Clinical Chemistry 60:6
812–817 (2014)

Q&A

Novel Biomarkers for Acute Myocardial Infarction: Is MicroRNA the New Kid on the Block?

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Early detection of acute myocardial infarction (AMI)[9] is crucial for deciding the course of treatment to preserve and prevent further damage to the myocardial tissue. Guidelines from the American College of Cardiology give the criteria for AMI as an increase or decrease in troponin with at least one value >99% percentile of a healthy population (with an imprecision of <10%) accompanied by either symptoms of ischemia or evidence of MI based on imaging or electrocardiography modalities. Over the last several years, creatine kinase MB testing has been replaced by newer generations of troponin assays. High-sensitivity assays are now considered the gold-standard marker for myocardial necrosis. Indeed, the improved analytical sensitivity of high-sensitivity troponin assays has lowered the threshold for myocardial injury, making it easier to detect baseline values in the picogram-per-milliliter range and thus expediting early diagnosis. The gains in diagnostic sensitivity have come at the cost of decreases in diagnostic specificity, however. A major challenge is that high-sensitivity assays quantify troponin in a greater proportion of healthy individuals. These assays also detect myocardial cell death associated with other pathophysiological conditions, such as cardiomyopathies, myocarditis, renal failure, congestive heart failure, and pulmonary embolism, and do not distinguish between mechanisms of tissue injury, thus limiting the ability to differentiate on the basis of MI severity [e.g., ST-segment elevation myocardial infarction (STEMI) vs. non-STEMI]. Consequently, the introduction of high-sensitivity troponin assays into clinical practice has caused confusion for physicians treating patients with acute chest pain. Therefore, the ideal biomarker to rule in and rule out AMI rapidly and reliably is still lacking.

During the last several years, there has been a burgeoning interest in circulating microRNAs (miRNAs) as potential novel biomarkers for AMI. miRNA is a type of single-stranded, noncoding small ribonucleic acid (about 22 nucleotides in length) located within introns of protein-coding genes that functions in suppressing protein synthesis via gene silencing. Recent animal and clinical studies have demonstrated that miRNAs increase in the plasma shortly after the onset of a coronary event. A recent explosion of clinical data has provided evidence that circulating miRNAs have utility not only in diagnosis but also in predicting survival outcome and atherosclerotic burden. Certain miRNA isoforms occur exclusively in the circulation after an AMI and appear to display faster release kinetics than seen for troponins detected with high-sensitivity assays. Furthermore, testing for circulating miRNA seems to match the benefits of high-sensitivity troponin testing in terms of clinical sensitivity and specificity. In some instances, miRNA testing improves the diagnostic potential when such testing is used together with cardiac biomarkers or as an independent test. Moreover, some groups have identified unique signature patterns of circulating miRNAs that accurately distinguish between non-STEMI and STEMI patients—a diagnosis that has important implications for early management in the acute-care setting.

In this Q&A, we ask 4 miRNA experts with an interest in coronary artery disease to shed light on the utility of circulating miRNA in testing for AMI and to provide insight into the benefits, applications, challenges, and limitations of this potentially powerful new tool.

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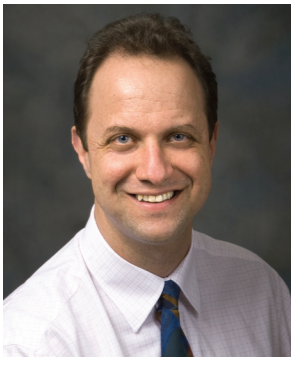
Received October 17, 2013; accepted October 22, 2013.
[9] Nonstandard abbreviations: AMI, acute myocardial infarction; STEMI, ST-segment elevation myocardial infarction; miRNA, microRNA; Ago2, argonaute 2.

Do you think an ideal biomarker for rapid and reliable diagnosis of AMI is still lacking? What are some of the challenges with the current pantheon of MI testing approaches used today?

Katey Rayner: The clinical necessity for rapid and accurate testing for an AMI is obvious, and the current use of troponin (and other cardiac enzyme–based measurements) confidently predicts the presence of an AMI. In contrast, a rapid and effective test for stable coronary disease and, more importantly, unstable coronary disease is lacking. It is my opinion that although miRNAs may associate strongly with cardiac enzyme values and may indeed indicate the occurrence of an AMI, these miRNA-based measurements will do nothing more than solidify the diagnosis and will not necessarily reveal any new information. On the other hand, there is an urgent need for markers that identify unstable plaques that are at risk for rupture, and miRNAs may provide this tool.

Stephanie Dimmeler: Reliable biomarkers for early and sensitive detection of AMI are available (high-sensitivity troponin assays), and I would not expect that circulating miRNAs would be better for routine practice. Perhaps the release of miRNAs is earlier than troponin (which would allow for an earlier detection of AMI); however, this is not yet clear.



George A. Calin: The lack of diagnostic specificity for many markers of MI is one factor. On the other hand, several miRNAs have tissue-specific expression (e.g., the miR-133 family), and therefore there are high expectations to identify more-specific markers that can be used as early-stage markers of myocardial necrosis. Also, if new markers unrelated to miRNAs were to be identified as highly specific, prediction of MI based on a combination of such markers with miRNA expression could be envisioned.

Thomas Thum: Generally speaking, I think it is difficult to beat the sensitivity and specificity of current approaches to detect MI, particularly with regard to that derived from use of high-sensitivity troponin assays. However, there are several circumstances where new biomarkers would be very helpful, for example in detecting other cardiac diseases that go along with myocardial damage (and rise of traditional biomarkers) such as Takotsubo cardiomyopathy or various forms of myocarditis.

Why has there been such an explosion of interest in the cardiovascular community in translating miRNA research at the bench into the clinical setting?

Katey Rayner: The excitement surrounding the use of miRNA-based therapies for cardiovascular disease treatment is in part owing to the relatively rapid development of anti-miRNA targeting strategies. Less than 5 years after miRNAs were discovered in mammals, "antagomirs" were used successfully in nonhuman primates to inhibit miR-122 and reduce total plasma cholesterol concentrations, and this led to the rapid advancement of anti-miR122 into clinical trials. One could almost argue that the *understanding* of miRNA biology has lagged behind the development of inhibitor technologies, but this has helped a large number of laboratories validate the role of specific miRNAs in cardiovascular diseases and supported their consideration for clinical trials. Unlike approaches that target one gene/molecule at a time (i.e., small molecules, pharmacologics), miRNA-based therapy has the potential to modulate an entire pathway by fine-tuning multiple genes at the same time, often resulting in a more lasting, sustained effect.

Stephanie Dimmeler: miRNAs can change gene expression networks and are often profoundly regulated during disease. Moreover, pharmacological small inhibitors are available to target miRNAs, allowing for easy testing of therapeutic strategies. In general, miRNA inhibitors have been safely used in a recent phase II study (for the treatment of hepatitis); there-

fore, one may expect that miRNA inhibitors might also be useful for the treatment of cardiovascular diseases.

George A. Calin: miRNAs are involved in any type of biologic process that has been investigated to date and also any type of disease analyzed. Therefore, large amounts of effort and funds have been dedicated to the identification of new therapeutic applications based on miRNAs, as well as other new biomarkers, to answer the many yet-unsolved clinical questions, such as the prediction of response to therapy. The recently published clinical trial in phase II with miravirsen (anti–miR-122, an antagonist to a highly specific liver miRNA) in patients with chronic hepatitis C virus genotype 1 infection showed prolonged dose-dependent reductions in hepatitis C RNA levels without evidence of viral resistance, certainly adding hope that such new therapies will be developed.

Thomas Thum: This explosion of interest is seen in the development of both novel diagnostic and therapeutic translational approaches. The fact that miRNA targets are often functionally grouped makes them very attractive therapeutic targets, and recent studies in many animal models of disease have shown enormous potential for such miRNA therapeutics. Indeed, within a short period of time we will see clinical studies testing miRNA therapeutics in humans.

miRNAs come in many different flavors. Given that different forms of circulating miRNAs have been discovered to reflect myocardial damage, what rigorous criteria should be used to determine their utility as biomarkers, and which miRNAs do you think fit into these standards?

Katey Rayner: The most important criterion for an miRNA to be considered as a biomarker for MI is that this miRNA is not found in the plasma/extracellular space as a result of any other pathology. That is, the miRNA used to diagnose a myocardial event must *not* be detectable in patients with other complications, such as liver disease, kidney dysfunction, or diabetes. This is especially important given that many patients with coronary artery disease suffer from multiple comorbidities that could complicate the miRNA-based diagnostic test. Currently, miR-1/133 and miR-208b lead the way with regard to their accuracy and sensitivity for distinguishing between AMI patients and healthy controls, although this awaits further confirmation in larger cohorts.

Stephanie Dimmeler: It is too early to make a final conclusion. Our current measurements and normalization protocols are far from ideal, and improvement in the methodology might be very helpful toward establishing miRNAs as biomarkers. The criteria to define the utility are not much different from any other biomarker and simply need extensive testing (and confirmation of the results) in large-scale clinical cohorts.

George A. Calin: As circulating miRNAs act as hormones, one important practical consequence derives from their large variation in expression in normal populations. Consequently, "normal" concentrations of circulating miRNAs vary widely among humans according to age, gender, and physiological events (such as menarche or pregnancy) and are influenced by various "extrinsic" factors, such as environmental temperature and stress. A practical consequence of this view is the fact that for any study comparing the expression of miRNAs in any type of body fluid from normal individuals and cancer patients, it is important to design the study in a "paired" way, at least for age, gender, and race, and to use at least twice as many controls as patients. In this way, the ample expression variation in the normal population can be assessed, and the comparisons with disease groups (AMI in this case) will be more meaningful and reproducible in independent cohorts. The best miRNA to use as a marker will be the one with exclusive expression in myocardial tissue; next-generation sequencing expression profiling could help in the identification of such miRNAs.

Thomas Thum: A first important issue is the normalization of the results. There is currently no real gold standard for normalization, as no "stable circulating" miRNA is known. Most researchers normalize by spiking in control miRNAs. I do not think that a single miRNA will be better than traditional MI biomarkers. However, a combination of miRNA markers, e.g., specific miRNAs for cardiomyocyte damage, inflammation, and alterations of endothelial cells and fibroblasts, may be able to tell us something about the underlying cause of the MI event that could result in therapeutic changes.

There is some controversy in the literature about which mechanisms determine the level of circulating miRNA under physiological conditions and in response to pathological stimuli. Could you highlight this controversy and explain what is known about some of their biological functions: Are they mediators of myocardial injury, or is their release a consequence of the injury itself?

Katey Rayner: There are many examples of miRNAs being secreted actively from cells in exosomes or microvesicles and associated with protein complexes in response to certain stimuli. Apoptotic bodies also con-

tain miRNAs, although it is not clear yet whether these miRNAs are specifically or nonspecifically packaged into these bodies. It is highly likely that miRNAs act as messengers to communicate in a paracrine manner with neighboring or distant cells, although the exact mechanisms of this have yet to be elucidated. In the case of MI, miRNAs are most likely released as a signal of damaged tissue from the cardiac muscle injury, perhaps to direct progenitor and/or inflammatory cells to home in on the site of injury. It is of little consequence whether this process is specific or nonspecific when such miRNAs are to be used as biomarkers. What is most important is their predictive value for diagnosis of MI. Mechanistically, if certain miRNAs alter the response to injury, then they may be manipulated for therapeutic benefit by use of either miRNA inhibition or replacement therapy.

Stephanie Dimmeler: Generally, miRNAs can be released via different coexisting mechanisms, but some miRNAs may be exported by preferential pathways. Based on our own studies, we would conclude that vesicle-embedded miRNAs are the preferential form for most miRNAs. However, we also could detect protein-bound [e.g., to argonaute 2 (Ago2)] miRNAs and lipoprotein-bound miRNAs, albeit to a lesser extent. The nature of the physiological long-distance communication function is unclear and hard to study in vivo. Certainly in cell culture, various groups have demonstrated transfer and biological activity of extracellular miRNAs; it is still an open question, however, whether low concentrations of extracellular miRNAs can elicit biological functions in vivo.

George A. Calin: There are 2 aspects to be discussed in relation to circulating miRNAs as hormones. One is the secretion step, the result of several mechanisms, including the presence of miRNAs in exosomes, microvesicles, or apoptotic bodies, as well as the interaction with the Ago2 protein and HDL complexes. All these forms make body-fluid miRNAs much more stable and therefore much easier to be quantified. Second is the question, what are the effects on effector cells? These effects are a result of the "classic" mechanism of action, meaning the targeting of mRNA via sequence complementarity, followed by mRNA degradation and/or the blocking of translation, both resulting in decreased protein expression. A new and unexpected mechanism was recently identified—the roles of miRNAs as direct agonists of Toll-like receptors in cancer cells and Alzheimer disease. Such mechanisms very probably are present also in patients with various myocardial diseases.

Thomas Thum: Many miRNAs are already bound to stabilizing Ago2 complexes and are released in this stable form after the cell membrane is ruptured, e.g., after an ischemic insult. In addition, miRNA can be actively secreted, e.g., via apoptotic bodies or smaller exosomes. These circulating miRNAs can be taken up into other target cells and may have physiological functions (like hormones) and thus have paracrine activity. This is intriguing, but many follow-up studies about the detailed mechanisms are needed.

As novel biomarkers for MI, how do you think miRNAs should be used as a diagnostic tool in management of patients with acute and chronic disease? How do you see miRNA fitting into or perhaps filling the holes of current diagnostic paradigms, such as troponins?

Katey Rayner: Most circulating miRNAs correlate closely with troponin concentrations in patients with AMI. There is some evidence that miR-208b might appear earlier than troponin T in the plasma, though this has yet to be confirmed. If the appearance of certain miRNAs precedes that of cardiac enzymes, then the use of these miRNAs (alone or, more likely, in combination) could be powerful. As mentioned above, however, the real potential of circulating miRNA biomarkers lies with their ability to detect/diagnose stable or unstable coronary disease *before* the occurrence of an MI. This is what is currently lacking in clinical practice.

Stephanie Dimmeler: Circulating miRNAs might be useful for stratification and risk prediction of diseases where currently good biomarkers are missing, such as the identification of patients with vulnerable plaques, which have a high risk for rupture and MI but are negative for high-sensitivity troponin.

George A. Calin: One aspect that is not well developed regarding the significance of quantification of miRNAs in plasma in acute or chronic disease of any type, including heart diseases, is the following: Concentrations of miRNAs, being hormones, are specific for each individual, and we are born with specific patterns of expression. Therefore, the measurement of plasma concentrations of miRNAs could represent a simple and easy way to predict the response to therapy, as this has an individual inherited component, too.

Thomas Thum: I think there is a huge benefit from miRNA diagnostics, mainly in chronic diseases. Focusing on the heart, I think there will be specific patterns of miRNAs for diagnosing various forms of heart failure and their underlying causes. The pattern of circulating miRNAs also may tell us something about the ongoing

pathological events inside the heart during chronic diseases such as heart failure, e.g., progressing intramyocardial fibrosis. Furthermore, there are already studies showing the prognostic power of circulating miRNAs. In the future, we will see results from larger patient cohorts, which will answer the question about the real clinical use of miRNAs in cardiovascular diseases.

Studies have shown that some miRNAs are significantly and exclusively increased in the plasma of patients with AMI. Should increased miRNA concentrations be interpreted quantitatively or qualitatively in the clinical setting, and why? Would upper cutoff limits or reference intervals in the normal and diseased population be useful in interpretation of results?

Katey Rayner: Ultimately, the range of "normal" concentrations of any miRNA needs to be determined empirically in a large population before its use as a biomarker for MI can be established. This should be done with a standardized procedure and used and validated across many centers, ideally in the form of a "bedside" test. Until this happens, it is impossible to predict what concentrations of miRNA to consider normal/abnormal. Moreover, a cardiac-specific miRNA (such as miR-208) would be ideal and would reduce any uncertainty over whether or not the presence of this miRNA in plasma is the result of some other tissue damage/pathology.

Stephanie Dimmeler: For routine clinical use, we need clear cutoff values and reliable measurements with acceptable day-to-day variations. With the present technologies, measurements of existing cohorts of samples are feasible, but standardization issues are not yet solved. The use of recombinant miRNAs for quantification or spiking of miRNAs for normalization in PCR-based measurement is not sufficient in our hands to allow for a bedside measurement of circulating miRNAs.

George A. Calin: The concentrations should be interpreted quantitatively, and reference intervals in the normal and disease population should be used. By quantitative reverse-transcription PCR, a very sensitive method of profiling, the miRNAs will also be present under specific conditions in normals (for example, would athletes have "pseudo-AMI" plasma miRNAs after heavy training, and at what concentrations?).

Thomas Thum: If these miRNAs are to be used in clinical medicine, there is a great need to define reference limits, which is quite an endeavor given the use of a PCR-based detection system, and comparability between different laboratories will be rather difficult. However, this is possible in a semiquantitative way. Reference intervals, not only in healthy controls but also in diseased individuals, are needed.

What are some of the state-of-the art technologies currently used to measure miRNA? What are the major analytical challenges or limitations, and what technologies need to be improved to reliably measure miRNA in the acute and nonacute clinical settings? Do you think point-of-care testing is a reality?

Katey Rayner: Currently, the most rapid way to measure miRNA is by quantitative PCR. An evolving diagnostic platform for measuring genetic material (including miRNA) is the Nanostring, although currently the turnaround time is >24 h, which is less than ideal for diagnosis of an MI and is better suited for more-chronic conditions. The recent use of the point-of-care "RAPID GENE" test to genotype individuals for the CYP2C19 allele (to guide antiplatelet therapy during coronary intervention) tells us that a rapid bedside test to measure miRNA concentrations is a very real possibility. In fact, the technologies used for genotyping and miRNA detection are not so different, so this may be a reality sooner than you think.

Stephanie Dimmeler: Not yet. Currently, most laboratories use PCR-based technologies, but other technologies are available as well. PCR is very sensitive but also difficult to standardize; other technologies may be better with respect to reproducibility and standardization; however, they are less sensitive. This field is in its early days, and surely the technologies need to be (and can be) further developed.

George A. Calin: The technology that is the most used in CLIA-based laboratories for translational medicine is quantitative reverse-transcription PCR, as it is specific, is easy to perform, is reproducible, and has a low cost. One limitation is the difficulty of finding useful normalizers that can be used in multiple independent sets of samples. The proposed alternative is to use spiking of nonhuman miRNAs, which assumes a uniformly equal quality of the samples, but this is not the case in the majority of instances. One alternative option—costly but more powerful—is small-RNA sequencing that is able to identify new miRNAs never before reported.

Thomas Thum: The most applied technology is real-time–based detection of miRNAs. Other technologies include microarray-based detection and bead technologies, as used for fluorescence-activated cell-sorting analysis. I think point-of-care testing may be developed

(2-h time), but as mentioned above, I think miRNAs will make their way into diagnostics more for chronic disease conditions.

Do you see circulating-miRNA testing for AMI a reality in 5 years down the road, and if not, what are the major hurdles to overcome in our understanding of miRNA testing?

Katey Rayner: In order for a circulating miRNA to become a widely used test for diagnosing AMI, the test needs to be better and faster (and perhaps less expensive) than what is used today. A single miRNA will likely not be more valuable than troponin measurements in this regard; however, a panel of many miRNAs may add confidence to cardiac marker measurements and may even be measured earlier. In contrast, if an miRNA or a panel of miRNAs that predicts unstable coronary disease can be identified, this would be highly valuable in the clinical setting to direct therapy. For either of these scenarios to become realities, the range of "normal" limits needs to be determined empirically for each circulating miRNA via a standardized and commercially available method. Ideally, this could be done with a point-of-care test that delivers rapid, accurate results in $<$1 h. If and when these criteria are met, circulating miRNAs may have enormous power in the clinical setting for diagnosis of both acute and chronic coronary diseases and may lead to novel therapies that target these dysregulated miRNAs.

Stephanie Dimmeler: Not likely for AMI, but possibly for other indications. Major hurdles are the establishment of a quick and sensitive, but robust and standardized, method that can be used in daily practice. Moreover, we have to test for and identify confounding factors that may influence measurement.

George A. Calin: With the advance of deep-sequencing technology toward clinical practice, many more miRNAs will be identified in the next few years, so it is a good possibility that over the next 5 years, miRNAs will be discovered as predictors of prognosis in both acute and stable disease. An important topic is the prediction of chronic disease, for which miRNAs could probably be used realistically in the next 5 years.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: E.P. Diamandis, *Clinical Chemistry*, AACC.
Consultant or Advisory Role: S. Dimmeler, MiRagen.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: S. Dimmeler, Deutsche Forschungsgemeinshaft (SFB834 B1).
Expert Testimony: None declared.
Patents: None declared.

DOI: 10.1373/clinchem.2013.215491