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

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Background—Small RNA molecules, called microRNAs, freely circulate in human plasma and correlate with varying pathologies. In this study, we 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: (1) acute myocardial infarction, (2) viral myocarditis, (3) diastolic dysfunction, and (4) acute heart failure. 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 polymerase chain reaction. Strikingly, in plasma from acute myocardial infarction patients, cardiac myocyte–associated miR-208b and -499 were highly elevated, 1600-fold ($P<0.005$) and 100-fold ($P<0.0005$), respectively, as compared with control subjects. Receiver operating characteristic curve analysis revealed an area under the curve 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 viral myocarditis, we observed a milder but significant elevation of these microRNAs, 30-fold and 6-fold, respectively. Plasma levels of leukocyte-expressed microRNAs were not significantly increased in acute myocardial infarction or viral myocarditis patients, despite elevated white blood cell counts. In patients with acute heart failure, only miR-499 was significantly elevated (2-fold), whereas 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, sex, body mass index, kidney function, systolic blood pressure, and white blood cell count.


Key Words: plasma microRNA ■ diagnosis ■ myocardial infarction ■ myocarditis ■ heart failure

MicroRNAs (miRNAs) form a class of small (~22 nucleotides) noncoding 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 fine-tune expression of up to 50% of protein-coding genes.2,3 MicroRNAs 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

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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 Although 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.14-16 The diagnostic potential of miRNA detection in human plasma for cardiovascular disorders is beginning to be explored: Ai et al17 studied plasmas of 93 patients with acute
myocardial infarction (AMI) patients and found miR-1 increased; Adachi et al\(^{16}\) found elevated miR-499 in plasmas of 9 AMI patients; increased plasma levels of miR-208a were reported by Wang et al\(^{19}\) for 33 AMI patients; Cheng et al\(^{20}\) reported increased plasma miR-1 levels in 31 AMI patients; and finally, in a total of 42 heart failure patients, miR-423 to 5p was found increased by Tijsen et al\(^{21}\) and correlated with pro–brain natriuretic peptide (proBNP) levels. Cardiovascular disease populations typically are elderly, overweight, and are associated with possible confounding factors such as age, sex, 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 ethics committees and were stored at \(-80^\circ\text{C}\). They were subjected to freeze-thawing once.

#### Acute Myocardial Infarction

Citrated plasma samples were obtained from patients presenting with AMI (n=32) and patients with atypical chest pain and positive stress testing but with normal coronary angiograms (n=36). All patients with AMI were enrolled in the Luxembourg AMI 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. AMI 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 the upper limit of normal. Blood samples were obtained at the time of mechanical reperfusion. More details are provided in online-only Data Supplement Table 1.

#### Viral Myocarditis

Ethylene-diaminetetraacetic acid (EDTA) plasma samples were obtained from patients with acute viral myocarditis (VM) during hospitalization in the acute phase (n=14) and post-VM phase (ranging from 1 to 12 months after the acute inflammatory phase, n=20), and from age-matched healthy control subjects (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 <0.01 \(\mu\text{g}/\text{L}\) and normal left ventricular ejection fraction, n=4), moderate VM (elevated troponin T or left ventricular ejection fraction <50%, n=5), and severe VM (left ventricular ejection fraction <30%, n=5). See online-only Data Supplement Table 2.

#### Diastolic Dysfunction

We examined EDTA plasma samples from 79 individuals at ages of 42 to 85 years, originally selected from a general Flemish population for the Flemisch Study on Environment, Genes, and Health Outcomes (FLEMENGHO\(^{22}\)) and thoroughly examined including echocardiographic analysis and detailed epidemiological characterization, as described previously.\(^{23}\) Asymptomatic individuals were age-matched and divided into 3 groups. Group 1 comprised the control subjects (n=20) with normal cardiac function and no risk factors, corresponding to American Heart Association (AHA) stage 0 heart failure;\(^{24}\) group 2, hypertensive individuals (n=20) without structural of 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 ventricular relaxation or elevated end-diastolic left ventricular pressures, corresponding to AHA stage B of heart failure. Full details are provided in online-only Data Supplement Table 3.

### Acute Heart Failure

EDTA plasma samples were collected from patients who were admitted with acute decompensation of congestive heart failure according to predefined Framingham-based criteria\(^{25}\) and an elevated plasma N-terminal (NT)-proBNP (\(>200\) pmol/L) (n=33), and from healthy staff members as control subjects (n=34) at the Cardiology Department of the Maastricht University Medical Center. For additional details, see online-only Data Supplement 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 \(\mu\text{L}\) of plasma, whole blood or urine, using the mirVana PARIS kit (Ambion, Warrington, United Kingdom) according to the manufacturer’s instructions and without enrichment for small RNAs and subsequently eluted in 50 \(\mu\text{L}\) nuclease-free water. Subsequently, potential genomic DNA contamination was eliminated using DNA-free kit (Ambion).

#### cDNA Synthesis and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

RNA (15 \(\mu\text{L}\)) was used per 20-\(\mu\text{L}\) reaction to generate cDNA using the miScript kit (Qiagen, Venlo, The Netherlands), which is designed to specifically detect mature microRNAs. The 20-\(\mu\text{L}\) reaction mix was subsequently diluted \(\times4\) in nuclease-free water, and 2 \(\mu\text{L}\) of cDNA was added per quantitative reverse transcriptase–polymerase chain reaction, using BR SYBR-green supermix for IQ (Quanta Bionsciences, Amsterdam, The Netherlands) in a MyIQ iCycler (Bio-Rad, Veenendaal, 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, and -499; VM: miR-1, -133a, -146a, -146b, -155, -208b, -223, and -499; Diastolic Dysfunction: miR-1, -21, -29b, -122, -126, -133a, -146a, -146b, -155, -208b, -223, -499; and acute heart failure (AHF): miR-1, -122, -133a, -208b, -223, and -499.

#### Normalization of Experimental Data

Quantitative polymerase chain reaction measurements of miRNA levels were normalized for isolation efficiency using a mix of 3 spiked-in synthetic Caenorhabditis elegans miRNAs (Eurogentec, Maastricht, The Netherlands), lacking sequence homology to human miRNAs, as described previously.\(^{13}\) Oligonucleotides were spiked into the samples during RNA isolation after plasma incubation with the provided \(\times2\) denaturing solution. We chose to correct for spikes rather than for endogenous “stable” miRNAs because there is no consensus in the literature regarding stable miRNAs for correction.

#### Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software Inc, La Jolla, Calif). 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 after normalization of nongaussian 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
Cardiomyocyte-Associated But Not Inflammatory miRNAs Are Significantly Elevated in the Acute Stage of VM

We subsequently investigated plasma miRNA levels in patients with acute VM, patients in the post-VM phase, and healthy control subjects. Again, levels of miR-208b and miR-499 were significantly elevated (30-fold and 6-fold, respectively; \(P<0.01\) versus control subjects for both) during the acute phase of VM (Figure 3 and online-only Data Supplement Figure 2). Their expression levels significantly correlated with the severity of VM: Subgroup analysis within the acute VM patients revealed normal values of miR-208b and -499 in patients with mild VM (with no troponin T elevation) and increasing levels for moderate and severe VM (\(\approx 20\)-fold and \(\approx 50\)-fold higher, respectively, than control levels for miR-208b and \(\approx 5\)-fold and 10-fold for miR-499) (online-only Data Supplement Figure 2). Of note, the more accentuated rise in miRNA plasma levels in AMI compared with VM paralleled the higher troponin T levels in AMI patients (online-only Data Supplement Tables 1 and 2). Levels of miR-1 and -133a and leukocyte-associated miR-146a, -146b, -155, and -223 were not significantly different between groups (Figure 3) despite significant leukocytosis in the acute VM group (online-only Data Supplement 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 control subjects, patients with hypertension without cardiac changes, and patients with evident myocardial diastolic dysfunction (Figure 4 and online-only Data Supplement 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,\(^{27–29}\) 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 because 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 AHF, where no correlation was found (Figure 5G). Tijsen et al\textsuperscript{21} 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 (online-only Data Supplement Table 5). Finally, no differences were found in plasma miRNA levels of smokers and nonsmokers, with the exception of miR-29b, which was significantly higher in smokers ($P<0.02$).

AHF 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 toward 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 (online-only Data Supplement Table 1, $R=-0.25$, $P=0.04$) and the higher mean age of AHF patients versus control subjects. Additionally, the liver-specific miR-122, which is known to correlate with hepatic damage,\textsuperscript{16} 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, Sex, and Renal Function

To determine to what 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 (online-only Data Supplement Table 5). Linear regression analyses revealed that plasma miRNA levels were not affected by the evaluated parameters. Patient sex and body mass index did not correlate with any of the plasma miRNAs (online-only Data Supplement 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 $80^\circ$C. Patient age did not significantly correlate with 10 of 12 miRNAs and only showed weak negative correlation with miRNAs -1 and -29b ($R=-0.25$ and $-0.26$, respectively, $P<0.05$ 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 whereas both miRNAs were well detected in both sample types, levels in plasma compared with urine were $\approx1000$-fold higher for miR-122 and $\approx100$-fold for miR-223 (online-only Data Supplement 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 /H9253-glutamyl transferase (online-only Data Supplement 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 WBC 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 with 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 (online-only Data Supplement Figure 5 and online-only Data Supplement Table 5). This lack of correlation was independently confirmed in our plasma databases of acute VM and AMI plasmas with leukocytosis (online-only Data Supplement 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 (online-only Data Supplement 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 (online-only Data Supplement...
their diagnostic potential.13,16 Our study demonstrates that in informative of human pathology has ignited wide interest in Recent evidence that freely circulating miRNAs may be compromised their diagnostic accuracy.

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Table 6A and 6B), pointing toward a common etiology. We also found highly identical patterns of these miRNAs in plasma and whole blood (online-only Data Supplement 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.13,16 Our study demonstrates that in humans, diverse conditions of myocardial damage are associated with striking perturbations of plasma levels of heart-associated miRNA-208b and -499. These are increasingly elevated during AHF (minimal), VM (marked), and AMI (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 $P<10^{-5}$ for both individual miRNAs. Encouragingly, these data are in agreement with a recent report published during the preparation of this report, that examined plasma levels of muscle-specific miRNAs in AMI patients and also identified miR-208(a) and -499 as the most sensitive biomarkers of AMI.19 Online-only Data Supplement 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 independent 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 fiber gene programs toward 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 after 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 miRNA-208b and -499 in patients with AMI (1600-fold and 100-fold, respectively) are in contrast with the mild elevations observed for heart-associated miR-1 and -133a (3-fold 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, because of 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 online-only Data Supplement Table 7). Our data generally support their conclusions but extend on their findings by identifying miR-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 NT-proBNP (extensive). In AMI, where cardiac damage was most severe, NT-proBNP (extensive) was not different for AHF patients and control subjects (n 33) than in control patients,17,19,20 but also in these studies this elevation was milder than observed for miR-208b and -499 (see online-only Data Supplement Table 7). Our data generally support their conclusions but extend on their findings by identifying miR-208b and -499 as far more sensitive markers for myocardial damage not only in AMI but also in VM.

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mass index, systolic blood pressure, smoking, and glomerular filtration rate. Further, the liver-specific miR-122 was the only miRNA mildly correlating with γ-glutamyltransferase levels, in agreement with the recent report that plasma miR-122 parallels levels of aminotransferase levels during hepatic damage.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 because their levels may be subject to variable leakage from circulating leukocytes, for example, caused by venipuncture. On the other hand, the significant decrease in miR-223 levels after AMI has potential biological and/or diagnostic significance, given the study by Wang et al.,17 who find decreased miR-223 levels during sepsis. Therefore, the value of miR-223 for AMI must be investigated further.

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

Sources of Funding
This work was supported by a Veni grant (016.096.126) from The Netherlands Organisation for Scientific Research to Dr Schroen, a Horizon grant (93519017) from The Netherlands Genomics Initiative (Dr Schroen), and a research grant from The Netherlands Heart Foundation (Dr Heymans) and research grants from the National AMI Registry (Dr Wagner), supported by the Ministry of Health, Luxembourg, and the Société pour la Recherche sur les Maladies Cardiovasculaires, Luxembourg.

Disclosures
None.

References
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, acute viral myocarditis, diastolic dysfunction, and acute heart failure. Most strikingly, 2 cardiomyocyte-specific microRNAs (miR-208b and miR-499) are markedly elevated in 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.
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Circ Cardiovasc Genet. 2010;3:499-506; originally published online October 4, 2010;
doi: 10.1161/CIRCGENETICS.110.957415
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue,
Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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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 the 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
Suppl. Figure 2. Plasma miR-208b and miR-499 elevation in viral myocarditis reflects VM severity.

A. miR-208b

B. miR-499
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
Suppl. Figure 4. Plasma miRNAs are not subject to efficient glomerular filtration

plasma vs urine miRNA levels

miRNA per volume

miR-122

miR-223

>1000-fold
p < 0.01

>100-fold
p < 0.01

plasma
urine
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

![Graph A]

B

![Graph B]
**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
hs-CRP High-Sensitivity C-Reactive Protein
N-terminal Prohormone Brain Natriuretic Peptide
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/m^2</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, x10^9/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/m^2</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>
</tbody>
</table>

**Underlying pathology**

- CAD 19 (57.5)
- Valvular 6 (18.2)

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>&lt;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 Cockroft-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></td>
<td>X</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></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-126</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146a</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-146b</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-223</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-133a*</td>
<td>0.24</td>
<td>0.69</td>
<td>0.11</td>
<td>0.17</td>
<td>0.34</td>
<td>0.30</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>miR-208b*</td>
<td>0.16</td>
<td>0.54</td>
<td>0.55</td>
<td>0.39</td>
<td>0.89</td>
<td>0.97</td>
<td>0.42</td>
<td>X</td>
</tr>
</tbody>
</table>

* Non-leukocyte associated microRNAs
### Supplemental Table 7. Reported plasma microRNAs changes in cardiac disease

<table>
<thead>
<tr>
<th>Disease</th>
<th>MicroRNA</th>
<th>Current study</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Myocardial Infarction</td>
<td>miR-208</td>
<td>1600-fold</td>
<td>up&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>miR-499</td>
<td>100-fold</td>
<td>up&lt;sup&gt;19&lt;/sup&gt;, up&lt;sup&gt;18&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>miR-1</td>
<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>
</tr>
<tr>
<td></td>
<td>miR-133a</td>
<td>4-fold</td>
<td>up&lt;sup&gt;19&lt;/sup&gt;, ns&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td>Viral Myocarditis</td>
<td>miR-208</td>
<td>30-fold</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miR-499</td>
<td>6-fold</td>
<td>-</td>
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<tr>
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All fold changes described represent elevations.

**Literature:** Ai Biochem Biophys Res Comm 2009<sup>17</sup>; Adachi et al. Clin Chem 2010<sup>18</sup>; Wang et al. EHJ 2010<sup>19</sup>; Cheng et al. Clin Science 2010<sup>20</sup>. 

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