Relationship Between The Temporal Profile of Plasma microRNA and Left Ventricular Remodeling In Patients Following Myocardial Infarction

Running title: Zile et al.; microRNA following MI

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Abstract:

**Background** - MicroRNAs (miRs) are small noncoding RNAs that recognize and bind to mRNAs and inhibit protein translation or degrade mRNA. Studies in animal models have suggested that miRs play a translational or post-translational regulatory role in myocardial growth, fibrosis, viability, and remodeling. However, whether specific temporal changes in miRs occur in patients during the LV remodeling process that follows a myocardial infarction (post-MI) remains unknown. The purpose of the current pilot study was to test the hypothesis that plasma miRs could be reliably measured in post-MI patients and that there is the relationship between temporal changes in specific miRs and post-MI LV structural remodeling.

**Methods and Results** - LV end-diastolic volume (EDV, echocardiography) and plasma miR were measured in age matched referent controls (CTL n=12) and post-MI patients (n=12) from day 2 through day 90 post-MI. Selected miRs (miR-1, -21, -29a, 133a, 208) were measured using quantitative rt-PCR and normalized for endogenous snRNA U6. Following MI, LVEDV increased progressively compared to CTL; this was accompanied by time dependent changes in specific miRs. For example, miR-21 initially fell 2 days post-MI (0.3±0.1 fold vs. CTL, p< 0.05), increased 5 days post-MI (2±1 fold vs. CTL, p< 0.05), and returned to CTL values at later post-MI time points. In contrast, miR-29a increased 5 days post-MI (4±1 fold vs. CTL, p< 0.05) and then fell to CTL at later time points. miR-208 increased 5 days post-MI (3±1 fold vs. CTL, p< 0.05) and remained elevated up to 90 days post-MI.

**Conclusions** - A time-dependent change in miRs occurred in post-MI patients that included an early and robust rise in miRs that have been shown to affect myocardial growth, fibrosis and viability. Thus, serially profiling miRs in the plasma of post-MI patients may hold both mechanistic and prognostic significance.

**Key words:** Myocardial Infarction, Remodeling, microRNA
Introduction

Left ventricular remodeling represents the aggregate effects of changes in cardiomyocytes, fibroblasts, and interstitial structure and function that result from cardiovascular disease processes such as a myocardial infarction. The molecular regulatory mechanisms that affect cellular and extracellular remodeling remain incompletely defined; however, recent publications suggest that microRNAs (miRs) may be one such mechanism (1-6). MiRs are small noncoding RNAs (~22 nucleotides) that recognize and bind to mRNAs and inhibit protein translation or degrade mRNA (1, 2). Studies in animal models have suggested that miRs play a translational or post-translational regulatory role in myocardial growth, fibrosis, viability, and remodeling (4, 7-15). For example, miR-1 has been suggested to blunt LV hypertrophy, augment apoptosis and facilitate progressive dilation, miR-208 augments hypertrophy and increases the extracellular matrix, miR-21 and miR-133a inhibit apoptosis and miR-29a inhibits changes in the extracellular matrix (4, 7-15).

Whether specific temporal changes in miRs occur in patients during the LV remodeling process that follows a myocardial infarction (post-MI) remains unknown. It is impractical to assess serial changes in miRs in post-MI patients using repetitive LV myocardial tissue biopsies. However, because miRs are actively exported into the interstitial space and enter the plasma in a form protected from degradation, it may be possible assessments of serial myocardial changes in miRs by using plasma sampling. Accordingly, the goals of the current study were first to develop a sensitive, reliable method to measure miRs in plasma in referent control subjects and post-MI patients, and second to measure serial changes in specific miRs following an MI to determine whether there is a relationship between temporal changes in specific miRs and LV structural remodeling in post-MI patients.
Methods

Subjects

Twelve patients with a confirmed MI and twelve referent age-matched control subjects were enrolled in this pilot study after providing informed consent. All of the studies described herein were reviewed and approved by the Medical University of South Carolina Institutional Review Board. An ECG and/or a positive cardiac enzyme panel confirmed the MI. Patients were excluded from enrollment if there was a history of a previous MI; previous coronary revascularization surgery within past 24 months; a history of active malignancy; significant renal or hepatic dysfunction; active rheumatological disease. MI patient were treated according to AHA/ACC guidelines. The referent control group consisted of subjects with no evidence of cardiovascular disease. Cardiovascular disease was excluded by performing a complete medical history, comprehensive physical examination, ECG, and echocardiogram.

Protocol

For the MI patients, studies were performed beginning at the time of enrollment (post-MI day 1). Plasma from a peripheral vein blood sample was used to measure miR profiles at post-MI days 2, 5, 28, and 90. At post-MI days 1, 5, 28, and 90 an echocardiogram was obtained. For both referent control and post-MI subjects, all plasma samples were drawn at the same time of the day, between 8 and 10 am. Therefore, potential diurnal variations which may be present were avoided. For the referent control subjects, an echocardiogram and plasma sample was performed once at the time of enrollment. All subjects fasted overnight before each study but took their morning medications as prescribed.
Echocardiographic Methods

Transthoracic echocardiography was performed using a Sonos 5500 system with an S-4 MHz transducer (Agilent Technologies, Andover, MA). Measurements were made with American Society of Echocardiography criteria (16). Two-dimensional echocardiographic studies were performed using standard short- and parasternal long axis views to obtain measurements of LV volumes. Images were coded and read in a blinded fashion, and this analysis remained unlinked to the miR levels until completion of the study.

Plasma miRNA Measurements

Small RNAs from plasma were isolated using the mirVana PARIS Kit (AM1556, Ambion) which is based upon a denaturing / phenol chloroform extraction approach. Briefly, 400 μL of plasma was added to an equal amount of denaturing solution, and incubated on ice for 5 minutes. Following which, 800 μL of an acid-phenol chloroform solution was added to the samples in order to inactivate RNAases and to create an aqueous RNA phase. This aqueous phase was removed, and passed through glass-fiber filters binding the RNA. The RNA was then eluted using a low ionic-strength solution, yielding a final volume of 100 μL. Then, 11.4 μL was reversed transcribed into cDNA (Applied Biosystems TaqMan MicroRNA RT Kit #4366579) using pre-specified miR sequences for: miR-1, miR-21, miR-29a, miR-125, miR-133a, miR-208, and snRNA U6 (Table 1). Next, 12.5 μL of the cDNA was preamplified (TaqMan PreAmp Master Mix Kit #4391128, Applied Biosystems) as well as the pooled miR primers. Finally, the preamplification product was subjected to real time PCR (CFX96 Real-Time System, BioRad). The relative cycle threshold (Ct) values for U6 snRNA were used as endogenous controls for
normalizing the respective miR Ct values (17) and were calculated as dCt (dCt = miRNA Ct - snRNA U6 Ct). Changes in miRNA deltaCT were reported as a fold change from the mean deltaCT of the referent controls calculated as FC = 2^((dCt post MI - dCt mean referent control)), where “dCt post MI” was calculated for each individual patient at each individual time point and each of these individual values was then subtracted from the mean value of all the referent controls. Using this method, a mean and SEM could be calculated for each miR. These normalized Ct values were computed for each sample, and these measurements were performed in duplicate. Referent control values were set at 1.0. Therefore, in post-MI patients fold change values less then 1.0 represented a fall in miRNA expression and fold change values greater then 1.0 represented an increase in miRNA expression compared to referent control. In initial assays performed in triplicate using referent control samples, the coefficient of variation for individual miR values was less than 10% (Table 2).

Six miRs and one endogenous control were chosen for this study. A representative miR was chosen to target a translational or post-translational molecular regulatory role in each aspect of post-MI remodeling including augmenting or inhibiting hypertrophy, extracellular matrix changes, apoptosis, and progressive dilation. miR-1 has been suggested to blunt LV hypertrophy, augment apoptosis and facilitate to progressive dilation, miR-208 augments hypertrophy and increases changes in the extracellular matrix, miR-21 and miR-133a inhibit apoptosis and miR-29a inhibits changes in the extracellular matrix.

In order to validate our extraction efficiency, eight human plasma samples were spiked with 10ng of a foreign C. elegans miR-39 sequence (cel-miR-39; Integrated DNA Technologies, Coralville, IA) prior to extraction. The extraction efficiency was calculated to be 76.8±0.6%. Also the coefficient of variation between the spiked samples was determined as 2.2%. Negative
controls were also run to verify the absence of genomic DNA contamination (reverse transcription control) and the absence of overall DNA contamination in the PCR system and working environment (template control). No genomic DNA contamination was detected.

Data Analysis

The echocardiographic and miRNA data given in this study were presented in an untransformed manner using parametric statistics. Comparisons between referent control values and post-MI values over time were examined by use of a 2-way ANOVA for repeated measures in which referent control/MI was the first treatment level and time after MI was the second treatment level. After the ANOVA, pair-wise comparisons were made using a Bonferroni method. The relationship between changes in miR levels and LV volumes in the post-MI period were examined by linear regression methods. Values of p < 0.05 were considered significant. All values are presented as the mean and SEM. All statistical procedures were performed with Stata Statistical Software (StataCorp, release 8.0, College Station, Tex). The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Demographics

By experimental design there were no differences in age between referent control and post-MI patients (Table 3). The ratio of men to women was higher in the post-MI group. Heart rate and blood pressure were comparable between groups. Differences in medications reflect expected ACC/AHA guideline based protocols for post-MI patients. In the referent control
subjects, β-adrenergic blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor antagonists were used to treat mild increases in systolic pressure. Aspirin or anti-inflammatory agents were used for management for arthritic pain.

**LV Structure**

LV end-diastolic volume increased in a time-dependent manner in the post-MI group as shown in Figure 1. LV end diastolic volume was already increased compared with referent control on day 1 post MI. LV end-diastolic volumes increased further from post-MI day 1 values at post-MI day 28 and 90.

**miR**

There were time dependent changes in the 5 measured miRs in the post MI patients compared to the referent control subjects (Figures 2-3). miR-1 and miR-21 fell at day 2 post MI, miR-29a, miR-133a and miR-208 were unchanged at day 2 post MI. miR-1, miR-133a, and miR-208 increased at day 5 and remained elevated through day 90 post MI. miR-21 and miR-29a were increased at day 5 but returned to normal by day 90 post MI. MiR-125 was not detectable at any time point in the post-MI patients. The value of snRNA U6 was unchanged from referent control at each time point examined in the post-MI patients.

There was a significant association between miR-29a early after MI (post MI day 5) and LVEDV late after an MI (post Mi day 90), \( r = 0.77 \) and \( p < 0.05 \) (Figure 3B). The greater the increase in miR-29a at 5 days post MI, the greater the increase in LVEDV at 90 days post-MI.
Discussion

Studies in animal models have suggested that miRs play a translational or post-translational regulatory role in myocardial growth, fibrosis, viability, and remodeling (1-6). However, whether specific temporal changes in miRs occur in patients during the LV remodeling process that follows a myocardial infarction (post-MI) remains unknown. The principle findings in this pilot study are two-fold. First, miRs can be reproducibly measured in the plasma of patients following a myocardial infarction using a sensitive, reliable method. Second, a time-dependent change in miR plasma levels occurs following a myocardial infarction, particularly in those miRs that are associated with myocardial growth, fibrosis and viability (4, 7-15). These new findings build upon recent studies in animal models of myocardial infarction that demonstrated that the specific miRs profiled in the present study had functional significance in rodent post-MI remodeling (5, 8, 10-13). In addition, the findings in the current study expand upon recent studies in patients in which measurements of selected miRs were made at a single point in time following a myocardial infarction. Taken together, data from the current and previous studies suggest that serially profiling miRs in the plasma of post-MI patients may hold both mechanistic and prognostic significance. The current study examined a relatively small number of patients following an MI for a 90 day period of time. For these reasons, the conclusions stated above must be interpreted as a pilot study. It is clear that these finding must be confirmed by further studies.

Plasma miRs
Given the fact that blood contains ribonucleases (RNases) it might be expected that neither serum nor plasma should contain any intact RNA. However, a number of recent studies have demonstrated the presence of miRs in normal subjects and patients with cancer and other disease processes (18, 19). These facts lead to studies that addressed the question of whether Qrt-PCR products found in plasma samples were the result of contamination by degraded products of large molecular weight RNA, tRNA, or genomic DNA. Studies indicate that there is stable reproducible population of miR exist in a form that is resistant to endogenase RNase, possibly because it is packaged inside an exosome or is associated with other molecules (18, 19). For example, some of the total RNA isolated from human plasma was degraded by treatment with exogenous RNase, however, miRs were not degraded. miRs were not degraded by treatment with DNase, multiple freeze thaw cycles, prolonged incubation, or a large range of pH (18). However, when miRs, not homologous to human miR, were added to human plasma, these miRs were degraded. In patients with known cancer in whom tissue samples demonstrate an increase in specific miRs, the plasma has also been shown to have increased miRs (18).

Changes in miRs Following Myocardial Infarction

To our knowledge, there have been no clinical studies in which serial changes in a targeted group of miRs have been measured in the plasma of patients following MI. Previous clinical studies have been done in patients with stable coronary artery disease, patients with ischemia induced cardiomyopathy, and at a single point in time early after a myocardial infarction (5, 6, 8-11, 20, 21). While significantly different in design and endpoints, these previous studies have important implications for the current study. Fichtlscherer and colleagues measured plasma miRs in patients with stable coronary artery disease (9). In their study, relative levels of miR 133a and
208a were marginally increased in patients with stable coronary artery disease and no evidence of myocardial dysfunction (9). This study examined patients potentially at risk to develop a myocardial infarction. Two more recent studies examined changes in miRs in patients early after a myocardial infarction (10, 11). In these studies, plasma miR-1 and miR-133a were increased and these miRs were postulated to be novel biomarkers with diagnostic and prognostic value (10, 11). Data from the current study markedly advanced these previous clinical studies by measuring plasma miR both early after an MI and for several months after MI, comparing data from MI patients to an age matched control subjects, examining a larger group of miRs chosen because they have been shown to effect myocardial growth, fibrosis and viability, and by relating changes in miRs to post MI alterations in structure and function. For example, in the current study, plasma levels of certain miRs, such as miR-21 and miR-29a increase early post-MI whereas other miRs such as miR-1, miR-133a and miR-208 remained persistently elevated up to 3 months post-MI. Furthermore, higher levels of miR-29a early post MI was related to greater remodeling late post MI.

Downstream biologically relevant determinants of collagen homeostasis such as matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) were examined in post-MI patients (24). In addition, collagen telopeptides have also been examined in post-MI patients (22, 23). In each case, following an MI, determinants of collagen homeostasis favor degradation and evidence of degraded collagen were present early following an MI but favored fibrosis late after an MI. Data from these previous studies and the current study suggest the hypothesis that changes in miRs may precede, determine and predict downstream collagen homeostatic events. However, proving a direct causal relationship between miRs and downstream collagen events must await further study.
However, the regional distribution of changes in miRs and the functional and mechanistic significance of the findings could not be determined from clinical studies using plasma samples. For these reasons, inferences from animal models of myocardial infarction must be made and the serial changes following an MI that occur in these animal models must be compared to the current study (5, 8, 11-13). In rodent studies of myocardial tissue miR profiling post-MI, van Rooij et al and other investigators demonstrated significant temporal and regional changes in miRs in the early (3-14 day) post MI period (8, 11-13). Indeed, these studies demonstrated early and dynamic changes in miR myocardial levels, such as miR-1, -21, 29a, and 133a which were similar to the early plasma miR profile obtained in the present study. However, they also demonstrated regional heterogeneity in the changes in myocardial miR profiles that differed within the remote, border, and MI regions in these rodent post MI models (8, 12). Taken together, the findings from previous and present studies underscore several considerations in terms of profiling plasma miRs following an acute cardiovascular event such as MI. First, single point measurements of plasma miRs may be insufficient to identify the relative magnitude and time dependent nature of the dynamic changes in miRs following an acute event. Second, plasma measurements of miRs will be a summation of release from local tissue compartments and therefore will not be sensitive to regional heterogeneity in miR expression.

While the present study demonstrated an association between certain plasma miR profiles and LV remodeling/dilation post-MI, the functional and mechanistic significance of the findings from the present study must be extrapolated primarily from rodent studies (5, 6, 8, 13-15) For example, in a transgenic mouse model over expressing miR-133a, reduced myocardial fibrosis was reported which was accompanied by abnormalities in myocardial repolarization (15). In the
present study miR-133a was persistently elevated in patients following an MI, but whether the magnitude of this increase was sufficient to alter myocardial ECM remodeling remains speculative. One observation from previous rodent studies was that predicted post-transcriptional regulatory targets for miR-133 based upon in-silico mapping, were not necessarily predictive of in-vivo findings (15). In the present study, the rationale for the selection of miRs for plasma profiling were based upon previous in-vivo observations in animal models, rather than based upon in-silico mapping algorithms. For example, miR-21 myocardial levels were shown to be increased early post-MI in rodents, and to regulate myocardial fibroblast matrix metalloproteinase-2 levels (13). Previous studies from this laboratory have demonstrated that dynamic changes in plasma MMP-2 levels occur in patients post-MI. Moreover, the temporal pattern of changes in plasma MMP-2 levels in this post-MI period were similar to those obtained in the present study with respect to miR-21. However, a recent study in transgenic mice, suggests that ablation of miR-21 is insufficient to significantly alter adverse myocardial matrix remodeling (14). Thus, the mechanistic significance of the temporal changes in miR-21 in post-MI patients which were observed in the present study remains unclear.

One of the miRs which appears to hold biological relevance to extracellular matrix remodeling and in particular, adverse post-MI remodeling is the miR-29 family (5, 6, 8). In the present study, a rapid surge in plasma miR-29a was observed early post-MI which rapidly fell at longer post-MI time points. An interaction between fibrillar collagen expression and pro-fibrotic signaling pathways and changes in the expression of the miR-29 family has been demonstrated in a number of animal model and human tissue studies (8). Data from the current and previous studies may suggest that early after a myocardial infarction, increased miR-29a expression may act to limit (or inhibit) a profibrotic ECM response, allow more ECM degradation, and result in
more extensive LV remodeling (particularly LV dilation) in both the short and long term following an MI. However, this postulated mechanisms remains to be proven in future clinical and animal studies.

Conclusions

miRs can be reproducibly measured in the plasma of patients following a myocardial infarction using a sensitive, reliable method. A time-dependent change in miR plasma levels occurred following a myocardial infarction, particularly in those miRs that are associated with myocardial growth, fibrosis and viability. Data from the current study suggest that serially profiling miRs in the plasma of post-MI patients may hold both mechanistic and prognostic significance.

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Conflict of Interest Disclosures: None

References:


### Table 1: Applied Biosystems miRNA primers

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<td>UGGAAUGUAAAGAAGAUGUAAU</td>
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<td>miR-21</td>
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<td>miR-125b-3p</td>
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<td>miR-133a</td>
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<td>miR-208a</td>
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<td>U6 snRNA</td>
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### Table 2: Ct Values and Coefficient of Variation for Twelve Referent Controls

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<tr>
<td>miR-1</td>
<td>28.55±0.69</td>
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<td>miR-21</td>
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<td>miR-29a</td>
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<td>miR-133a</td>
<td>27.54±0.94</td>
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<td>miR-208a</td>
<td>37.75±1.44</td>
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<tr>
<td>U6 snRNA</td>
<td>27.46±1.06</td>
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Table 3: Demographics for Referent Control Subjects and Myocardial Infarction Patients

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<td>Number</td>
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<tr>
<td>Age (years)</td>
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<td>58±3</td>
</tr>
<tr>
<td>Males</td>
<td>5 (42%)</td>
<td>9 (75%)</td>
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<tr>
<td>Body Surface Area (m2)</td>
<td>1.88±0.06</td>
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</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>66±2</td>
<td>68±2</td>
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<tr>
<td>Arterial Systolic Pressure (mmHg)</td>
<td>124±3</td>
<td>119±4</td>
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<tr>
<td>Arterial Diastolic Pressure (mmHg)</td>
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</tr>
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</table>

Data are Mean ± SEM, * = p < 0.05 vs. referent control

Figure Legends:

Figure 1: Left Ventricular (LV) end diastolic volume increased progressively in patients over 90 days following a myocardial infarction (closed circles) compared with age matched referent controls (the gray shaded box represents the normal reference range for LV End Diastolic Volume for our laboratory; the mean ± 2 times the standard deviation for an age-matched referent control group was used to develop the reference range). In addition, mean ± SD (open circle) for the actual referent control subjects used in this current study were plotted. * = p<0.05 vs. control group.

Figure 2: Serial changes in miRs in the patients following a myocardial infarction (MI). Data for post-MI patients are presented as a fold change from referent controls (CTL) set at 1. Data are mean ± SEM, * = P < 0.05 vs. CTL; # = P < 0.05 vs. day 2.

Figure 3: Panel A-Serial changes in miR-29 in the patients following a myocardial infarction (MI). Data for post-MI patients are presented as a fold change from referent controls (CTL) set at 1. Data are mean ± SEM, * = P < 0.05 vs.CTL; # = P < 0.05 vs. day 2. Panel B-Relationship between early changes in miR-29a values at day 5 post-MI versus late changes in left ventricular (LV) end diastolic volume 90 days post-MI. The larger the early increase in miR-29a at 5 days, the larger the late increase LV end diastolic volume following an MI. y = - 5.5 + 0.07x, r = 0.77
A. Fold change in miR-1 levels from Control

B. Fold change in miR-208 levels from Control

C. Fold change in miR-133a

D. Fold change in miR-21 levels from Control

Days Following MI

* indicates statistically significant difference from control
# indicates statistically significant difference from previous time point
miR-29a

Fold change in miR-29a levels from Control

A

Days Following MI

B

LV End Diastolic Volume (ml) 90 days Post-MI
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