Circulating MicroRNA-208b and MicroRNA-499 Reflect Myocardial Damage in Cardiovascular Disease

Running title: Corsten et al.; Circulating microRNAs as cardiovascular biomarkers

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Abstract:

Background - Small RNA molecules, called microRNAs, freely circulate in human plasma and correlate with varying pathologies. In this study, we have explored their diagnostic potential in a selection of prevalent cardiovascular disorders.

Methods and Results - MicroRNAs were isolated from plasmas from well-characterized patients with varying degrees of cardiac damage: a) acute myocardial infarction (AMI), b) viral myocarditis (VM), c) diastolic dysfunction, and d) acute heart failure (AHF). Plasma levels of selected microRNAs, including heart-associated (miR-1, -133a, -208b and -499), fibrosis-associated (miR-21 and miR-29b) and leukocyte-associated (miR-146, -155 and -223) candidates, were subsequently assessed using real-time PCR. Strikingly, in plasma from AMI patients, cardiac myocyte-associated miRs-208b and -499 were highly elevated, 1600-fold (p<0.005) and 100-fold (p<0.0005) respectively, as compared with controls. ROC-curve analysis revealed an AUC of 0.94 (p<10^-10) for miR-208b and 0.92 (p<10^-9) for miR-499. Both microRNAs correlated with plasma troponin T, indicating release of microRNAs from injured cardiomyocytes. In VM, we observed a milder but significant elevation of these microRNAs, 30- and 6-fold respectively. Plasma levels of leukocyte-expressed microRNAs were not significantly increased in AMI or VM patients, despite elevated white blood cell counts. In AHF patients, only miR-499 was significantly elevated (2-fold), while no significant changes in microRNAs studied could be observed in diastolic dysfunction. Remarkably, plasma microRNA levels were not affected by a wide range of clinical confounders, including age, gender, BMI, kidney function, systolic blood pressure, and white blood cell count.

Conclusions - Cardiac damage initiates the detectable release of cardiomyocyte-specific microRNAs-208b and -499 into the circulation.

Key words: plasma microRNA, diagnosis, myocardial infarction, myocarditis, heart failure
Introduction

MicroRNAs (miRNAs) form a class of small (~22 nucleotides) non-coding RNAs that negatively regulate gene expression by recognizing complementary messenger RNAs (mRNAs) and prohibiting their translation into functional protein\(^1\). Their critical (patho)physiological importance is evidenced by their marked evolutionary conservation and current estimates are that they fin-tune expression of up to 50% of protein-coding genes\(^2, 3\). MiRNAs are crucial for virtually all cellular processes and are a prerequisite for normal cardiac function\(^4-7\). Consequently, aberrant miRNA expression profiles are associated with various cardiovascular conditions such as hypertrophy, fibrosis, heart failure, and arrhythmias\(^8-11\).

Recently, different groups reported that non-cell associated miRNAs are circulating freely in mammalian blood with marked biostability, and can be detected with high sensitivity and specificity in human plasma and serum\(^12, 13\). Though the biological meaning of these miRNAs is unclear, pioneer profiling studies have attributed specific serum miRNA signatures to conditions varying from pregnancy to drug-induced liver injury, sepsis, and a variety of malignancies\(^12-16\). The diagnostic potential of miRNA detection in human plasma for cardiovascular disorders is beginning to be explored: Ai et al. studied plasmas of 93 AMI patients and found miR-1 increased\(^17\); Adachi et al. found elevated miR-499 in plasmas of 9 AMI patients\(^18\); increased plasma levels of miR-208a were reported by Wang et al. for 33 AMI patients\(^19\); Cheng et al. reported increased plasma miR-1 levels in 31 AMI patients\(^20\); and finally in a total of 42 heart failure patients, miR-423-5p was found increased by Tijsen et al. and correlated with proBNP levels\(^21\). Cardiovascular disease populations typically are elderly, overweight, and are associated with possible confounding factors such as age, gender,
body-mass-index, and kidney and liver function. The importance of these clinical confounders for multiple plasma miRNA levels has, to our knowledge, not been investigated to date.

Methods

Clinical samples All clinical samples were obtained with approval of the local Human Research Ethical Committees and stored at -80 degrees Celsius. They were subjected to freeze-thawing once.

Acute myocardial infarction (AMI): Citrate plasma samples were obtained from patients presenting with AMI (n=32) and patients with atypical chest pain, positive stress testing but with normal coronary angiograms (n=36). All patients with acute MI were enrolled in the Luxembourg acute MI registry and treated with primary percutaneous coronary intervention. All patients had successful mechanical reperfusion and stenting of the infarct artery within 12 hours of chest pain onset. All patients received Aspirin, Clopidogrel, Heparin and Abciximab. Acute MI was defined by the presence of chest pain < 12 hours with significant ST elevation and increase in creatine kinase and troponin I to greater than 2-fold upper limit of normal. Blood samples were obtained at the time of mechanical reperfusion. More details are provided in Supplementary Table 1.

Viral myocarditis (VM): Ethylenediaminetetraacetic acid (EDTA) plasma samples were obtained from patients with acute VM during hospitalization in the acute phase (n=14), post-VM phase (ranging from 1 to 12 months after the acute inflammatory phase, n=20) and from age-matched healthy controls (n=20) at the Cardiology Department of the Maastricht University Medical Center between 2007 and 2009. Acute VM patients were further subdivided into mild VM (troponin T<0.01μg/L and normal left ventricular ejection fraction (LVEF), n=4), moderate VM (elevated troponin T or LVEF<50%, n=5) and severe VM (LVEF<30%, n=5). See Supplementary Table 2.
**Diastolic dysfunction:** We examined EDTA plasma samples from 79 individuals at ages of 42-85, originally selected from a general Flemish population for the FLemisch Study on Enviroment, Genes, and Health Outcomes (FLEMENGHO\textsuperscript{22}), and thoroughly examined including echocardiographic analysis and detailed epidemiological characterization, as described previously\textsuperscript{23}. Asymptomatic individuals were age-matched and divided into three groups. Group 1: controls (n=20) with normal cardiac function and no risk factors, corresponding to AHA stage 0 of Heart Failure\textsuperscript{24}. Group 2: hypertensive individuals (n=20) without structural or functional cardiac involvement, corresponding to AHA stage A of HF. Group 3: hypertensive individuals with clear signs of cardiac involvement (n=39) with impaired left ventricle (LV) relaxation, or elevated end-diastolic LV pressures, corresponding the AHA stage B of HF. Full details are provided in Supplementary Table 3.

**Acute heart failure (AHF):** EDTA plasma samples were collected from patients that were admitted with acute decompensation of congestive heart failure according to predefined Framingham-based criteria\textsuperscript{25} and an elevated plasma NT-proBNP (>200pmol/L) (n=33), and from healthy staff-members as controls (n=34) at the Cardiology Department of the Maastricht University Medical Center. For additional details, see Supplementary Table 4.

**Whole blood samples:** To compare miRNA levels in matched plasma and leukocyte-rich whole blood, whole blood samples were used from 12 individuals from the Diastolic dysfunction cohort.

**Urine samples:** To investigate whether plasma miRs are excreted from the blood through glomerular filtration, simultaneous urine and EDTA plasma samples were taken from healthy volunteers (n=5) from the VM healthy control group in Maastricht in November 2009.
**RNA extraction** Total RNA was extracted from 100 µL of plasma, whole blood or urine, using the mirVana PARIS kit (Ambion, Warrington, UK), according to the manufacturer’s instructions and without enrichment for small RNAs, and subsequently eluted in 50 µL nuclease-free water. Subsequently, potential genomic DNA contamination was eliminated using DNA-free kit (Ambion).

**cDNA synthesis and qRT-PCR** 15 µL of RNA was used per 20-µL reaction to generate cDNA using the miScript kit (Qiagen, Venlo, The Netherlands) that is designed to specifically detect mature microRNAs. The 20 µL reaction mix was then diluted 4x in nuclease free water and 2 µL of cDNA was added per qPCR reaction, using BR SYBR-green supermix for IQ (Quanta Biosciences, Amsterdam, The Netherlands) in a MyIQ iCycler (Bio-Rad, Veenendal, The Netherlands) device using MiScript primers sets (Qiagen). The selection of miRNA candidates to be tested was based on intellectual choice for each disease cohort. The following miRNAs were assayed: AMI: miR-1, -122, -133a, -208b, -223, -499; VM: miR-1, -133a, -146a, -146b, -155, -208b, -223, -499; Diastolic Dysfunction: miR-1, -21, -29b, -122, -126, -133a, -146a, -146b, -155, -208b, -223, -499 and AHF: miR-1, -122, -133a, -208b, -223, -499.

**Normalization of experimental data** Quantitative qPCR measurements of miRNA levels were normalized for isolation efficiency using a mix of three spiked-in synthetic *C. elegans* miRNAs (Eurogentec, Maastricht, The Netherlands), lacking sequence homology to human miRNAs, as described previously13. Oligonucleotides were spiked into the samples during RNA isolation following plasma incubation with the provided 2x denaturing solution. We have chosen to correct for spikes rather than for endogenous “stable” miRNAs, since there is no consensus in the literature regarding stable microRNAs for correction.
Statistical analyses Statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± SEM unless indicated otherwise and plasma miRNA levels are presented as fold-change relative to controls. Linear regression analyses were used to correlate quantitative variables following normalization of non-Gaussian variables by log-transformation. Regression analyses of miRNA levels versus clinical parameters in the diastolic dysfunction database involved n=79 patient plasmas, resulting in a power of 80% to demonstrate a Pearson correlation of 0.31. Comparisons between 2 groups were performed with Student’s t tests for Gaussian data or Mann-Whitney tests for non-Gaussian data. For comparisons of more than 2 groups, one-way ANOVA was used, followed by post hoc testing using Bonferroni correction for more groups. Receiver operating characteristic curves were generated with PASW 18.0 for Macintosh, using nonparametric analyses to estimate error margins for the area under the curve.

Results

Plasma miRNA-208b and -499 levels are highly elevated after acute myocardial infarction and correlate with cardiac injury markers

We first established miRNA plasma levels in AMI patients as compared to patients presenting with atypical chest pain and no cardiac disease. Heart-associated miRNAs, but not liver-specific miR-122 or leukocyte-associated miR-223, were elevated in AMI patients compared to controls (Figure 1). While miR-133a (p<0.05) and miR-1 (non significant) levels were mildly increased, we found a robust elevation of plasma miR-208b and miR-499 levels by ~1600-fold (p<0.005) and ~100-fold (P<0.0005), respectively. Receiver operating
characteristic (ROC) curves generated for both miRNAs revealed an area under the curve of 0.944 for miR-208b (95% confidence interval = 0.863-1.000, p<10^{-10}) and 0.918 for miR-499 (95% confidence interval = 0.842-0.995, p<10^{-9}) (Figure 2). Subsequently, we assessed the correlation between plasma levels of miR-208b and miR-499 with serum levels of cardiac injury markers troponin T (Figure 2C and D) and creatine phosphokinase (CPK) in the AMI group (Supplementary Figure 1). Both miR-208b and miR-499 correlated significantly to troponin T and CPK levels, with the highest degree of correlation observed for miR-499 (CPK: regression coefficient R=0.41, p<0.0001; and troponin T: R=0.69, p<0.0001).

Intriguingly, miR-223 was mildly but significantly decreased in AMI patients as compared to controls. We found no correlation between miR-223 levels and Troponin T levels or WBC counts (Supplementary Figure 1). Wang et al. also report decreased miR-223 levels in patients with sepsis. Future research will need to establish if there is a rationale for the diminished presence of this predominantly granulocyte-originating miRNA in inflammation-related pathologies.

**Cardiomyocyte-associated but not inflammatory microRNAs are significantly elevated in the acute stage of viral myocarditis**

We subsequently investigated plasma miRNA levels in patients with acute VM, patients in the post VM phase, and healthy controls. Again, levels of miR-208b and miR-499 were significantly elevated (30- and 6-fold, respectively, p<0.01 versus controls for both) during the acute phase of VM (Figure 3 and Supplementary Figure 2). Their expression levels significantly correlated with the severity of VM: subgroup analysis within the acute VM patients revealed normal values of miRs-208b and -499 in mild VM patients (with no troponin T elevation), and increasing levels for moderate and severe VM (~20-fold and 50-fold higher, respectively, than control levels for miR-208b, and ~5-fold and 10-fold for miR-
Of note, the more accentuated rise in miRNA plasma levels in AMI compared to VM paralleled the higher troponin T levels in AMI patients (Supplementary Tables 1 and 2). Levels of miR-1, -133a and leukocyte-associated miRs-146a, -146b, -155 and -223 were not significantly different between groups (Figure 3), despite significant leukocytosis in the acute VM group (Supplementary Table 2).

**Cardiomyocyte- and leukocyte-expressed plasma miRNAs are not diagnostic for diastolic dysfunction**

None of the muscular or leukocytic miRNAs studied showed significantly different levels between the healthy controls, patients with hypertension without cardiac changes and patients with evident myocardial diastolic dysfunction (Figure 4 and Supplementary Figure 3). Also levels of miR-21 and -29b, which are both involved in the pathophysiology of cardiac fibrosis and upregulated in failing human hearts, were not elevated in diastolic dysfunction plasmas. We then investigated correlations of the individual miRNA levels to cardiovascular indices. Intriguingly, the myocardium-associated miR-133a correlated significantly and positively to serum NT-proBNP levels (R=0.43, p=0.001, see Figure 4H). However, this correlation was independent of cardiac diastolic function, as miR-133a was not elevated in patients with diastolic dysfunction (Figure 4D). In addition, the NT-proBNP levels in this cohort were very mildly elevated, as opposed to the NT-proBNP levels in the acute heart failure cohort, where no correlation was found (Figure 5G). Tijsen et al. also found no association between miR-133a plasma levels and NT-proBNP levels in heart failure patients. No correlations were observed between miRNA plasma levels and systolic blood pressure, or left ventricular mass index (Supplementary Table 5). Finally, no differences were found in plasma microRNA levels of smokers and non-smokers, with exception of miR-29b, which was significantly higher in smokers (p=0.02).
Acute heart failure provokes mild differences in heart-associated miRNAs

We subsequently assessed plasma miRNA levels in patients with acutely decompensated AHF. All heart-associated miRNAs showed a trend towards mildly higher plasma levels in the AHF group, reaching significance for miR-499 (2-fold; p<0.05) (Figure 5). Of note, significance might have been masked for miR-1, by the mild negative correlation of miR-1 with age (Supplementary Table 1, R=-0.25, p=0.04) and the higher mean age of AHF patients versus controls. Additionally, the liver-specific miR-122, that is known to correlate with hepatic damage, was significantly elevated in AHF patients (Figure 5), possibly reflecting hepatic venous congestion. In this patient group, we found no positive correlation of miR-133a with NT-pro-BNP levels (Figure 5G), nor with troponin T levels (Figure 5H).

Circulating plasma miRNAs are markedly robust against clinical confounders including age, gender, and renal function

To determine to which extent plasma miRNA levels are confounded by the baseline characteristics of plasma donors, we selected 79 samples from the extensively characterized diastolic dysfunction patient database to evaluate the potential impact of a set of clinical confounders on miRNA levels (See Supplementary Table 5). Linear regression analyses revealed that plasma miRNA levels were not affected by the evaluated parameters. Patient gender and body mass index did not correlate with any of the plasma miRNAs (Supplementary Table 5). Plasma sample age, ranging from 11 to 49 months also had no influence on detected levels, indicating that miRNAs are not degraded when stored at -80 degrees Celsius. Patient age did not significantly correlate with 10 out of 12 miRNAs, and only showed weak negative correlation with miRNAs -1 and -29b (R=-0.25 and -0.26,
respectively and p<0.05 for both). Interestingly, glomerular filtration rate did not correlate with plasma miRNA levels. In addition, we investigated matched plasma and urine levels of miR-122 (liver) and -223 (granulocytes), and found that while both miRNAs were well detected in both sample types, levels in plasma compared to urine were ~1000-fold higher for miR-122 and ~100 fold for miR-223 (Supplementary Figure 4). Together, these data indicate that freely circulating miRNAs are not renally cleared.

Plasma levels of the liver-specific miR-122 correlated with levels of the liver-specific marker gamma glutamyl transferase (gamma GT) (Supplementary Table 5), in agreement with the elevated levels of miR-122 in AHF (Figure 5E). Finally, levels of miR-133a correlated with NT-proBNP levels in this patient group, as described above.

Leukocyte-associated miRNAs do not reflect white blood cell count or leukocytic miRNA levels

To investigate whether leukocyte-associated miRNAs could serve as markers for mild inflammatory processes, we compared plasma levels of inflammatory miRNAs to WBC counts. Plasma levels did not correlate with circulating numbers of leukocytes, indicating that secretion of miRNAs from circulating leukocytes is not the main determinant of their plasma levels (Supplementary Figure 5 and Supplementary Table 5). This lack of correlation was independently confirmed in our plasma databases of acute VM and AMI plasmas with leukocytosis (Supplementary Figure 1 and data not shown). We subsequently compared the levels of plasma miRNA with whole blood miRNA levels (containing leukocytes) across patients, but again did not find a significant correlation (Supplementary Figure 4), indicating that plasma miRNAs reflect neither WBC count nor leukocytic miRNA expression. However, we did notice a striking quantitative correlation between leukocyte-associated but not cardiac miRNAs within samples (Supplementary Table 6A and B), pointing towards a common
etiology. We also found highly identical patterns of these miRNAs in plasma and whole blood (Supplementary Figure 6) within individuals, with levels always 1000-fold higher in whole blood, suggesting that leukocytes are indeed the primary source of these miRNAs in plasma. The paradoxical lack of correlation with WBC counts and whole blood miRNA might be explained by variable leakage between patients, compromising their diagnostic accuracy.

Discussion

Recent evidence that freely circulating miRNAs may be informative of human pathology has ignited wide interest in their diagnostic potential. Our study demonstrates that in humans, diverse conditions of myocardial damage are associated with striking perturbations of plasma levels of heart-associated miRNAs-208b and -499. These are increasingly elevated during acute heart failure (minimal), viral myocarditis (marked), and acute myocardial infarction (extensive). In AMI, where cardiac damage was most severe, ROC-curves for AMI detection by miR-208b or -499 levels revealed a pronounced diagnostic accuracy, evidenced by an area under the curve of >0.91 with p<10^-9 for both individual miRNAs. Encouragingly, these data are in agreement with a recent report published during the preparation of this manuscript, that examined plasma levels of muscle-specific miRNAs in AMI patients and also identified miRs-208(a) and -499 as the most sensitive biomarkers of AMI. Supplementary Table 7 provides an overview of the plasma miRNA changes reported in cardiovascular disease thus far. In addition, our study uniquely shows that miR-208b and -499 release is not exclusive for AMI and occurs independently of ischemia and ejection fraction. Biologically, these data evoke tantalizing questions about potential functions for circulating miR-208b and -499, which modulate cardiomyocyte performance by shifting the
balance between slow and fast muscle fibre gene programs towards the slow type \(^7\) \(^{30}\). There is currently no evidence demonstrating a functional role for plasma miRNAs through affecting distant gene expression in vivo. Interestingly, it was reported that following drug-induced liver injury, miRNAs elevated in plasma were consistently downregulated in corresponding livers \(^{16}\) suggesting the intriguing possibility of a cellular survival mechanism in which, during stress, undesirable miRNAs are actively excreted. However, miR-208 is elevated in acutely infarcted human hearts \(^{31}\) and additional research is required to address the biological role of plasma miRNAs after cardiac damage.

Interestingly, the profound increases of miRNAs -208b and -499 in patients with AMI (1600- and 100-fold, respectively) are in contrast with the mild elevations observed for heart-associated miRs-1 and -133a (2- and 4-fold, respectively). This could be a reflection of the higher specificity of miR-208b and -499 for cardiac muscle, or higher baseline levels of miR-1 and -133a in serum, due to turnover of skeletal muscle \(^{19}\). An alternative possibility is that these miRNAs might be trapped in structural complexes in cardiomyocytes, preventing leakage. Three groups previously reported that plasma miR-1 levels are elevated in AMI patients \(^{17,19,20}\), but also in these studies this elevation was milder than observed for miR-208b and -499 (see Supplementary Table 7). Our data generally support their conclusions but extend on their findings by identifying miRs -208b and -499 as far more sensitive markers for myocardial damage not only in AMI but also in VM.

An intriguing observation from our study was the correlation of miR-133a plasma levels with with NT-proBNP in asymptomatic patients with diastolic dysfunction, which was not observed in AHF patients. Additional research in larger patient populations will be required to unveil the significance of this phenomenon.
The influence of potentially confounding patient characteristics has so far remained largely unexplored. We present evidence that the investigated plasma miRNA levels are highly robust to a variety of patient parameters including age, gender, body mass index, systolic blood pressure, smoking, and glomerular filtration rate. Further, the liver-specific miR-122 was the only miRNA mildly correlating with gamma GT levels, in agreement with the recent report that plasma miR-122 parallels levels of aminotransferase levels during hepatic damage\textsuperscript{16}. These findings are of vital importance for the diagnostic applicability of plasma miRNAs in clinical settings, and in particular cardiovascular medicine, where skewed distributions of age and biometry, combined with comorbidities such as renal failure are common. Our data indicate caution to use leukocyte-expressed plasma miRNAs as diagnostic targets in conditions of mild inflammation, as their levels may be subject to variable leakage from circulating leukocytes, eg caused by venipuncture. On the other hand, the significant decrease in miR-223 levels following AMI has potential biological and/or diagnostic significance, given the study by Wang et al.\textsuperscript{26} who find decreased miR-223 levels during sepsis. Therefore, the value of miR-223 for AMI needs to be investigated further.

In conclusion, we report that cardiac damage in diverse cardiac diseases initiates massive release of cardiomyocyte-specific microRNAs into the circulation, and that these microRNAs are highly insensitive to clinical characteristics in a cardiovascular patient population.

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Conflict of Interest Disclosures: none

References:

7. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ, Jr., Olson EN. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell. 2009;17:662-673.


Figure Legends:

Figure 1. Cardiomyocyte miRNA levels mark acute myocardial infarction (AMI). Compared to microRNA levels in controls with atypical chest pain and no cardiac disease (n=36), AMI patient plasmas (n=32) showed a sharp rise in levels of heart-associated microRNAs miR-208b (1600-fold, p<0.005), -499 (100 fold, p<0.0005) and to a lesser extent miR-133a (p<0.05) and miR-1 (p=0.12) (panels A through D). Plasma levels of liver-specific miR-122 were not different between groups (F) and miR-223 levels were even decreased in AMI plasmas (p<0.005) (E).

Figure 2. MiR-208b and miR-499 are potential circulating biomarkers for acute myocardial infarction (AMI). (A and B) Scatter plots of plasma miR levels in controls (n=36) versus AMI patients (n=32), revealing a 1500-fold increase for miR-208b (p<0.005) and 90-fold increase for miR-499 (p<0.001) in AMI. MiR-208b was not detected in 32 out of 36 controls and 3 our of 32 AMI patients. (C and D) Within the AMI groups levels of miR-208b and miR-499 correlated significantly with circulating troponin T levels (miR-208b:
R=0.59, p=0.0005 and miR-499: R=0.69, p<0.0001). Panels A-D show that a few AMI patients had relatively low miR-208 and miR-499 levels. This was reproducible and not due to technical issues. (E and F) Receiver operating characteristic (ROC) curves revealing an area under the curve of 0.944 for miR-208b (95% confidence interval: 0.863-1.00, p<10^-10) and 0.918 for miR-499 (95% CI: 0.842-0.995, p<10^-9).

**Figure 3. Plasma miRNA levels reflect myocardial damage but not inflammation in acute viral myocarditis.** (A and B) MiR-208b and -499, which were the strongest indicators of myocardial damage in myocardial infarction, are significantly elevated in plasmas from acute VM patients (n=14), compared to post VM patients (n=20, p<0.05 for miR-208b and p<0.01 for miR-499) or healthy controls (n=20, p<0.01 for both miRs). MiRs-1 and -133a levels were not significantly different between groups (C and D). (E-H) Leukocyte-associated miRs-146a, -146b, -155, and -223 showed a non-significant trend to higher levels during the acute phase of VM.

**Figure 4. Plasma miRNA levels in a cohort of diastolic dysfunction.** Levels of our plasma microRNA panel did not discriminate between diastolic dysfunction patients (n=39) and hypertensive (n=20) or normotensive (n=20) controls. Panels A through G depict levels of the muscle-associated miRs-208b, -499, -1 and -133 (A-D), fibrosis associated miRs-21 and -29b (E and F), and the leukocyte-associated miR-223 (G). (H) The striated muscle associated miR-133a correlates significantly to serum N-terminal prohormone brain natriuretic peptide (NT-proBNP) levels in patients with diastolic dysfunction (R=0.43, p=0.001). Color-coding of data points in 4H is as follows: controls black, hypertensive patients grey, and patients with diastolic dysfunction white.
Figure 5. Plasma miRNA levels in patients with acutely heart failure AHF. (A-D) Plasma levels of the heart-associated miRs-208b, -499, -1, and -133a were higher in AHF patients (n=33) than in controls (n=20), although only miR-499 level differences were significantly elevated (p<0.05). The liver-specific miR-122 was elevated in AHF patients (E), while granulocyte-specific miR-223 was not different for AHF patients and controls (F). (G) In AHF patients, miR-133a failed to correlate with NT-proBNP (R=-0.32, p=0.09) as was observed in diastolic dysfunction patients. This lack of correlation could not be explained by a correlation with circulating troponin T levels (R=0.27, p=0.23) (H).
miR-208b level

miR-208b

p < 0.005

n=36  n=32

Controls  AMI

miR-499 level

miR-499

p < 0.0005

n=36  n=32

Controls  AMI

Trop T vs miR-208b in AMI

Log miR-208b

R = 0.59

p = 0.0005

Log Troponin T (μg/L)

Trop T vs miR-499 in AMI

Log miR-499

R = 0.69

p < 0.0001

Log Troponin T (μg/L)

ROC-curve miR-208b

AUC = 0.944

(0.863–1.000)

1 - Specificity

ROC-curve miR-499

AUC = 0.918

(0.842–0.995)

1 - Specificity
A) miR-208b

\[ p < 0.01 \quad p < 0.05 \]

B) miR-499

\[ p < 0.01 \quad p < 0.01 \]

C) miR-1

\[ p > 0.05 \quad p > 0.05 \]

D) miR-133a

\[ p > 0.05 \]

E) miR-146a

\[ p > 0.05 \quad p > 0.05 \]

F) miR-146b

\[ p > 0.05 \quad p > 0.05 \]

G) miR-155

\[ p > 0.05 \quad p > 0.05 \]

H) miR-223

\[ p > 0.05 \quad p > 0.05 \]
Circulating MicroRNA-208b and MicroRNA-499 Reflect Myocardial Damage in Cardiovascular Disease

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SUPPLEMENTAL MATERIAL

Clinical perspective

It was recently discovered that small RNAs, called microRNAs, circulate freely and stably in human plasma. This finding has sparked interest in the potential of microRNAs as biomarkers, because microRNAs are strongly implicated in cardiovascular disease and RNA molecules can be detected with high specificity and sensitivity using novel molecular techniques. We report profiles of microRNAs in varying cardiovascular disorders: acute myocardial infarction (AMI), acute viral myocarditis (VM), diastolic dysfunction and acute heart failure. Most strikingly, 2 cardiomyocyte-specific microRNAs (miR-208b and miR-499) are markedly elevated upon cardiac damage and correlate to circulating levels of troponin T. Our findings suggest a potential role for microRNAs as biomarkers in cardiology and mandate subsequent investigations to define their clinical applicability in early detection of myocardial damage.

Supplementary Figure Legends

Suppl. Figure 1. Plasma miRNA levels in AMI. Plasma levels of miR-208b (panel A) and miR-499 (B) correlate with circulating levels of cardiac damage marker creatine phosphokinase (CPK) in AMI patients (miR-208b: R=0.40, p=0.03 and miR-499: R=0.41, p=0.01). Regression analysis between plasma levels of the granulocyte-specific miR-223 and white blood cell count (panel C) revealed no correlation (R=0.10, p=0.63). MiR-223 levels also did not correlate with levels of troponin T (panel D; R=0.03, p=0.88).

Suppl. Figure 2. Plasma miR-208b and miR-499 elevation in viral myocarditis reflects VM severity. Plasma levels of miR-208b (panel A) and miR-499 (panel B) are elevated in acute viral myocarditis in relation to disease severity, as classified into mild VM (troponin
T<0.01μg/L and normal left ventricular ejection fraction (LVEF), n=4), moderate VM (elevated troponin T or LVEF<50%, n=5) and severe VM (LVEF<30%, n=5). MiR-208b was detected in only 4 out of 20 control patients and none of the patients in the mild VM group (that per definition does not include patients with troponin T elevation). MiR-499 was detected in all patients and levels were comparably low in the control and mild V group. Moderate VM samples display a trend towards elevation of both microRNAs and in severe VM, both miR-208b and miR-499 are significantly elevated (p<0.001).

Suppl. Figure 3. Plasma miRNA levels in patients with myocardial Diastolic Dysfunction. Panels A through L depict plasma miRNA levels in controls without hypertension or cardiovascular risk factors, hypertensive patients without cardiac involvement, and patients with evident diastolic dysfunction. MiRNAs studies included miRNAs associated with myocardium (miRs-, -133a, -208b, and -499), leukocytes (miRs-146a, 146b, -155, 223), fibroblasts (miRs-21, and -29b), endothelium (miR-126) and liver (miR-122). Panel M depicts NT-proBNP levels for all three groups (not significantly different).

Suppl. Figure 4. Plasma miRNAs are not subject to efficient glomerular filtration. MicroRNA detection in matched plasma and urine samples revealed substantially higher levels in plasmas for all investigated microRNAs, which, together with he lack of correlation of with glomerular filtration rate, indicates that microRNAs are not renally cleared. Displayed microRNAs were selected for predominant preglomerular origin (miR-122: liver-specific; miR-223: granulocyte-specific). Error bars represent standard deviations.

Suppl. Figure 5. Plasma microRNAs do not reflect white blood cell count or leukocytic microRNA fraction in Diastolic Dysfunction. Plasma levels of leukocyte-associated microRNAs-146a, -155 and -223 fail to correlate with circulating white blood cell counts (n=79) (A, C and E). Plasma levels of leukocyte-associated microRNAs additionally do not correlate with measured microRNA levels from whole blood (n=12) (B, D and F).
Suppl. Figure 6. Plasma microRNA profiles mirror microRNA profiles in the leukocyte blood fraction and might be skewed by leakage. (A) Average threshold cycle (Ct) values for qRT-PCR detection of microRNAs in paired plasma and whole blood samples (n=12), showing the highest whole blood levels (lowest Ct values) in leukocyte-associated microRNAs-21, -29b, -126 and -223. Panel (B) displays the difference between plasma and whole blood Ct per patient. The liver-specific miR-122 is not detected earlier in whole blood, as expected due to its exclusive liver specificity. Interestingly, leukocyte-associated microRNAs show highly similar patterns in plasma and whole blood (all were detected ~11 cycles earlier in whole blood than plasma regardless of expression level), indicating that these plasma microRNA are predominantly derived from leukocytes. Since plasma microRNAs did not correlate with leukocyte counts at the individual patient level (Suppl. Fig. 5), these findings may suggest that variable leakage of microRNAs from leukocytes distorts the diagnostic accuracy of plasma microRNAs. Error bars represent standard deviations.
Suppl. Figure 1. Plasma miRNA levels in AMI

A

CPK versus miR-208b in AMI

B

CPK versus miR-499 in AMI

C

WBC count vs miR-223 in AMI

D

Trop T vs miR-223 in AMI

Log miR-208b

Log CPK (units/L)

R = 0.40
p = 0.03

Log miR-499

Log CPK (units/L)

R = 0.41
p = 0.01

miR-223 level

white blood cell count (10^6/L)

R = 0.10
p = 0.63

Log miR-223

Log Troponin T

R = 0.03
p = 0.88
Suppl. Figure 2. Plasma miR-208b and miR-499 elevation in viral myocarditis reflects VM severity.
Suppl. Figure 3. Plasma miRNA levels in patients with myocardial Diastolic Dysfunction

A. miR-1

B. miR-21

C. miR-29b

D. miR-122

E. miR-126

F. miR-133a

G. miR-146a

H. miR-146b

I. miR-155

J. miR-208b

K. miR-223

L. miR-499

M. NT-proBNP

\( p > 0.05 \)
Suppl. Figure 4. Plasma miRNAs are not subject to efficient glomerular filtration

plasma vs urine miRNA levels

>1000-fold  
$p < 0.01$

>100-fold  
$p < 0.01$

miRNA per volume

miR-122  miR-223
Suppl. Figure 5. Plasma microRNAs do not reflect white blood cell count or leukocytic microRNA fraction in Diastolic Dysfunction

A. miR-146b

B. miR-146b

C. miR-155

D. miR-155

E. miR-223

F. miR-223
Suppl. Figure 6. Plasma microRNA profiles mirror microRNA profiles in the leukocyte blood fraction and might be skewed by leakage

A

B

ΔCt [plasma - WB]
Supplemental Table 1. Acute Myocardial Infarction Database Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute Myocardial Infarction (n=36)</th>
<th>Controls (n=36)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>62 ± 13</td>
<td>62 ± 13</td>
<td>0.95</td>
</tr>
<tr>
<td>Women</td>
<td>12 (33.3)</td>
<td>13 (36.1)</td>
<td>0.86</td>
</tr>
<tr>
<td>Sample age, months</td>
<td>80 ± 10</td>
<td>94 ± 1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum Troponin T, μg/L</td>
<td>3.5 ± 4.8</td>
<td>&lt; 0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NT-proBNP, pmol/L</td>
<td>615 ± 678</td>
<td>366 ± 835</td>
<td>0.36</td>
</tr>
<tr>
<td>Serum hs-CRP, mg/L</td>
<td>0.74 ± 0.80</td>
<td>0.46 ± 0.79</td>
<td>0.23</td>
</tr>
<tr>
<td>PTCA, n</td>
<td>36 (100)</td>
<td>1 (2.8)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, or n (%)

* P-values based on student t-test or Chi-square analysis

CPK Creatine Phosphokinase
NT-proBNP N-terminal Prohormone Brain Natriuretic Peptide
hs-CRP High-Sensitivity C-Reactive Protein
PTCA Percutaneous Transluminal Coronary Angioplasty
### Supplemental Table 2. Viral Myocarditis Database Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute VM (n=14)</th>
<th>Post VM (n=20)</th>
<th>Controls (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>32.7 ± 16.3</td>
<td>37.3 ± 19.4</td>
<td>32.1 ± 7.3</td>
<td>0.51*</td>
</tr>
<tr>
<td>Women</td>
<td>2 (14.2)</td>
<td>3 (15)</td>
<td>8 (40)</td>
<td>0.96†</td>
</tr>
<tr>
<td>Sample age, months</td>
<td>19 ± 13</td>
<td>22 ± 6</td>
<td>6 ± 0.4</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum CRP, mg/L</td>
<td>95 ± 92</td>
<td>11 ± 14</td>
<td>ND</td>
<td>&lt;0.005‡</td>
</tr>
<tr>
<td>Serum troponin T, μg/L</td>
<td>1.1 ± 1.6</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>&lt;0.05‡</td>
</tr>
<tr>
<td>Blood leukocytes, x10⁹/L</td>
<td>12.5 ± 5.5</td>
<td>7.1 ± 3.1</td>
<td>ND</td>
<td>&lt;0.005‡</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>48.3 ± 14.1</td>
<td>57.2 ± 7.6</td>
<td>ND</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, or n (%)

P-values based on *One-way ANOVA, † Chi-square analysis or ‡ Student’s t-test or Mann-Whitney test.

CRP C-Reactive Protein

LVEF Left Ventricular Ejection Fraction

ND Not determined
### Supplemental Table 3. Diastolic Dysfunction Patient Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n=20)</th>
<th>Hypertension (n=20)</th>
<th>Diastolic Dysfunction (n=39)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63 ± 7</td>
<td>60 ± 8</td>
<td>69 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Women</td>
<td>9 (45)</td>
<td>8 (40)</td>
<td>17 (44)</td>
<td>0.95</td>
</tr>
<tr>
<td>Sample age, months</td>
<td>33 ± 11</td>
<td>33 ± 11</td>
<td>37 ± 11</td>
<td>0.31</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.8 ± 2.3</td>
<td>27.3 ± 4.3</td>
<td>28.8 ± 3.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>121 ± 8</td>
<td>157 ± 15</td>
<td>150 ± 13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>86 ± 17</td>
<td>90 ± 14</td>
<td>90 ± 16</td>
<td>0.86</td>
</tr>
<tr>
<td>GFR†, mL/min</td>
<td>73 ± 17</td>
<td>80 ± 16</td>
<td>72 ± 21</td>
<td>0.29</td>
</tr>
<tr>
<td>γGT, units/L</td>
<td>31 ± 20</td>
<td>36 ± 21</td>
<td>32 ± 25</td>
<td>0.78</td>
</tr>
<tr>
<td>WBC count, x10⁹/L</td>
<td>6.1 ± 2.1</td>
<td>5.6 ± 1.4</td>
<td>5.7 ± 1.7</td>
<td>0.66</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.9 ± 0.3</td>
<td>5.1 ± 0.8</td>
<td>5.2 ± 1.7</td>
<td>0.55</td>
</tr>
<tr>
<td>NT-proBNP, pmol/L</td>
<td>245 ± 118</td>
<td>268 ± 155</td>
<td>337 ± 159</td>
<td>0.09</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>88 ± 12</td>
<td>100 ± 18</td>
<td>117 ± 22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current smoker</td>
<td>6 (30)</td>
<td>5 (25)</td>
<td>4 (10)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, or n (%)

* P-values based on One-way ANOVA or Chi-square analysis

† Glomerular filtration rates were calculated using the Cockroft-Gault formula

BMI Body Mass Index
EDP End Diastolic Pressure
GFR Glomerular Filtration Rate
LVMI Left Ventricular Mass Index
NT-proBNP N-terminal Prohormone Brain Natriuretic Peptide
SBP Systolic Blood Pressure
## Supplemental Table 4. Acute Heart Failure Database Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute Decompensation (n=33)</th>
<th>Healthy Controls (n=24)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>76.3 ± 8.4</td>
<td>34.8 ± 17.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women</td>
<td>20 (60.1)</td>
<td>16 (59.6)</td>
<td>0.82</td>
</tr>
<tr>
<td>Sample age, months</td>
<td>6.5 ± 2.4</td>
<td>6.9 ± 2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>NT-proBNP, pmol/L</td>
<td>1590 ± 2022</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LVEF, %</td>
<td>37.0 ± 15.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Preserved LVEF &gt; 45%</td>
<td>15 (45)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Underlying pathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>19 (57.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valvular</td>
<td>6 (18.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, or n (%)

* P-values based on student t-test or Chi-square analysis

- CAD Coronary Artery Disease
- CRP C-Reactive Protein
- LVEF Left Ventricular Ejection Fraction
- ND Not determined
- NT-proBNP N-terminal Prohormone Brain Natriuretic Peptide
### Supplemental Table 5. Correlations (R) of clinical characteristics with plasma microRNA levels in Diastolic Dysfunction

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Distribution</th>
<th>miR-1</th>
<th>miR-21</th>
<th>miR-29b</th>
<th>miR-122</th>
<th>miR-126</th>
<th>miR-133a</th>
<th>miR-146a</th>
<th>miR-146b</th>
<th>miR-155</th>
<th>miR-208b</th>
<th>miR-223</th>
<th>miR-499</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age, years</td>
<td>65 ± 9</td>
<td>-0.25 *</td>
<td>-0.21</td>
<td>-0.24 *</td>
<td>-0.13</td>
<td>-0.19</td>
<td>-0.09</td>
<td>-0.21</td>
<td>-0.22</td>
<td>-0.09</td>
<td>0.04</td>
<td>-0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Sample age, months</td>
<td>35 ± 11</td>
<td>0.05</td>
<td>-0.06</td>
<td>-0.14</td>
<td>-0.10</td>
<td>&lt;0.01</td>
<td>-0.20</td>
<td>-0.01</td>
<td>-0.10</td>
<td>-0.01</td>
<td>-0.16</td>
<td>-0.03</td>
<td>-0.19</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28 ± 4</td>
<td>-0.07</td>
<td>-0.06</td>
<td>-0.03</td>
<td>0.14</td>
<td>-0.04</td>
<td>-0.03</td>
<td>-0.01</td>
<td>-0.03</td>
<td>0.08</td>
<td>-0.03</td>
<td>0.05</td>
<td>-0.08</td>
</tr>
<tr>
<td>GFR, mL/min §</td>
<td>74 ± 19</td>
<td>0.10</td>
<td>0.05</td>
<td>0.07</td>
<td>0.15</td>
<td>0.02</td>
<td>-0.05</td>
<td>0.09</td>
<td>0.05</td>
<td>0.10</td>
<td>0.07</td>
<td>&lt;0.01</td>
<td>-0.10</td>
</tr>
<tr>
<td>Gamma GT, units/L</td>
<td>33 ± 23</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.28 *</td>
<td>&lt;0.01</td>
<td>0.12</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>-0.01</td>
<td>-0.11</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>WBC count, x10⁹/L</td>
<td>5.8 ± 1.7</td>
<td>0.16</td>
<td>0.15</td>
<td>0.13</td>
<td>0.05</td>
<td>0.16</td>
<td>-0.11</td>
<td>0.15</td>
<td>0.13</td>
<td>0.13</td>
<td>-0.08</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>145 ± 20</td>
<td>-0.07</td>
<td>0.09</td>
<td>0.14</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
<td>0.18</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>-0.02</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>NT-proBNP, pmol/L</td>
<td>299 ± 152</td>
<td>-0.10</td>
<td>&lt;0.01</td>
<td>0.03</td>
<td>-0.17</td>
<td>-0.06</td>
<td>0.42 †</td>
<td>-0.10</td>
<td>-0.19</td>
<td>-0.10</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>105 ± 22</td>
<td>-0.07</td>
<td>0.09</td>
<td>0.13</td>
<td>0.15</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.12</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>-0.03</td>
<td>0.16</td>
</tr>
<tr>
<td>Gender, women ‡</td>
<td>39 (49)</td>
<td>0.75</td>
<td>0.80</td>
<td>0.93</td>
<td>0.99</td>
<td>0.94</td>
<td>0.20</td>
<td>0.77</td>
<td>0.49</td>
<td>0.44</td>
<td>0.59</td>
<td>0.81</td>
<td>0.21</td>
</tr>
<tr>
<td>Current smoker ‡</td>
<td>15 (19)</td>
<td>0.41</td>
<td>0.06</td>
<td>0.02</td>
<td>0.17</td>
<td>0.27</td>
<td>0.86</td>
<td>0.20</td>
<td>0.13</td>
<td>0.53</td>
<td>0.64</td>
<td>0.31</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Distribution values are presented as mean ± SD, or n (%).

Correlations are presented as correlation coefficients (R) and significance (p<0.05) is indicated in **bold** as follows: * p < 0.05, † p < 0.001.

‡ Categorical variable: P value is provided instead of R

§ Glomerular filtration rates were calculated using the Cockcroft-Gault formula

GFR Glomerular Filtration Rate

LVMI Left Ventricular Mass Index

NT-proBNP N-terminal Prohormone Brain Natriuretic Peptide

SBP Systolic Blood Pressure
Supplemental Table 6a. MicroRNA level intercorrelations in Diastolic Dysfunction Plasmas (correlation coefficient R)

<table>
<thead>
<tr>
<th>R values</th>
<th>miR-21</th>
<th>miR-29b</th>
<th>miR-126</th>
<th>miR-146a</th>
<th>miR-146b</th>
<th>miR-223</th>
<th>miR-133a*</th>
<th>miR-208b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-29b</td>
<td>&gt;0.75</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-126</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146a</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146b</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-223</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>&gt;0.75</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-133a*</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>miR-208b*</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>X</td>
</tr>
</tbody>
</table>

* Non-leukocyte associated microRNAs
### Supplemental Table 6b. MicroRNA level intercorrelations in Diastolic Dysfunction Plasmas (P values)

<table>
<thead>
<tr>
<th>P-value</th>
<th>miR-21</th>
<th>miR-29b</th>
<th>miR-126</th>
<th>miR-146a</th>
<th>miR-146b</th>
<th>miR-223</th>
<th>miR-133a*</th>
<th>miR-208b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-29b</td>
<td>&lt;0.0001</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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* Non-leukocyte associated microRNAs
### Supplemental Table 7. Reported plasma microRNAs changes in cardiac disease

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<th>Disease</th>
<th>MicroRNA</th>
<th>Current study</th>
<th>Literature</th>
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<td>up&lt;sup&gt;19&lt;/sup&gt;, up&lt;sup&gt;18&lt;/sup&gt;</td>
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<td>3-fold (ns)</td>
<td>up&lt;sup&gt;19&lt;/sup&gt;, 20-fold&lt;sup&gt;20&lt;/sup&gt;, up&lt;sup&gt;17&lt;/sup&gt;</td>
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All fold changes described represent elevations.