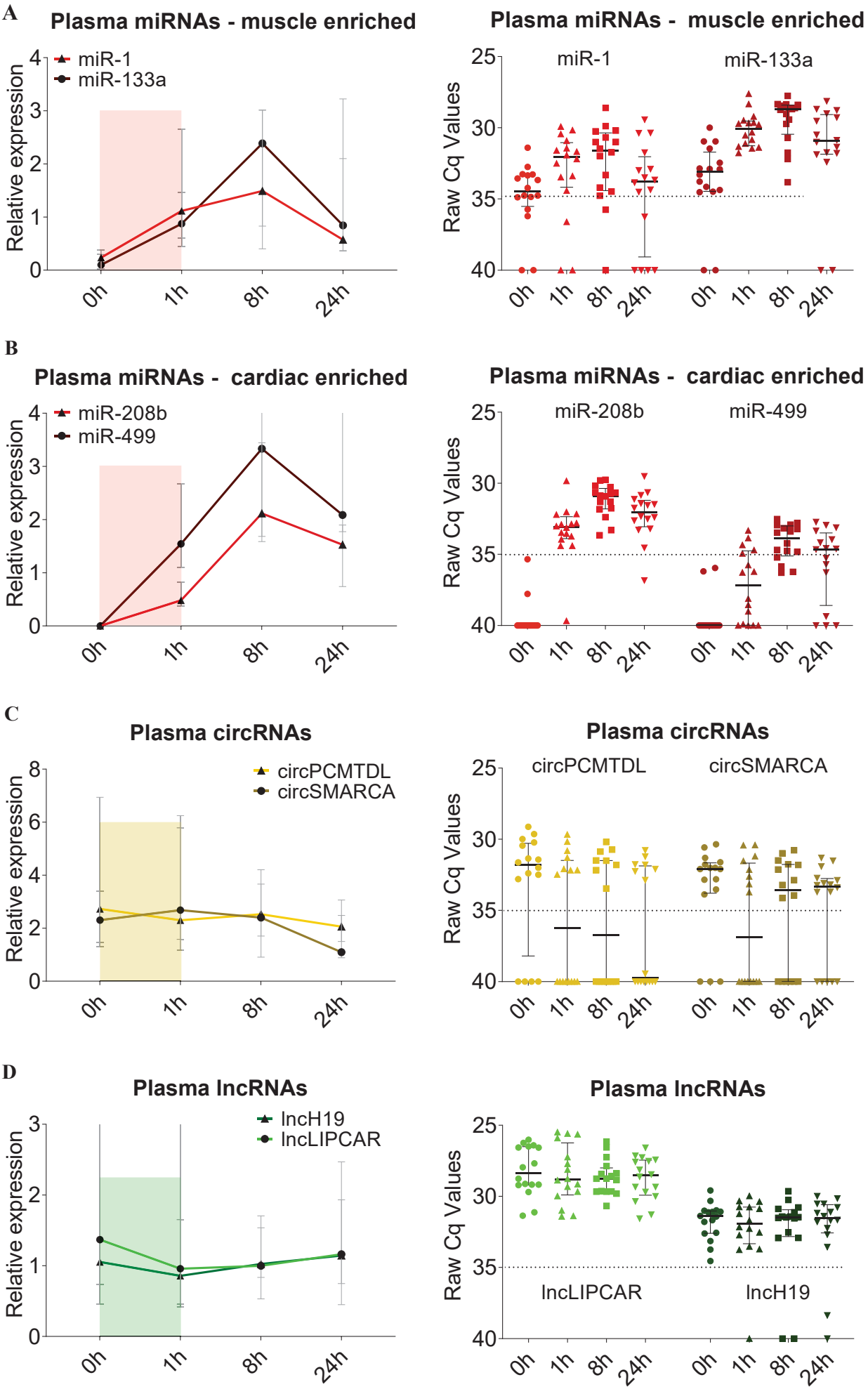


Figure 4

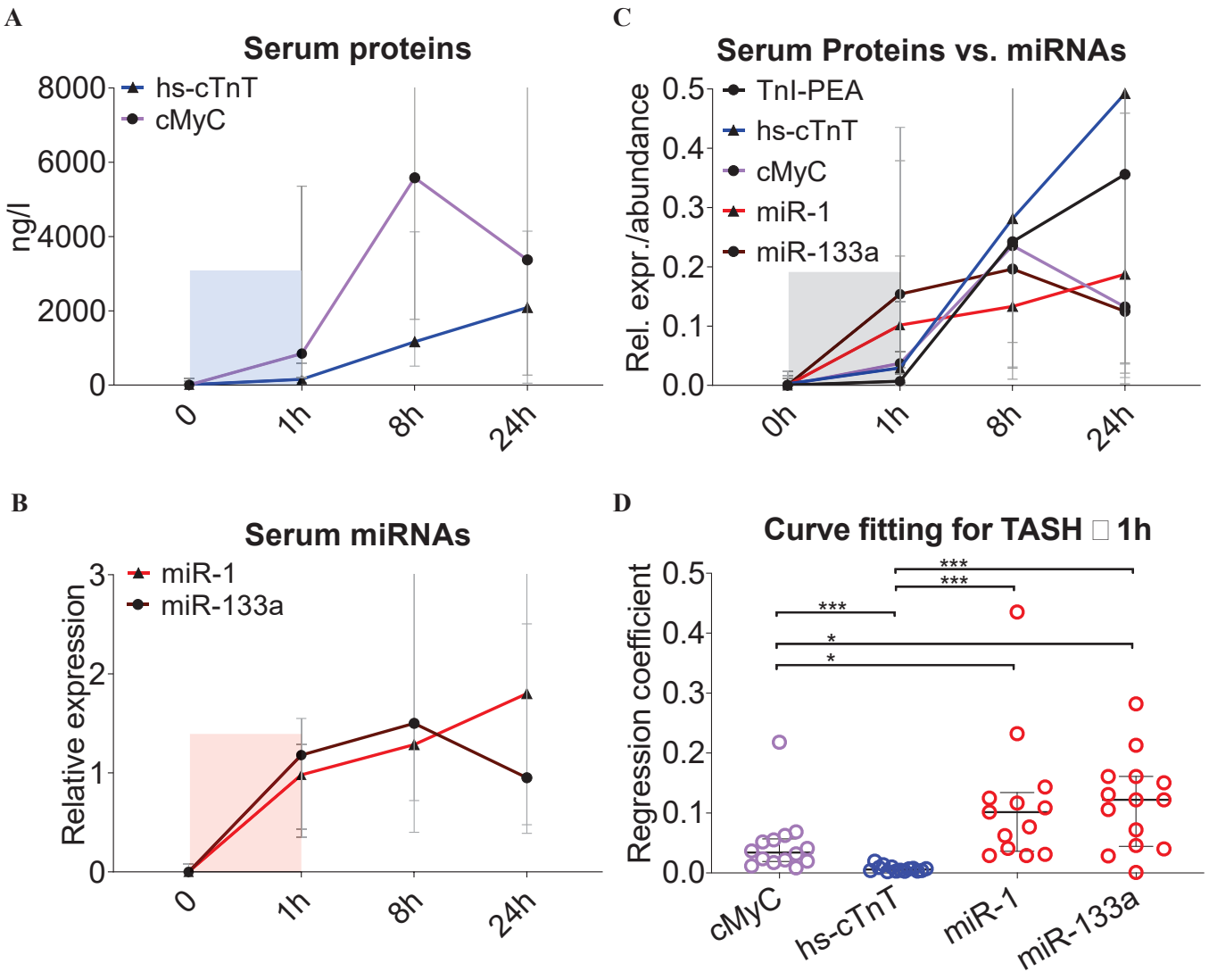


Figure 5

	hs-cTnT	hs-cTnI	cMyC	TnI-PEA	CK-MB	CK	miR-1	miR-133a	miR-208b	miR-499
hs-cTnT	1,0000	0,8305	0,8656	0,8328	0,8624	0,6043	0,665	0,6701	0,8141	0,8762
hs-cTnI		1,0000	0,8449	0,7802	0,8707	0,6074	0,6994	0,6493	0,7518	0,8123
cMyC			1,0000	0,8048	0,8627	0,6282	0,7334	0,722	0,8047	0,88
TnI-PEA				1,0000	0,8709	0,7358	0,7096	0,6632	0,7945	0,8251
CK-MB					1,0000	0,7897	0,7731	0,8253	0,8184	0,8218
CK						1,0000	0,5999	0,5835	0,7914	0,8397
miR-1							1,0000	0,7868	0,7965	0,8381
miR-133a								1,0000	0,8387	0,8899
miR-208b									1,0000	0,9112
miR-499										1,0000

Figure 6

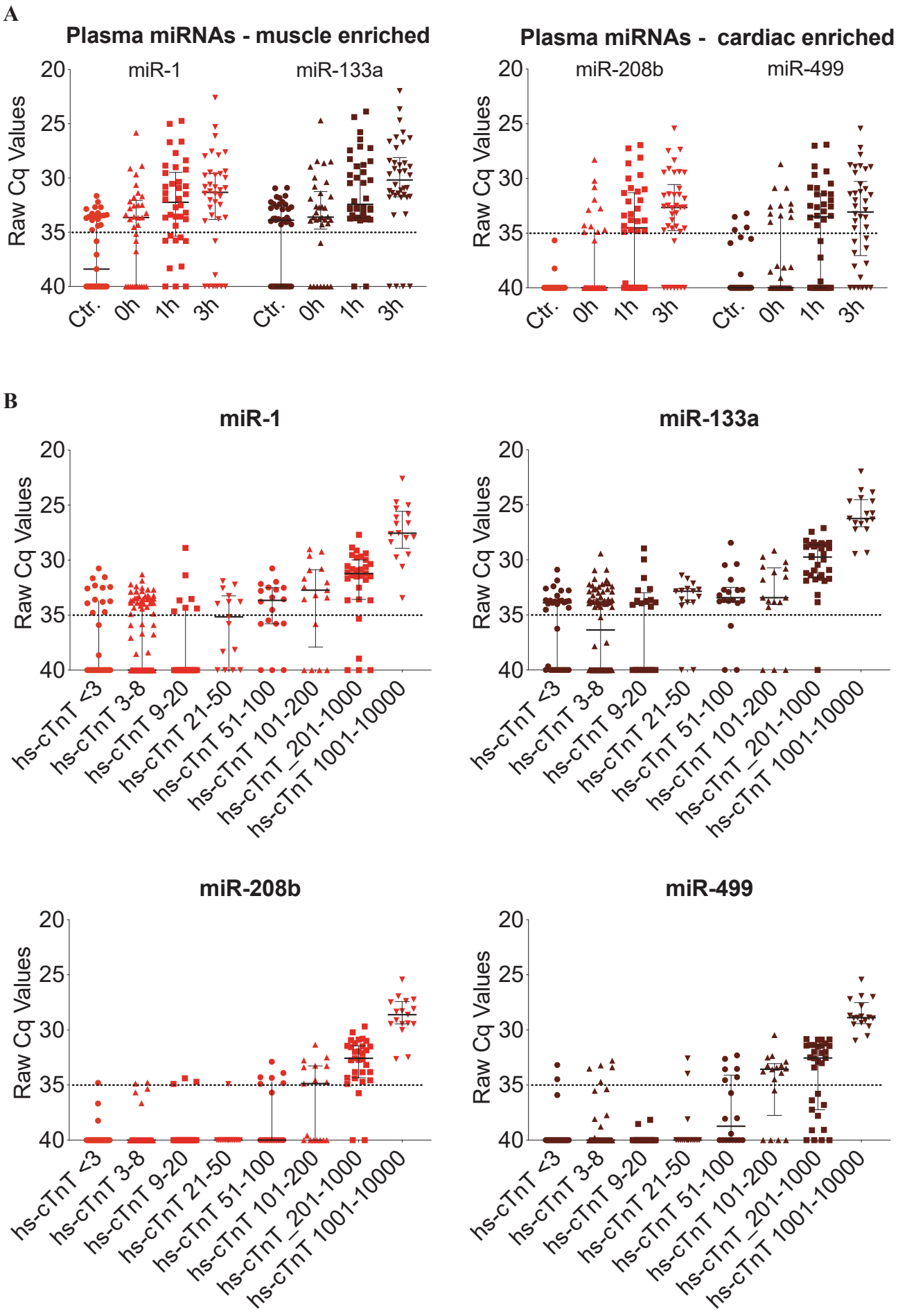


Figure 7

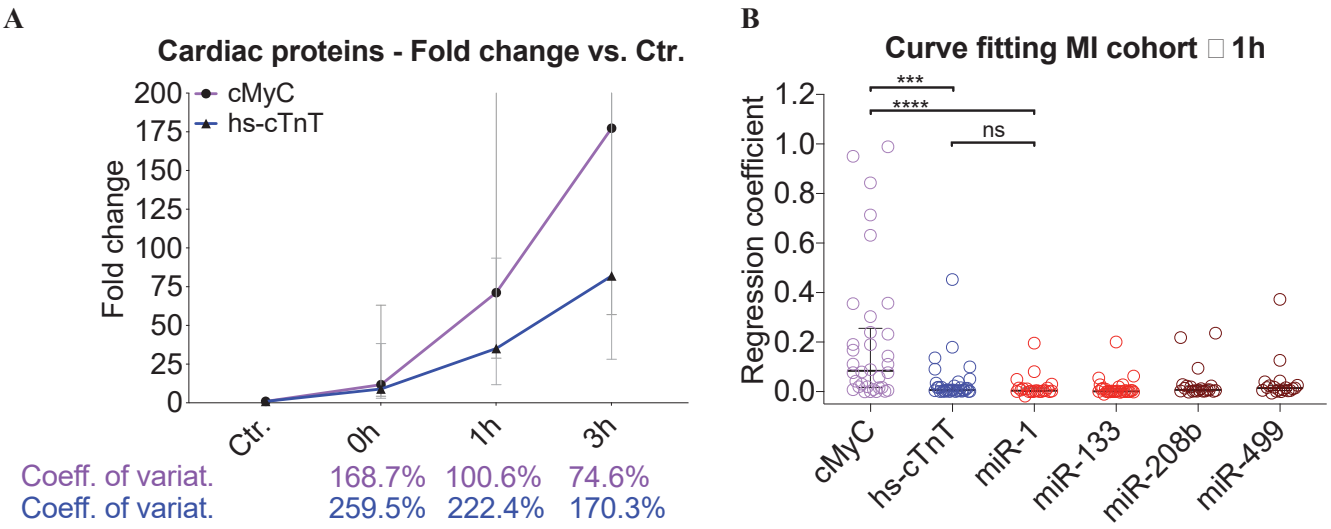
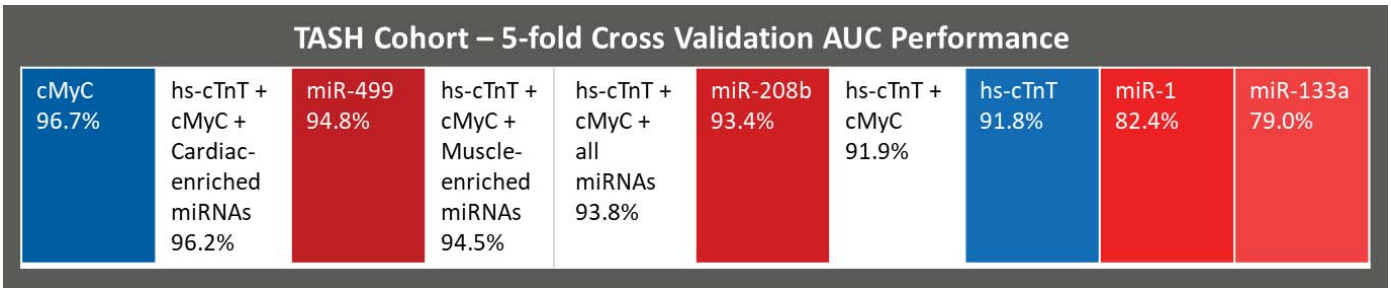


Figure 8

A



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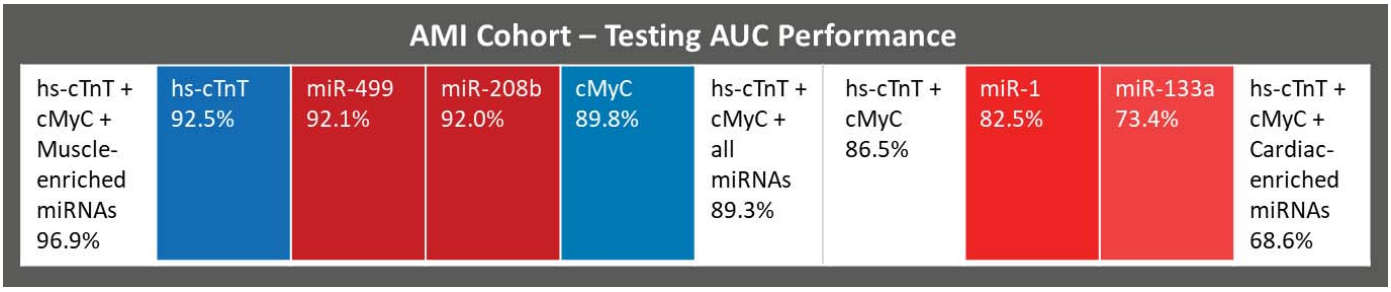
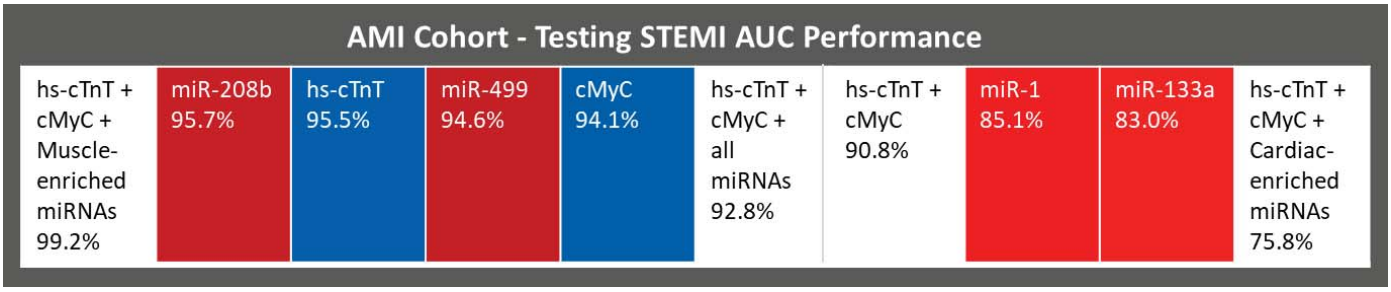


C



D