MicroRNAs in cardiac diseases: The devil is in the details
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CHAPTER 10
CARDIAC HANDLING OF CIRCULATING MIRNAS:
THE PORCINE HYPERTROPHY MODEL
ABSTRACT

Circulating miRNAs are promising candidates as biomarkers for a wide range of diseases. We recently identified circulating miR-423-5p and 6 other miRNAs being increased in plasma of heart failure patients and therefore as potential biomarkers for heart failure. Interestingly, the expression of miR-423-5p was also increased in failing human myocardium, which suggests that the increase of miR-423-5p in plasma of heart failure patients is derived from the myocardium. To investigate this hypothesis, we studied whether miR-423-5p and 8 other miRNAs were released by the myocardium in pigs after 1, 3 or 8 weeks of ascending aortic banding (AoB). In these pigs we were able to determine circulating miRNA levels in both arterial and selective coronary venous sampling. We calculated transmyocardial concentration gradients by subtracting circulating miRNA levels in the arterial blood from the levels in coronary venous blood, where a positive gradient indicates release by the myocardium and a negative gradient indicates uptake. In addition, we determined expression of these miRs in myocardial samples from the epi- and endocardial site of the left ventricular free wall.

AoB induced left ventricular hypertrophy but did not cause manifest heart failure, so that this model may be limited in representing our initial findings in human subjects with heart failure. In line with this concept, we did not detect regulation of any of the 9 miRNAs in the hypertrophic myocardium. Second, we also did not detect any significant changes in circulating miRNA levels in AoB compared to sham operated pigs and no significant gradients over the myocardium were detected, which indicates that these miRNAs are not released or taken up by the hypertrophic myocardium. As a separate finding, we noticed that use of heparin introduced a large variation, which could be importantly reduced by treating samples with heparinase.

In conclusion, miRNAs recently identified to be enriched in plasma of heart failure patients are not clearly regulated in the hypertrophic non-failing porcine myocardium. We found no clear release or uptake by the myocardium.
INTRODUCTION

Circulating miRNAs are attractive candidates to serve as biomarkers for a wide range of diseases, because they fulfill a number of criteria for biomarkers. They are easy accessible in the circulation, where they are very stable even under harsh conditions as boiling, low or high pH, long-term storage at room temperature and in multiple freeze-thaw cycles.1,2 Furthermore, they are often regulated in a tissue- and pathology-specific manner and therefore expected to show disease-specific profiles in the circulation. And since detection of miRNAs is based on sequence-specific amplification, this detection is also highly sensitive and specific. These qualities suggest that the discovery-validation pipeline for miRNA biomarkers will be more efficient than for protein-based biomarkers, where bottlenecks at the point of specific antibody generation are often encountered.3

In the case of heart failure circulating miRNAs emerged as promising biomarker candidates. In a cohort of 39 healthy controls, 30 dyspneic patients with heart failure and 20 dyspneic patients due to other causes, we identified seven miRNAs to be increased in plasma of heart failure patients (miR-423-5p, miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, and miR-1254), among which miR-423-5p was most strongly related to the clinical diagnosis of heart failure (chapter 9).3 This elevation of circulating miR-423-5p levels in heart failure was confirmed by Goren et al.4 and Fan et al.5, who respectively determined circulating miRNA levels in 30 chronic heart failure patients compared to 30 healthy controls and 45 dilated cardiomyopathy patients compared to 39 healthy controls. Goren et al.4 also identified 3 other circulating miRNAs (miR-320a, miR-22 and miR-92b) to be increased in heart failure patients. Furthermore the increase of circulating miR-423-5p in heart failure was also confirmed by Dickinson et al.6 They performed serial blood drawings in Dahl salt-sensitive rats, where heart failure was hypertension-induced by a high-salt diet, and found among others miR-423-5p to be positively correlated to disease progression. They also found that circulating miR-423-5p levels could be repressed after treatment with antimiR-208a, which improved cardiac function and survival in these rats.

Despite the consistent increase of circulating miR-423-5p in human and rodent heart failure, it is unknown whether this increase is linked to myocardial changes and caused by myocardial production and release. In the pig, miR-423-5p was shown to be ubiquitously expressed, with high levels in heart, liver and brain.7 Together with the reported upregulation of miR-423-5p in human failing myocardium,8 this suggests that circulating miR-423-5p in heart failure is derived from the myocardium. To investigate this hypothesis we made use of a porcine hypertrophy model, where hypertrophy was induced by ascending aortic banding (AoB). In these pigs we determined the expression of miR-423-5p and 8 other miRNAs in myocardial samples from the epi- and endocardial site of the left ventricular free wall. In addition, we were able to determine circulating miRNA levels in plasma samples derived from either arterial or selective great cardiac vein sampling, where coronary vessels that drain the heart assemble before entering the main circulation. We compared circulating miRNA levels in AoB pigs to miRNA levels in sham-operated pigs. Moreover, we calculated transmyocardial concentration...
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gradients by subtracting circulating miRNA levels in the arterial blood from the levels in coronary venous blood, where a positive gradient indicates release by the myocardium and a negative gradient indicates uptake. We show that these pigs develop left ventricular hypertrophy but do not develop heart failure and that none of the measured miRNAs is regulated in the hypertrophic porcine myocardium. Furthermore, we were not able to detect any release or uptake of these miRNAs by the myocardium.

METHODS

Porcine ascending aortic banding

Experiments were performed on pigs with a mixed background of Finnish and Dutch Landras with Tempo or Duroc of either sex. This study was performed in accordance with the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus Medical Center.

Pigs were randomly assigned into two groups, AoB or sham-operated. They were sedated with an intramuscular (im) injection of Zoletil (5 mg/kg tiletamine and 5 mg/kg zolazepam), Rompun (2.25 mg/kg xylazine) and Atropine (1 ml). This was followed by intubation and ventilation with a mixture of O₂ and N₂ (1:3) to which 1-2% (v/v) isoflurane was added to maintain anesthesia. Antibiotics were administered prophylactically (1 ml Streptoprocpen (im), containing 200 mg procaine benzylpenicillin and 250 mg dihydrostreptomycin sulphate). The chest was opened under sterile conditions via the second right intercostal space and a Ty-rap was placed around the ascending aorta. Two fluid-filled polyvinylchloride catheters were inserted into the aorta, one proximal and one distal to the Ty-rap, for the measurement of aortic pressure. Aortic banding was performed by tightening the Ty-rap until a peak systolic pressure gradient of 40-70 mmHg, which results in left ventricular (LV) pressure overload. Catheters were tunneled subcutaneously to the back, the chest was closed and animals were allowed to recover. Animals received analgesia (0.3 mg Buprenorphine) for two days. Sham-operated animals underwent the same procedure without implantation of the Ty-rap.

One day after surgery aortic pressure and systolic pressure gradients were measured under awake and resting conditions to confirm successful AoB. The fluid-filled catheters were flushed three times a week and filled with Heparin (5000 IE) to prevent blood clots.

Echocardiography

One week (8 sham and 11 AoB), three weeks (13 sham and 14 AoB) or eight weeks (6 sham and 10 AoB) after surgery, pigs were sedated with Zoletil (5 mg/kg tiletamine and 5 mg/kg zolazepam) and Atropine (1 ml). 2D echocardiographic recordings of the LV short axis were obtained (ALOKA ProSound SSD-4000; Japan). LV cross-sectional area and LV wall thickness were determined and ejection fraction and wall thickening were calculated.
Sample collection
Subsequently to the echocardiography, pigs were anesthetized (5 ml Sodium Pentobarbital, intravenously), intubated and ventilated with a mixture of O\textsubscript{2} and N\textsubscript{2} (1:3) for monitoring of hemodynamic parameters. After monitoring of these parameters, blood was drawn from the aorta (arterial) and the great cardiac vein (venous) in EDTA tubes. Blood was centrifuged 10 min at 1500 x g at 4°C, aliquoted and stored at -80°C. Afterwards the heart was arrested and immediately excised. The LV was divided into myocardial tissue from the epicardial and endocardial site of the LV free wall, snap-frozen in liquid nitrogen and stored at -80°C.

Human heparin experiment
Blood was collected via a direct venous puncture into 2.7 ml sodium EDTA containing tubes (BD Biosciences). This blood was processed by spinning at 1550 x g for 10 minutes at room temperature. Plasma was carefully transferred to a fresh RNAse/DNase free tube, and heparin was added in the following end-concentrations: 0, 0.15, 1.5, 15, 30, and 60 international units (IU)/ml. RNA isolation, heparinase treatment and real-time PCR were performed as described below.

RNA isolation
Total RNA from myocardial tissue was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. Circulating miRNAs were isolated from 400 µl plasma using the mirVana PARIS kit (Ambion) according to the manufacturer's protocol for liquid samples. The protocol was modified such that samples were extracted twice with an equal volume of acid-phenol:chloroform and the columns were dried for 3 minutes after the last washing step and before elution. Furthermore after 5 minutes incubation of plasma with the denaturing solution of the mirVana PARIS kit 5 µl of synthetic RNA oligonucleotides (5 fmol/µl mixture of cel-miR-54, cel-miR-39, cel-miR-238) were spiked in.

Heparinase treatment
We used a fixed volume of 13.9 µl of isolated RNA for heparinase treatment. This was performed in the RT-buffer of the miScript reverse transcription kit (Qiagen) with addition of 2U of Superscript (Ambion) and 1U of Heparinase I (Sigma). Samples were incubated for 2 hours at 25°C. In the human heparin experiment non-heparinase treated samples were processed comparably, where Heparinase I was substituted by nuclease-free water.

cDNA preparation
For plasma samples 1 µl of RT enzyme, derived from the miScript reverse transcription kit (Qiagen), was added to the heparinase-treated RNA samples and RNA was reverse transcribed according to the manufacturer's protocol.
From total RNA isolated from myocardial tissue 400 ng was used as input in the reverse transcription reaction using the miScript reverse transcription kit according to the manufacturer’s protocol.

**Real-time PCR**

Real-time PCR was performed using High Resolution Melting Master (Roche), with addition of MgCl\(_2\) in an end-concentration of 2.5 mmol/L. We used 2 μl of 8-times diluted cDNA in a total volume of 10 μL. The forward primers had the same sequence as the mature miRNA sequence, with all U’s changed into T’s and the sequence of the reverse primer was 5’-GAAGCGACCCGTTACC-3’. Real-time PCR reactions were performed on a LightCycler480 system II (Roche) using the following program: 10 minutes pre-incubation at 95°C and 40 cyl of 45 seconds 95°C, 45 seconds 55°C, and 45 seconds 72°C.

**Data analysis**

Real-time PCR data were analyzed using LinRegPCR quantitative PCR data analysis software. The starting concentration of miRNAs calculated by this software were corrected for the calculated starting concentration of the reference gene HPRT for tissue samples and the spike-in miRNA cel-miR-54 for plasma samples. Data are depicted as mean +/- standard error of the mean, between-group comparisons were tested using Student’s t-test and p-values<0.05 were considered significant.

**RESULTS**

**AoB-induced pressure-overload in pigs results in cardiac hypertrophy**

To investigate whether the increase of specific miRNAs in the circulation is derived from the myocardium we used pigs subjected to 1, 3 or 8 weeks of pressure-overload induced hypertrophy by AoB. Hypertrophic growth of the myocardium is confirmed at all 3 time-points as shown by an increase in heart weight corrected for body weight (Figure 1A). Furthermore it is evidenced by the lack of decrease in cardiac function, as measured by the ejection fraction in echocardiography, that these pigs do not develop heart failure (Figure 1B). We collected myocardial tissue at the epi- and endocardial site of the LV free wall and confirm at the mRNA level the upregulation of the hypertrophic marker gene BNP at all three time points and in myocardium derived from both the epi- and endocardial site of the LV (Figure 1C). Together these data indicate that the AoB surgery was successful to induce hypertrophy in the porcine myocardium but failed to induce heart failure.

**Circulating miRNAs are not regulated in the hypertrophic pig myocardium**

We further investigated whether the 9 miRNAs (miR-423-5p, miR-129-5p, miR-622, miR-18b-3p [former miR-18b*], HS_202.1, miR-302d, miR-22-3p, miR-92b, and miR-320a) increased in the circulation of heart failure patients, as identified by our group or Goren et al., were regulated
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in the porcine hypertrophic myocardium. Therefore we measured the expression levels of these 9 miRNAs, which were all conserved in the pig genome, in the myocardial samples. We used miR-21 as a ‘positive control’ miRNA, because it is identified previously as upregulated in the hypertrophic and failing rodent and human myocardium\(^{10}\) and therefore expected to be also upregulated in the porcine hypertrophic myocardium. As shown in Figure 2 we indeed found upregulation of miR-21 in the hypertrophic myocardium from both the epicardial and endocardial site of the LV. Strikingly this upregulation was highest and statistically significant at 3 weeks after AoB, while at 1 and 8 weeks after AoB there was a relatively small, non-significant upregulation. In Figure 2 we also show the regulation of miR-423-5p, miR-129-5p and miR-622 as an example for all 9 other miRNAs measured in the hypertrophic pig myocardium. As can be appreciated from these examples, we found no up- or downregulation of these 9 miRNAs in the hypertrophic

Figure 1. AoB results in cardiac hypertrophy in pigs 1, 3 and 8 weeks after surgery. A) Heart weight corrected for body weight is significantly increased after AoB at all 3 time-points. B) AoB does not result in a decrease in cardiac function at any of the 3 time-points, as evidenced by ejection fraction measurement with echocardiography. C) The hypertrophic marker gene BNP is upregulated at the mRNA level at all 3 time-points in myocardium derived from the epicardial and endocardial site of the LV free wall. BNP levels are normalized for HPRT and data are shown relative to epicardial sham expression. N=8 sham and 11 AoB at 1 week, n=13 sham and 14 AoB at 3 weeks, and n=6 sham and 10 AoB at 8 weeks. *p<0.05 compared to sham-operated animals.
porcine myocardium regardless of duration of AoB or site of tissue collection. The only miRNA that showed some regulation was miR-622, which was significantly downregulated specifically in samples from the endocardial site 3 weeks after AoB, the same time-point where miR-21 our positive control was increased.

![Figure 2. Circulating miRNAs are not regulated in the hypertrophic pig myocardium. A) Here we confirm the upregulation of the positive control miRNA, miR-21, in hypertrophic myocardium derived from the epi- and endocardial site of the left ventricular free wall at all 3 time-points. Furthermore we show B) miR-423-5p, C) miR-129-5p and D) miR-622 as example of all miRNAs identified in plasma of heart failure patients. None of these miRNAs was regulated in hypertrophic porcine myocardium at any time-point. miRNA levels are normalized for HPRT and data are shown relative to epicardial sham expression. N=8 sham and 11 AoB at 1 week, n=13 sham and 14 AoB at 3 weeks, and n=6 sham and 10 AoB at 8 weeks. *p<0.05 compared to sham-operated animals.](image)

**Heparinase-treatment rescues miRNA detection in heparin-contaminated samples**

To investigate whether the circulating miRNA levels were increased in AoB compared to sham-operated pigs, we determined the miRNA levels in plasma of these pigs. We first determined whether RNA isolation and miRNA measurement were successful by investigating the stability of the *c. elegans* spike-in miRNA (cel-miR-54) and of an endogenous miRNA expected to be stable (miR-15b). As shown in Figure 3A for the animals of the 1 week AoB group and cel-miR-54 as an example there was substantial variation of these miRNAs across the samples; in some samples...
miRNAs were detected at a high level and in others they were almost absent. The levels did not correlate with type of blood (arterial vs venous) or with specific animals. For example in pig 23 the venous levels are high and arterial levels almost not detectable, while in pig 25 the arterial levels are high and the venous levels almost not detectable (Figure 3A).

During surgery we inserted catheters flushed with heparin into the aorta to measure aortic pressure. Heparin is previously shown to inhibit reverse transcriptase and DNA polymerase enzymes and thus to interfere with RNA measurements by real-time PCR. Therefore we hypothesized that traces of heparin in our plasma samples interfered with our miRNA measurements and thus caused the variability between the samples. We also hypothesized that heparinase treatment would be able to break down this heparin and rescue our miRNA measurements. To test the effect of heparin contamination on our miRNA measurements and whether we were able to rescue these measurements by heparinase treatment we set up an experiment with human plasma. We collected plasma of healthy volunteers in EDTA tubes.

Figure 3. Heparinase treatment reduces variability of reference miRNAs. A) MiRNA levels in arterial and venous plasma, determined without heparinase treatment of the RNA samples, show a huge variability in the spiked in miRNA, cel-miR-54. B) Heparinase treatment reduces variability of cel-miR-54 in arterial and venous plasma samples. In this Figure we only show the animals of the 1 week AoB group (n=8 sham, n=11 AoB) as example. Results were comparable for the 3 and 8 week group.
and added heparin in different end-concentrations, where the end-concentration of 1.5 IU/ml corresponds to the dose in which heparin is injected in patients and 15 IU/ml corresponds to the end-concentration when blood is drawn using heparin-tubes. We compared the detection of two endogenous miRNAs and one *c. elegans* spike-in in these samples with and without heparinase treatment. This revealed that already the lowest amount of heparin (0.15 IU/ml) was sufficient to completely inhibit detection of these three miRNAs (Figure 4). Furthermore this experiment showed that heparinase treatment was able to rescue miRNA measurements of cel-miR-39 and miR-423-5p when heparin was present up to 15 IU/ml and that the measurements were slightly inhibited at higher concentrations of heparin. On the other hand, detection of miR-1249 was still slightly decreased after heparinase treatment in samples with low amounts of heparin and here a dose-response of increasing amounts of heparin is visible (Figure 4).

As the blood of our pigs was expected to contain only traces of heparin, the above-described experiment indicated that heparinase treatment might reduce the variability detected in our arterial and venous blood. Therefore we treated all plasma RNA samples with heparinase and determined the miRNA levels of cel-miR-54 and miR-15b again. We compared the detected miRNA levels after heparinase treatment with the detected levels before heparinase treatment and confirm that heparinase treatment was able to reduce the variability in these miRNA measurements and rescue them in most of the plasma samples (Figure 3B). Some samples still showed very low amounts of the measured miRNAs (e.g. arterial blood of pig 17, Figure 3B), these pigs (venous and arterial samples) are excluded from further analysis.

Figure 4. Heparinase treatment rescues miRNA detection after addition of heparin. We added several amounts of heparin to human plasma of 2 healthy control subjects and show that already very low amounts inhibit miRNA detection by real-time PCR. Heparinase treatment rescues miRNA detection with a miRNA-specific efficiency, which is highly efficient for miR-423-5p and cel-miR-39 and less efficient for miR-1249.
Circulating miRNAs levels are not changed in the plasma of hypertrophic pigs

After heparinase treatment we were able to reliably determine the levels of the 9 selected miRNAs in the arterial and venous blood samples. We not only compared the miRNA levels between sham and AoB-operated animals in arterial and venous blood, but we also calculated the gradient over the myocardium by subtracting the arterial miRNA levels from the venous levels, which implicates that a positive gradient represents release and a negative gradient uptake by the myocardium. In Figure 5 we show the miRNA levels in arterial and venous blood and the calculated gradients of miR-21, miR-423-5p, miR-129-5p and miR-622 at the 3 time-points after AoB again as an example for all determined miRNAs. What can be appreciated from these figures is that none of the miRNAs was significantly regulated in arterial or venous blood in AoB compared to sham-operated animals. Furthermore there were no significant changes in gradients between sham and AoB-operated animals, indicating no difference in release or uptake of these miRNAs in the hypertrophic compared to the healthy myocardium. Most of the calculated gradients showed such high variation that they are not significantly deviating from zero gradient, indicating that there is no uptake or release at all by the heart. Only at 3 weeks after AoB there is a trend towards an increase of miR-423-5p, miR-129-5p and miR-622 (which was not detected for the other measured miRNAs) in venous blood of AoB compared to sham animals, which is accompanied by an increase in the positive gradient indicating that this increase might result from release by the myocardium.

DISCUSSION

Here we investigated whether increase of certain circulating miRNAs in plasma of heart failure patients might result from release by the myocardium, which was suggested by the previously identified upregulation of the prime candidate, miR-423-5p, in human failing myocardium. To investigate this hypothesis we used a porcine model of pressure-overload induced hypertrophy by AoB, where we investigated the myocardial and plasma miRNA levels 1, 3 and 8 weeks after AoB. Unfortunately we were not able to detect myocardial regulation of miR-423-5p, nor any other miRNA (except for the positive control miRNA, miR-21) in the hypertrophic myocardium, nor were we able to detect changes in miRNA levels in the blood.

The lack of regulation of these miRNAs in the porcine hypertrophic myocardium compared to the regulation of miR-423-5p in the failing human myocardium might be related to the stage of the disease. Since cardiac hypertrophy reflects an early stage of cardiac remodeling, it may be quite distant from heart failure which is the end-stage of the disease. This difference in hypertrophy compared to heart failure might also underlie the lack of changes in circulating miRNAs in AoB versus sham-operated animals. Therefore this model may be better suited to detect more hypertrophy-specific circulating miRNA profiles in the plasma samples by micro-array or deep-sequencing methods. So in other words, the lack of changes in circulating miRNAs in this study does not implicate that these miRNAs would not be increased in the plasma of
pigs with heart failure. Therefore we cannot determine whether this possible increase would be derived from the failing myocardium on the base of the lack of changes in circulating miRNAs in this study.

Having said that, we do detect a trend towards an increase of miR-423-5p, miR-129-5p, and miR-622 in the venous blood of 3 weeks AoB compared to sham-operated pigs and the accompanying small increase in the positive gradient further suggests that these miRNAs might be released by the failing myocardium. Furthermore we did not detect an increase of circulating miRNAs in AoB compared to sham-operated animals without changes in the gradient over the heart, which excludes that these circulating miRNAs are released by other organs already early in
the disease progression during hypertrophy. However this does not indicate that the increase of these circulating miRNAs during heart failure might not be derived from other organs.

A limitation of this study is that we cannot be sure whether this model is suitable to measure gradients over the heart. This is mainly due to the long half-life of miRNAs, which might result in accumulation of miRNAs in plasma. As a result of this accumulation miRNA levels in arterial blood might be relatively high compared to the amount of miRNAs released by the myocardium, which results in a lack of detection of this gradient as the small gradient will be regarded as noise compared to the arterial miRNA level. We do not regard this as a major concern in our study, as we did not detect an increase of miRNA levels in arterial blood of AoB compared to sham-operated animals, which indicates that the measured miRNAs are not accumulating in the blood. Unfortunately there is no literature about circulating miRNAs in hypertrophy and therefore we could not include a positive control in the plasma part of the study. The positive control miRNA, miR-21, we used for the tissue regulation is not suitable as a positive control for the plasma samples as it was not identified before as in- or decreased in plasma of heart failure patients. However, the miRNAs that are identified to be increased in the plasma of heart failure patients differ from the miRNAs regulated in the failing myocardium and therefore no better positive control was available.

It was previously shown that heparin inhibits reverse transcriptase and DNA polymerase enzymes and thus interferes with RNA measurements by real-time PCR. Because our pig plasma samples were contaminated with heparin, we investigated whether heparin also interferes with miRNA measurements by real-time PCR. We show that addition of already very low amounts of heparin inhibits the detection of miRNAs by real-time PCR dramatically. We also show that heparinase treatment of heparin contaminated plasma samples rescues the detection of miRNAs by real-time PCR. However, the success of this rescue appears to be miRNA-specific, as for some miRNAs rescue was complete, while others still showed some inhibition in the samples were heparin was added compared to samples without heparin (Figure 4). A reason for this miRNA-specific rescue might be the efficiency of amplification in the real-time PCR reaction, which is primer and amplicon dependent. This indicates that in case heparin breakdown by heparinase treatment is not complete, tiny amounts of heparin might still be able to inhibit the detection of miRNAs with less efficient amplification, while miRNAs with efficient amplification can be reliably measured. In general these results indicate that large plasma databases, in which patients received heparin as part of clinical practice, can still be used for miRNA research after heparinase treatment of RNA samples. Although some miRNAs, which are sensitive to heparin inhibition might be missed or non-reliably detected in these studies. Therefore one should test before whether the miRNA of interest is sensitive to heparin inhibition.
REFERENCES


